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中国农业科学院兰州畜牧与兽药研究所　主编

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编 委 会

前　言

近年来，在中国农业科学院科技创新工程的引领下，兰州畜牧与兽药研究所的科研水平快速提升。全所科研人员和管理人员不但有工作上的热情，更有对工作认识上的高度和对学科理解上的深度。他们在紧张繁忙的实践活动中，笔耕不辍，将自己的研究成果写成论文。这不单是科研人员和管理人员的工作总结、过程记录，更是他们智慧的结晶，最终成为研究所的一笔宝贵财富。

为了珍惜这笔财富，加强优秀论文的交流与传播，营造更加浓厚的学术氛围，促进科研水平和管理水平的提升，切实推进研究所的科技创新，科技管理处搜集了2013年研究所科研人员公开发表的论文编印成《中国农业科学院兰州畜牧与兽药研究所科技论文集》第二卷，共109篇。由于时间仓促，可能还有论文未能收录，敬希见谅！

编　者

2015年12月

目　录

RT-qPCR analysis of *dexB* and *galE* gene expression of *Streptococcus alactolyticus* in *Astragalus membranaceus* fermentation

Gui J. Hao, Kai Zhang, Jing Y. Zhang, Xu R. Wang, Zhe Qin, Xue Z. Wang, Lei Wang, Jia R. Meng, Zhi Q. Yang, Jian X. Li

(Engineering and Technology Research Center of Traditional Chinese Veterinary Medicine of Gansu Province, Key Lab of New Animal Drug Project of Gansu Province, Lanzhou Institute of Husbandry and Pharmaceutics Sciences of Chinese Academy of Agricultural Sciences, Lanzhou, China)

Abstract: *Streptococcus alactolyticus* strain FGM, isolated from chicken cecum, was used to increase the extract yield of polysaccharides during *Astragalus membranaceus* fermentation. It was previously demonstrated that polysaccharides from fermented *A. membranaceus* by *S. alactolyticus* had some similar properties to those from *A. membranaceus* in terms of its ability to help heal hepatic fibrosis in rats and modulate immunopotentiation of broiler chicken. However, methods to increase the yield of the polysaccharides during fermentation of *A. membranaceus* are not well understood. In this paper, we investigated the involvement of uridine diphosphate (UDP) – glucose 4 – epimerase (*galE*) and glucan – 1, 6 – α – glucosidase (*dexB*) during *A. membranaceus* fermentation through realtime reverse transcription quantitative PCR. The *galE* and *dexB* genes of *S. alactolyticus* were cloned by homologybased cloning and the genome walking method for the first time, and the 3D structure of *dexB* was analyzed by Swiss – Pdb-Viewer 4. 0. 1 software. The expression of both the *galE* and dexB genes in *A. membranaceus* fermentation was studied using the determined ideal reference gene *ldh* for transcript normalization. The results showed that these two genes were both highly induced and peaked after 12 h of fermentation. The expression level of *galE* was stepwise increased from 48 to 72 h, while *dexB* transcripts were markedly increased at 60 h and decreased by 72 h. These data suggested that *dexB* and *galE* of *S. alactolyticus* strain FGM were involved in the regulation of *A. membranaceus* fermentation and they might play some roles in the increase of polysaccharides.

Key words: *Streptococcus alactolyticus*; *Astragalus membranaceus*; UDP – glucose 4 – epimerase; Glucan – 1, 6 – α – glucosidase; Genome walking . RT – qPCR

Introduction

Streptococcus alactolyticus, a member of the *Streptococcus bovis/Streptococcus* equinus complex

(SBSEC), was originally isolated from the gastrointestinal tract of pigs and chickens (Farrow et al., 1984) and reported as a commonmember of the commensal gut microbiota in these animal species, as well as pigeons, ducks, and others (Devriese 1991; Devriese et al., 1994; Gong et al., 2002a, b; Højberg et al., 2005; Kurzak et al., 1998; Baele et al., 2002). On the basis of the 16S rRNA - derived phylogeny of the SBSEC strains, Chadfield et al., (2007) suggested that *S. alactolyticus* was a monophyletic group. Although the SBSEC was associated with various diseases such as endocarditis, bacteremia, sepsis, bovine mastitis, and bloat (Herrera et al., 2009), the pathogenic status of *S. alactolyticus* in infections is unknown and there are few reports on *S. alactolyticus* to date. Rinkinen et al., (2004) suggested that *S. alactolyticus* was the dominating culturable lactic acid bacterium (LAB) species in canine jejunum and feces of dogs. Interestingly, Czerwiński et al., (2010) demonstrated that the caecal microbiota in chicken was also dominated by the LAB *S. alactolyticus*. In our studies, the *S. alactolyticus* strain FGM (GenBank accession No. JX435470) was isolated from chicken cecum and applied to improve ingredients and increase the extract yield of polysaccharides from *Astragalus membranaceus* fermentation.

A. membranaceus (leguminous) has attracted extensive interest because of its active ingredients and biological function. Many chemical constituents in *A. membranaceus* have been isolated including polysaccharides, saponins, flavonoids, and aminophenols (Yan et at., 2010). In China, *Astragalus* polysaccharides have been applied as immunostimulants and antiviral drugs in poultry and swine. Previous studies showed that the concentration of polysaccharides was 4 - fold after A. *membranaceus* was fermented with *S. alactolyticus* strain FGM, and it was concluded that there were similar bioequivalence effects from the fermented *A. membranaceus* extraction as the *A. membranaceus* extraction on the body weight gains and immunopotentiation in broiler chicken (Zhu et al., 2008a; Zhang et al., 2011). Moreover, fermented *Astragalus* polysaccharides (FAPS) showed some ability to inhibit hepatic stellate cell activation and produce an obvious antagonism against hepatic fibrosis induced by CC14 exposure (Qin et al., 2012; Qin 2012). These findings indicated the presence of genetic specialization in *S. alactolyticus*, which enabled enhanced and enzymatic biotransformation during *A. membranaceus* fermentation.

The reason for an increase in polysaccharides in the fermented production was generally correlated with polysaccharides synthesis and decomposition. The exopolysaccharides (EPS) produced by some bacterial species used in food applications include *Streptococci*, *Lactobacilli*, *Lactococci*, and *Bifibobacteria* (Prasanna et al., 2012). The uridine diphosphate (UDP) - glucose 4 - epimerase (*galE*; EC 5. 1. 3. 2), also known as UDP - galactose 4 - epimerase, catalyzes the interconversion of UDP - galactose (UDP - Gal) and UDP - glucose (UDP - Glc), a pivotal step in the Leloir pathway of galactose metabolism (Grossiord et al., 1998). Studies showed that *galE* was a potential bottleneck in sugar nucleotide biosynthesis and subsequent EPS biosynthesis in LAB (Mozzi et al., 2003; Zhao et al., 2011). Gram - positive bacteria from the genus *Streptococcus* were also known to produce glucan - 1, 6 - α - glucosidase (*dexB*) whichwas active on isomaltooligosaccharides (IMO) and in some cases also on dextran (α - 1, 6 - linked glucan) (Møller et al., 2012). *dexB* (EC 3. 2. 1. 70), which belongs to glycoside hydrolase family 13 subfamily 31 (GH13_ 31) according to the CAZy database classification, catalyzes 1, 6 - α - D - glucosidic

linkages in 1, 6 – α – D – glucans and derived oligosaccharides. The only other biochemically and structurally characterized GH13_ 31 with *dexB* specificity were from *Streptococcus mutans* (Hondoh et al., 2008; Saburi et al., 2006) and *Lactobacillus acidophilus* NCFM (Møller et al., 2012).

The present study investigates *dexB* and galE gene expression of *S. alactolyticus* strain FGMto try to explain the possible functions of the *dexB* and *galE* during *A. membranaceus* fermentation. *DexB* and *galE* genes from the *S. alactolyticus* strain FGM were cloned by homology – based cloning and genome walking, and the 3D structure of *dexB* was analyzed by Swiss – PdbViewer 4. 0. 1 software for the first time. A set of reference genes was evaluated to make comparison and normalization meaningful and accurate in real – time reverse transcription quantitative PCR (RT – qPCR), and the expression levels of both genes in *A. membranaceus* fermentation were quantified versusan appropriate reference gene.

Materials and methods

Bacterial strains

S. alactolyticus strain FGM (GenBank accession No. JX435470; China Patent No. 20120141827. 5) was isolated from chicken cecum and preserved in the China General Microbiological Culture Collection Center as No. 4227. A. *membranaceus* fermentation medium (China Patent No. 201210141832. 6) had been optimized by neural network and genetic algorithm with *A. membranaceus* powder mass fraction of 8%.

S. alactolyticus strain FGM was grown anaerobically at 37℃ in MRS broth. The anaerobic fermentation was carriedout in 250 mL erlenmeyer flasks with 100 mL working volume at 37℃ in a shaker incubator at 120 rpm for 68 h, original pH value of 7. 2, and the volume fraction of inoculums was 4% (4.5×10^8 CFU/mL). In a 10^{-L} fermentation tank with 6 L working volume, the conditions were same as above.

Genome DNA isolation and dexB and galE gene cloning

Genomic DNA was isolated using a bacterial DNA kit (OMEGA, USA) in accordance with the manufacturer's instructions. In reference to S. bovis and *S. equinus dexB* and *galE* gene sequences, gene – specific primers (Table 1) weredesigned by Primer premier V5. 0 software. The PCR amplification was carried out in a 50μL reaction mixture containing 3 μL ($30ng \cdot \mu L^{-1}$) of DNA, 1 μL ($20\ \mu mol \cdot L^{-1}$) of forward and reverse primers, 25 μL of Premix Taq® (TaKaRa), and 20 μL ddH_2O. PCR reaction conditions on Applied Biosystems Veriti 96 well Thermal Cycle (Gene Company Limited) were as follows: initial denaturation at 95℃ for 4 min, followed by 35 cycles of 95℃ for 15 s, 52℃ for 15 s, and 72℃ for 30 s; final extension step at 72℃ for 7 min.

Table 1 Primers used for *dexB*, *galE*, *recA*, and *ldh* gene amplification of *S. alactolyticus* strain FGM

Primers	Sequence (5′ – 3′)	Size	PCR Products (bp)	Gen Bank accession numbers
galE – F	TAGTGCTTGGTGGGGCTGGTTA	22	410	JX898029
galE – R	GATTGGGCGTTGTGGTGTTGTT	22		

(continued)

Primers	Sequence (5′ - 3′)	Size	PCR Products (bp)	Gen Bank accession numbers
dexB - 1F	CAATTTGAACATATCGGTCT	20	894	JX898028
dexB - 1R	ATGCGAATACTGTCTAGCAC	20		
SP1	TCCAGCATTCTTGCCATTATCCC	23	1 264	
SP2	TGCCGATTTCTTCTCCTTGGTAG	23		
SP3	TCCAGTTCTGTCTGCCATTTGTTG	24		
recA - F	GTGCTGTTGACCTCGTCGTTGT	22	655	JX947343
recA - R	GTGCTGTTGACCTCGTCGTTGT	22		
ldh - F	GGTGCCGTAGGTTCATCT	18	857	JX947344
ldh - R	TGTTGTTCAGCGTCATTCA	19		

To obtain unknown *dexB* gene sequences adjacent to the known region, the genome walking technique was applied. Three gene - specific primers (SP1, SP2, and SP3) derived from the known sequence were designed (Table 1). Three nested PCR reactions used the same degenerate primer AP2 provided in the Genome Walking Kit (TaKaRa). The primary and secondary PCR reaction mixtures were diluted 100 - fold and used as a template for the subsequent walk. Nested PCR conditions and other reaction mixtures were performed in accordance with the manufacturer's protocol. All PCR products were purified, ligated to pGEM - T easy vector (Promega) and sequenced. The results of sequencing were subjected to BLAST analysis. The obtained sequence of *dexB* was assembled and analyzed with the help of DNAMAN software and a neighbour joining tree based on the *dexB* of *S. alactolyticus* was constructed using ClustalW 2.0 function of MEGA5.05. The 3D structure of the deduced proteins was predicted using SWISSMODEL on the website (http://www.expasy.org) and analyzed by Swiss - PdbViewer 4.0.1.

Cloning of reference genes

The *ldh* and *recA* genes were selected as candidate reference genes to normalize mRNA levels between different samples and cloned with specific primers (Table 2) to obtain their partial sequences for subsequent real - time PCR analysis. PCR reaction mixtures and conditions were same as above. The amplified PCR products were purified, ligated to pGEM - T easy vector (Promega) and sequenced.

Total RNA isolation and cDNA library construction

Fermentation broths (-80℃) at different stages (0, 12, 24, 36, 48, 60, and 72 h) were centrifuged at 1 000 × g, 4℃ for 3 min to collect the supernatant and separate the insoluble precipitated impurities, then the total RNA was isolated using a bacterial RNA kit (OMEGA, USA) according to the manufacturer's instructions. Contaminating DNA was further removed with RNase - free DNase I (OMEGA, USA) during the extraction. Isolated RNA was quantified and assessed for purity using a Thermo Scientific NanoDrop 2000 spectrophotometer and was visualized on agarose gel to check RNA integrities. Samples with $A_{260\ nm}/A_{280\ nm}$ values ranging from 1.9 to 2.1 and $A_{260\ nm}/A_{230\ nm}$ values ranging from 1.7 to 2.1 were taken as acceptable. The reverse transcription re-

action for real – time PCR was performed with a PrimeScript® RT Reagent Kit Perfect Real – Time (TaKaRa). A total of 10 μL of the reaction system contained 2 μL of 5 × PrimeScript® Buffer (for Real Time), 0.5 μL of PrimeScript® RT Enzyme Mix I, 0.5 μL of both oligo dT (50 μmol · L^{-1}) and six randomprimers (100 μmol · L^{-1}), and 500 ng of total RNA. The reaction mixture was incubated at 37℃ for 15 min and then heat inactivated at 85℃ for 5 s.

Reference gene selection and real – time PCR

In reference to known and obtained relative genes sequences of *S. alactolyticus*, gene – specific primers (listed in Table 2) of reference genes and *dexB* and *galE* genes were designed. Five reference genes (16S, *gapdh*, *ldh*, *rpoB*, and *recA*) were analyzed for their expression stability during growth in A. *membranaceus* fermentation by qbase^{PLUS} 2.4 software using the method described by Hellemans et al., (2007). The SYBR® Green I real – time PCR assay was performed in an optical 96 – well thin wall plate sealed with optical quality sealing tape on an iCycler iQ™5 Multi – Color Real – Time PCR Detection System (Bio – Rad Laboratories). PCR amplicons for each gene at first were visualized on mass fraction of 1% agarose gel to confirm that there was no nonspecific amplification, nor formation of heterodimer or self – primer dimers. Serial 10 – fold dilutions of DNA diluted with Easy dilution (TaKaRa) were used as standard curves to check for relevant amplification efficiency (E) and nonspecific amplicons. Each real – time PCR reaction was performed in triplicate with a final volume of 25 μL containing 12.5 μL of SYBR® Premix Ex Taq™ Ⅱ, 1 μL of each primer (10 μmol · L^{-1}), 2 μL of DNA template and 8.5 μL of sterile ddH_2O, and two negative controls without template or primers. The two step real – time PCR reaction was run at 95℃ for 30 s followed by 40 cycles of 5 s at 95℃ and 30 s at 60℃. The melting curve program consisted of temperatures between 60 and 95℃ with a heating rate of 0.05℃/s with a continuous fluorescence measurement. Additionally, the expected size of the amplicon and the absence of nonspecific products were confirmed again in agarose gels. Executions of the PCR program and data collection were facilitated by Bio – Rad iQ™ 5 Optical System Software, version 2.0.

Table 2 qPCR primers for *S. alactolyticus* strain FGM

Primers	Sequence (5′ – 3′)	Size	PCR products (bp)	Amplification Efficiencies (%)	The highest variation Coefficients (%)
16S – F	GCGACGATACATAGCCGACCT	21	92	100.9	2.14
16S – R	TGATTCCCTACTGCTGCCTCC	21			
gapdh – F	GGCAAACGATGGCGTAGAAAT	21	120	104.0	2.31
gapdh – R	CCACCAGGAGCAGTGATAACAAC	23			
ldh – F	CAAGAAGACTCCGTTGAAACCAGA	24	291	104.8	1.56
ldh – R	CTCGTAAACCAAGGAATCGCACA	23			
rpoB – F	CACGGTGGTGATGGTGTTGTTC	22	213	102.3	1.98
rpoB – R	TGTTCGGTCTGGTAGGTAAGGCA	23			
recA – F	CGAAAAGATAGCAGCATCGGTAAAG	25	251	106.2	1.87
recA – R	AAGGTGTCAGGGTGGTCACTCAAG	24			

(continued)

Primers	Sequence (5′ - 3′)	Size	PCR products (bp)	Amplification Efficiencies (%)	The highest variation Coefficients (%)
dexB - F	GCAATGGGATAATGGCAAGA	20	133	108.4	3.25
dexB - R	GCGGTAAGTGTAGAAAATGGAA	22			
galE - F	GCTGCTTACATCGGCTCTCAC	21	92	99.7	2.12
galE - R	CGCTGTCCTGTCACCAAACTAT	22			

Once the appropriate reference gene was determined, mRNA levels were determined for different stages (0, 12, 24, 36, 48, 60, and 72 h) using the $2^{-\Delta\Delta CT}$ method. To analyze the differential expressions, the mRNA levels evaluated for each gene were measured compared with the zero time point.

GenBank accession numbers

All obtained genes sequences were deposited in GenBank and *dexB*, *galE*, *recA*, *and ldh* gene accession No. were JX898028, JX898029, JX947343, and JX947344, respectively.

Results

Characteristics of *S. alactolyticus dexB*

Primers of the *dexB* gene generated a major product 894 bp in length which was inconsistent with the predicted 404 bp derived from the dexB gene of *S. bovis* ATCC 700338 (ENA, EFM28271.1). Interestingly, the results of sequencing subjected to BLAST suggested that it showed the highest, with 87% identity with dexB of *S. infantarius* subsp. *infantarius* CJ18 (GenBank, CP003295.1). To characterize the unknown genomic DNA sequences flanking the known region, genome walking was applied and a 1, 264 - bp segment was successfully obtained. BLAST analysis showed that it was homologous (89%) to the *S. macedonicus* ACA - DC 198 genome (GenBank, HE613569.1). Additionally, it also demonstrated that a partial gene sequence of sucrose phosphorylase was located upstream of the *dexB* gene in *S. alactolyticus*. The assembled complete sequence was 1, 853 bp in length and it contains a large open reading frame of 1, 611 bp (GenBank accession No. JX898028), encoding a protein of 536 amino acid residues with a predicted molecular weight of 62 kD. The neighbour joining tree constructed using MEGA5.05 revealed that the *dexB* gene from *S. alactolyticus* was the closest (88.8% identity) to that of *S. equinus* (Figure 1). The deduced amino acid sequence showed 77.4, 90.5, 90.9, and 92.4% overall identity with *S. mutans* ATCC 700610 (UniProtKB, Q99040), *S. equinus* ATCC 9812 (UniProtKB, E8JR30), *S. bovis* ATCC 700338 (UniProtKB, EOPBE5), and *S. infantarius* subsp. *infantarius* ATCC BAA - 102 (UniProtKB, B1SDT5) *dexB*, respectively. The identity of the protein sequences between *S. alactolyticus* and *L. acidophilus* NCFM (UniProtKB, Q5FMB7) assigned to GH13 _ 31 was 57.5%.

A multiple sequence alignment of top hits from BLASTP with *S. alactolyticus dexB* (*Sa - dexB*) combined with the enzyme from L. acidophilus NCFM showed that *Sa - dexB* aligned very well with