J Ind Microbiol Biotechnol (2015) 42:197–206
DOI 10.1007/s10295-014-1555-8

BIOENERGY/BIOFUELS/BIOCHEMICALS

SIMB
Society for Industrial Microbiology and Biotechnology

Constructing a recombinant hyaluronic acid biosynthesis operon and producing food-grade hyaluronic acid in *Lactococcus lactis*

Juzheng Sheng · Peixue Ling · Fengshan Wang

Received: 26 September 2014 / Accepted: 20 November 2014 / Published online: 2 December 2014

Abstract Hyaluronic acid (HA), a natural high molecular weight polysaccharide, is produced by *Streptococcus zooepidemicus*. However, *Streptococcus* has several drawbacks including its potential to produce exotoxins, so there is demand for an alternative HA source. Here, a recombinant HA biosynthesis operon, as well as the HA biosynthesis operon of *S. zooepidemicus* were introduced into *L. lactis* using the nisin-controlled expression system, respectively. HA was successfully synthesized by recombinant *L. lactis*. Furthermore, overexpression of the endogenous enzymes directing the synthesis of precursor sugars was effective at increasing HA production, and increasing the supply of UDP-activated monosaccharide donors aided synthesis of monodisperse HA polysaccharides. Besides GRAS host strain (*L. lactis*) and NICE system, the selecting marker (*lacF* gene) of the recombinant strain is also food grade. Therefore, HA produced by recombinant *L. lactis* overcomes the problems associated with *Streptococcus* and provides a source of food-grading HA appropriate for widespread biotechnological applications.

Keywords Food grade · Hyaluronic acid · Hyaluronan synthase · UDP-glucose-6-dehydrogenase · *Streptococcus zooepidemicus* · *Lactococcus lactis* · The nisin-controlled gene expression (NICE) system

J. Sheng (✉) · F. Wang
Key Laboratory of Chemical Biology of Natural Products (Ministry of Education), Institute of Biochemical and Biotechnological Drug, School of Pharmaceutical Sciences, Shandong University, Jinan 250012, China
e-mail: shengjuzheng@sdu.edu.cn

F. Wang
e-mail: fswang@sdu.edu.cn

P. Ling
Shandong Academy of Pharmaceutical Science, Jinan 250101, China
e-mail: lingpxha@yanhoo.com.cn

F. Wang
National Glycoengineering Research Center, Shandong University, Jinan 250012, China

Introduction

Hyaluronic acid (HA) is a natural high molecular weight polysaccharide and plays structural, recognition and signaling roles in the living body [8, 28, 31]. HA has been used in medicines, cosmetics, drug delivery systems, vaccine aids and health foods [13, 27]. In industry, HA is produced using the Gram-positive bacterium *Streptococcus zooepidemicus*. However, *Streptococcus* is a less-than-ideal source because of its potential to produce exotoxins, the difficultly in fermentation control and the expensive medium required for growth [30]. Therefore, an alternative HA production source would be desirable.

Lactococcus lactis, one of the most widely used Gram-positive lactic acid bacteria in food fermentations, has been generally regarded as safe (GRAS) strain and is increasingly used in modern biotechnology [5, 6, 18, 22]. The nisin-controlled gene expression (NICE) system is one of the most successful and widely used tools in *L. lactis* [15, 16, 21] and the *lacF* gene is commonly used as a food-grading selecting marker in the NICE system [5, 21, 32]. To overcome the shortcomings of *S. zooepidemicus* as an HA production host, we planned to construct some GRAS recombinant strains based on *L. lactis* containing the HA biosynthesis operon and the *lacF* selectable marker.

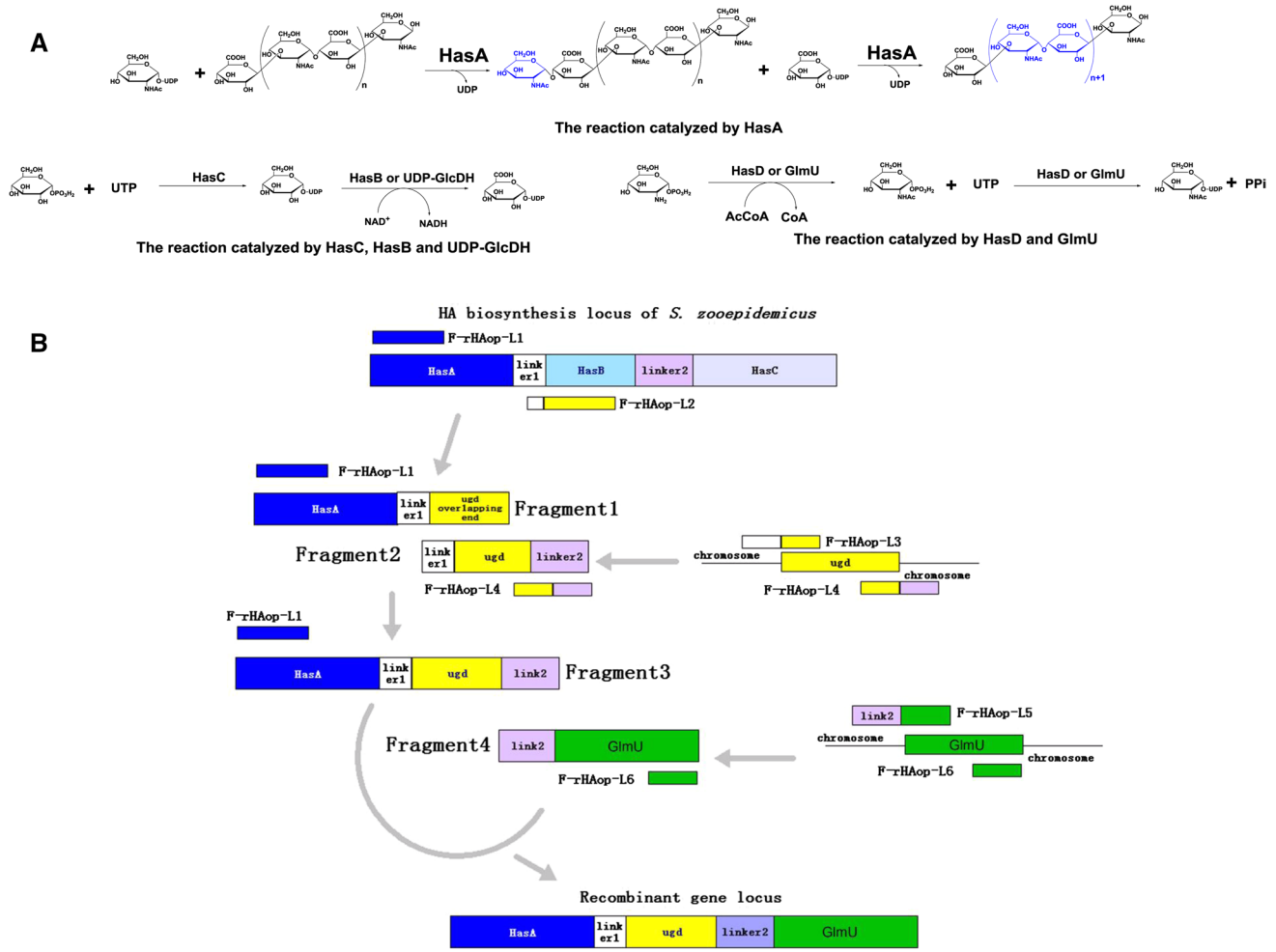

Fig. 1 Schematic synthesis of HA and the construction of the recombinant HA biosynthesis operon. **a** The reaction catalyzed by HasA, HasB, HasC, HasD, UDP-GlcDH and GlmU. **b** The location of the primers and the mechanism of triple fusion. In rHAop, the ORFs sz*HasB* and sz*HasC* were displaced by the ORFs *ugd* and *glmU* from *L. lactis*, respectively. ORF *szHasA* and the DNA fragments (linker1 and linker2) between each of the two genes in the HA biosynthesis operon of *S. zooepidemicus* were conserved. The primers and overlapping ends with identical sequences to the fragments are indicated by the *same colors*. Fragment 1 was amplified from *S. zooepidemicus* genomic DNA using primers rHAop-L1/L2. Fragment 2 (linker1 overlapping ends-ugd-linker2) and Fragment 4 (linker2-GLMU) were amplified from *L. lactis* genomic DNA using primers rHAop-L3/L4. Fragment 3 was amplified using rHAop-L1/L4 as the primers and a mixture of Fragment 1 and Fragment 2 as the template. DNA fragment rHAop was amplified using rHAop-L1/L6 as the primers and a mixture of Fragment 3 and Fragment 4 as the template. Each stage roughly corresponded to a PCR cycle (denaturation, annealing and extension) and reactions were performed using the *Pfu* polymerase mix

A series of enzymes are involved in the biosynthesis of HA in *S. zooepidemicus*, including hyaluronan synthase (HasA), uridine-diphosphate (UDP)-glucose-6-dehydrogenase (HasB), UDP-glucose pyrophosphorylase (HasC) and glucosamine-1-phosphate (GlcN-1-P) *N*-acetyltransferase/UDP-*N*-acetylglucosamine (UDP-GlcNAc) pyrophosphorylase (HasD) (Fig. 1a). HasA, the key enzyme, catalyzes the polymerization of glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc) to produce HA. UDP-glucuronic acid (UDP-GlcA) and UDP-GlcNAc were the activated monosaccharide donors in the biosynthesis of HA [19, 20]. HasB, HasC and HasD are the enzymes involved in the synthesis pathway of these precursor donors [1]. UDP-glucose-6-dehydrogenase (UDP-GlcDH) and GlcN-1-P acetyl transferase/UDP-GlcNAc pyrophosphorylase (GlmU) from *L. lactis* catalyze the same reactions as HasB and HasD (Fig. 1) [2].

In this study, we constructed a recombinant HA biosynthesis operon and introduced it into *L. lactis* using the NICE system. We produced a food-grade HA producing recombinant strain expressing HasA and overexpressing endogenous UDP-GlcDH and GlmU. Both the disaccharide analysis and radioimmunoassay (RIA) data showed that recombinant *L. lactis* strains biosynthesized food-grade

HA successfully. In addition, HasA was essential for high heterologous HA production, and overexpression of the endogenous enzymes directing the synthesis of the UDP sugars had positive impacts on HA production in *L. lactis*. Meanwhile, increasing the supply of the UDP-activated monosaccharide donors was beneficial for the synthesis of monodisperse HA polysaccharides.

Materials and methods

Strains and cultivation conditions

Escherichia coli strain MC1061 [3] was used as intermediate host and handled by standard techniques [29]. *Streptococcus zooepidemicus* FHA0 (Shandong Freda Biochem Co. Ltd., Jinan, China) was used as the genetic reference strain. *L. lactis* strains NZ9000 and NZ3900 [5, 15, 21] were used as the host strains and were obtained from NIZO Food Research (Ede, The Netherlands). *Escherichia coli* was grown in LB medium (10 g l^{-1} NaCl, 10 g l^{-1} peptone, 5 g l^{-1} yeast extract) at 37 °C. *Streptococcus zooepidemicus* was cultivated in THYB [Todd–Hewitt (TH) broth supplemented with 0.5 % yeast extract] at 37 °C. *L. lactis* was grown at 30 °C in GM17 medium [M17 broth supplemented with 1 % (wt vol^{-1}) glucose] or LM17 medium [M17 broth supplemented with 1 % (wt vol^{-1}) lactose]. Chloramphenicol was used at a concentration of 10 μg ml^{-1} for *E. coli* and *L. lactis*. Elliker medium [20 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 4 g l^{-1} sodium chloride, 1.5 g l^{-1} sodium acetate (water free), 0.5 g l^{-1} L(+)-ascorbic acid supplemented with 0.5 % (wt vol^{-1}) lactose and 0.004 % (wt vol^{-1}) bromocresol purple] was used for selection of lac+*L. lactis* colonies.

The host strain *L. lactis* NZ3900 cannot grow on lactose because of a *lacF* gene deletion on the genome. Vector pNZ8149 containing the *lacF* gene as a food-grading selecting marker was selected for its ability to grow on lactose [7]. On the rich Elliker medium, all *L. lactis* cells can grow, with or without the *lacF* gene. Because lactose was the only carbon source in Elliker medium, recombinant strains containing pNZ8149, lactose fermenting cells, gave yellow colonies.

DNA manipulation and transformation

Plasmid DNA was extracted and purified from *E. coli* by the alkaline lysis method [24] or from *L. lactis* as described previously [29]. Restriction endonucleases, T4 DNA ligase and *Pfu* polymerase, purchased from Fermentas (Vilnius, The Lithuania), were used according to the recommendations of the manufacturer. The SDS-PAGE standard was also purchased from MBI. Electroporation of *E. coli* MC1061 and *L. lactis* was performed according to the methods of Dower et al. [9] and Wells et al. [29] using a Gene Pulser (Berkeley, CA, United States, Bio-Rad).

Construction of recombinant strains

The strains and plasmids used in this study are listed in Table 1. And the primers used in this work are described in Table 2.

The genes *szHasA, szHasB* and *szHasC* were amplified from the chromosomal DNA of *S. zooepidemicus* using primers HasA-L1/L2, HasB-L1/L2 and HasC-L1/L2 containing *Nco*I and *Xba*I or *Pst*I restriction sites (underlined). The *szHasABC* DNA fragment was amplified from the chromosomal DNA of *S. zooepidemicus*, using primers P-HasA-L1 and P-HasC-L2. The final PCR products, *szHasA*, *szHasC* and *szHasABC*, were digested with *Nco*I and *Xba*I, and *szHasB* was digested with *Nco*I and *Pst*I. The DNA fragments were inserted into plasmid pNZ8148, with a *nisA* promoter and terminator, resulting in plasmids pNZ8148-szHasA, pNZ8148-szHasB, pNZ8148-szHasC and pNZ8148-szHasABC. The DNA fragment *szHasABC* was inserted into plasmid pNZ8149, resulting in plasmid pNZ8149-szHasABC.

The artificial recombinant operon was reconstructed by fusion PCR and named rHAop. The location of the primers and the mechanism of triple fusion are shown in Fig. 1. Fragment 1 (szHasA-linker1-ugd overlapping end) was amplified from *S. zooepidemicus* genomic DNA using primers rHAop-L1/L2. Fragment 2 (linker1 overlapping ends-ugd-linker2) and Fragment 4 (linker2-GLMU) were amplified from *L. lactis* genomic DNA using primers rHAop-L3/L4 and rHAop-L5/L6, respectively. The PCR products were extracted and purified from the agarose gel using a Gel Purification Kit (Promega, Fitchburg, Wisconsin, USA). Then, Fragment 3 was amplified using rHAop-L1/L4 as primers and a mixture of Fragment 1 and Fragment 2 as the template. The final fragment of the recombinant operon, rHAop, was amplified using rHAop-L1/L6 as primers and a mixture of Fragment 3 and Fragment 4 as the template. All reactions were performed using the *Pfu* polymerase mix.

The recombinant operon, rHAop, was cloned into pGEM-T vector (Promega) and sequenced by Shanghai Bioasia Engineering Co. (Shanghai, China). Then, this DNA fragment without any sequence variations was amplified with the 5′ primer rHAop-F with an added *Nco*I restriction site (underlined) and the 3′ primer rHAop-R with an added *Xba*I restriction site (underlined). The amplified fragments were digested with *Nco*I and *Xba*I, and inserted into pNZ8149, producing the expression plasmid pNZ8149-rHAop.

The plasmids pNZ8148, pNZ8148-szHasA, pNZ8148-szHasB, pNZ8148-szHasC and pNZ8148-szHasABC were

Table 1 Strains and plasmids used in this study

Strain/plasmid	Relevant genotype	References
Strain		
NZ9000	Model strain MG1363 with *nisRK* genes integrated in the chromosome. Host strain for nisin inducible vectors	[15]
NZ9000-pNZ8148	NZ9000, pNZ8148	[26]
NZ9000-A	NZ9000, pNZ8148-szHasA	[26]
NZ9000-B	NZ9000, pNZ8148-szHasB	This study
NZ9000-C	NZ9000, pNZ8148-szHasC	This study
NFHA01	NZ9000, pNZ8148-szHasABC	This study
NZ3900	NZ9000 with *lacF* deletion	[5]
NZ3900-pNZ8139	NZ3900, pNZ8149	This study
NFHA02	NZ3900, pNZ8149-szHasABC	This study
NFHA03	NZ3900, pNZ8149-rHAop	This study
Plasmid		
pNZ8148	nisA promoter and multiple cloning site, pSH71 replicon, Cm resistance	[21]
pNZ8149	Like pNZ8148, with the *cat*-gene replaced by the food-grade *lacF* gene as selection marker	[5]
pNZ8148-szHasA	pNZ8148, PnisA-szHasA	[26]
pNZ8148-szHasB	pNZ8148, PnisA-szHasB	This study
pNZ8148-szHasC	pNZ8148, PnisA-szHasC	This study
pNZ8148-szHasABC	pNZ8148, PnisA-szHasABC	This study
pNZ8149-szHasABC	pNZ8149, PnisA-szHasABC	This study
pNZ8149-rHAop	pNZ8149, PnisA-rHAop	This study

Table 2 Primers used for amplification of different gene fragments in this study

Primers	Sequences
HasA-L1	5′-CAGCCCATGGGCAGAACATTAAAAAACCTCA-3′
HasA-L2	5′-CATTTCTAGATTATAATAATTTTTTACGTGTT-3′
HasB-L1	5′-CAGCCCATGGGCAAGTGAAAATTTCTGTAGC-3′
HasB-L2	5′-CATTCTGCAGCTAGTCTCTTCCAAAGACATC-3′
HasC-L1	5′-CAGCCCATGGGCACAAAGGTC AGAAAAGC-3′
HasC-L2	5′-CATTTCTAGACCAAACTT GTCTCCAAC-3′
rHAop-L1	5′-CAGCATGAGAACATTAAAAAACCT-3′
rHAop-L2	5′-TGCTATTTTAGTCATTTCATTTTTTCCTTTGATACCTT-3′
rHAop-L3	5′-AAGGTATCAAAGGAAAAAATGAAATGACTAAAATAGCA-3′
rHAop-L4	5′-TGCCATTCGTT TTCTGACTAGTCCCTTCTATACAAATC-3′
rHAop-L5	5′-CAGAAAACGAATGGCACTCATAAGGAACCACAAATCAAGG AGGAACTCATGAATAAATTCGCTATTGTC-3′
rHAop-L6	5′-CCTTTATTGTCCACGATAATGTGG-3′
rHAop-F	5′- CAGCCCATGGGCAGAACATTAAAAAACCTCA-3′
rHAop-R	5′-CCTTTCTAGATTATTGACCACGATAATG-3′

The restriction sites in the primers have been underlined

introduced into *L. lactis* strain NZ9000. The transformants were selected on GM17 plates containing chloramphenicol and were designated NZ9000-pNZ8148, NZ9000-A, NZ9000-B, NZ9000-C and NFHA01. NZ3900 was transformed with the plasmids pNZ8149-szHasABC and pNZ8149-rHAop. The transformants were selected on Elliker medium and named NFHA02 and NFHA03.

Induction

Nisin (Sigma-Aldrich, St. Louis, MO, USA) was processed according to the method of Eichenbaum et al. (1998). The induction of recombinant *L. lactis* strains was performed as described previously [5, 11]. Briefly, after a 1 in 20^{-1} inoculation (vol vol^{-1}) of fresh medium with a 12 h

culture, cells were grown at 30 °C to an OD_{600} of 0.5. Then, 10 ng ml^{-1} nisin was added and cells were incubated for another 12 h at 30 °C to induce the expression of target proteins.

Cell extraction and SDS-PAGE

For enzyme activity measurements, the strains were cultivated in GM17 containing chloramphenicol (10 μg ml^{-1}) or LM17 medium and harvested after induction for 12 h. Cells were washed with sterile water and disrupted using an ultrasonic processor. Proteins in the crude extract were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 9 %). The protein concentration was measured using a Coomassie blue protein assay, with bovine serum albumin as the standard.

Hyaluronic acid analysis

HA was isolated from the fermentation broth according to a previously described method [25]. Briefly, a 1 % solution of cetylpyridinium chloride was added to the supernatant fluid containing HA, whereupon HA precipitated as cetylpyridinium-HA. The precipitate was dissolved in 0.5 mol l^{-1} NaCl solution containing 4 % ethanol. Then, three volumes of ethanol were added to one volume of the solution to precipitate HA and after centrifugation, the precipitate was dissolved in distilled water.

HA polysaccharides (100 mg) were degraded by hyaluronate lyase as previously described [12] and desalted on a Bio-Gel P-2 column (0.5 × 200 cm) in 0.1 M NH_4HCO_3. The eluent was monitored at a UV wavelength of 232 nm. Fractions containing HA disaccharide were pooled and dried. Then, disaccharides were analyzed on a C18 reversed-phase column (0.45 × 25 cm; Vydac) with UV 232 detection, as previously described [10].

The HA concentration in the fermentation broth was estimated using an HA RIA kit (Shanghai Navy Institute of Medical Sciences, Shanghai, China). Multiple dilutions of medium samples were prepared and assayed in triplicate along with HA standards according to the manufacturer's instructions. This assay currently provides the most sensitive and specific way to quantify HA [30].

The number average molecular weight (M_n) and the weight-average molecular weight ($\overline{M}_w$) were measured by SEC–MALLS analysis as previously described [14].

Mass spectrometry analysis

MS analyses were performed on a Thermo LCQ-Deca. The disaccharide was dissolved in 50 % methanol. Experiments were performed in negative ionization mode with a spray voltage of 5 kV and a capillary temperature of 275 °C [25].

Results and discussion

Construction of the recombinant *L. lactis* strains

Six plasmids (Table 1) were constructed and introduced into *L. lactis*. Genes *szHasA*, *szHasB* and *szHasC* were carried on pSH71-derived replicons under the control of the *PnisA* promoter in the expression plasmids pNZ8148-szHasA (Fig. 2Aa), pNZ8148-szHasB (Fig. 2Ab) and pNZ8148-szHasC (Fig. 2Ac), respectively. The recombinant *L. lactis* strains harboring pNZ8148-szHasA, pNZ8148-szHasB and pNZ8148-szHasC were designated N9000-A, N9000-B and N9000-C, respectively.

The NICE system cannot only be used to drive expression of single genes, but also of whole operons [21]. In this study, the HA biosynthesis operon of *S. zooepidemicus* containing the genes *szHasA*, *szHasB* and *szHasC* was introduced into *L. lactis* strains NZ9000 and NZ3900 using the vectors pNZ8148-szHasABC (Fig. 2Ad) and pNZ8149-szHasABC (Fig. 2Ae), respectively, producing strains NFHA01 and NFHA02.

HA biosynthesis involves synthesis of the monosaccharide donors, followed by polymerization of GlcA and GlcNAc. We hypothesized that the endogenous enzymes from *L. lactis* should work more efficiently than szHasB and szHasC in the synthesis pathway of the UDP sugars in the recombinant strain. Therefore, a recombinant HA biosynthesis operon was constructed and designated rHaop (Fig. 1B). In this recombinant DNA fragment, open reading frames (ORFs) sz*HasB* and *szHasC* were displaced by ORFs *ugd* and *glmU* from *L. lactis*, respectively. The *szHasA* gene and the DNA fragments (linker1 and linker2) between each of the two genes in the HA biosynthesis operon of *S. zooepidemicus* were conserved. Then, rHAop was introduced into *L. lactis* NZ3900 using the vector pNZ8149-rHAop (Fig. 2Af), resulting in strain NFHA03.

Food-grade HA production in the recombinant strains

Several studies have shown that HasA plays a critical role during HA synthesis and that the enzymes involved in monosaccharide donor synthesis also have an effect on the yield and the molecular weight (M_w) of HA in recombinant strains [20, 26]. To determine the roles of HasA and the UDP-sugar synthetic enzymes in the synthesis of HA in *L. lactis*, six recombinant strains with different metabolic combinations were constructed (Fig. 2B).

Strains NZ9000-A, NZ9000-B, NZ9000-C and NFHA01 were incubated for 12 h at 30 °C with 10 ng ml^{-1} nisin. Total cell extracts were prepared, and equal amounts of proteins were analyzed by SDS-PAGE (Fig. 3A). SDS-PAGE image analysis showed that HasA (M_w: 47.7 kDa), HasB (M_w: 43.5 kDa) and HasC (M_w: 28.9 kDa) were

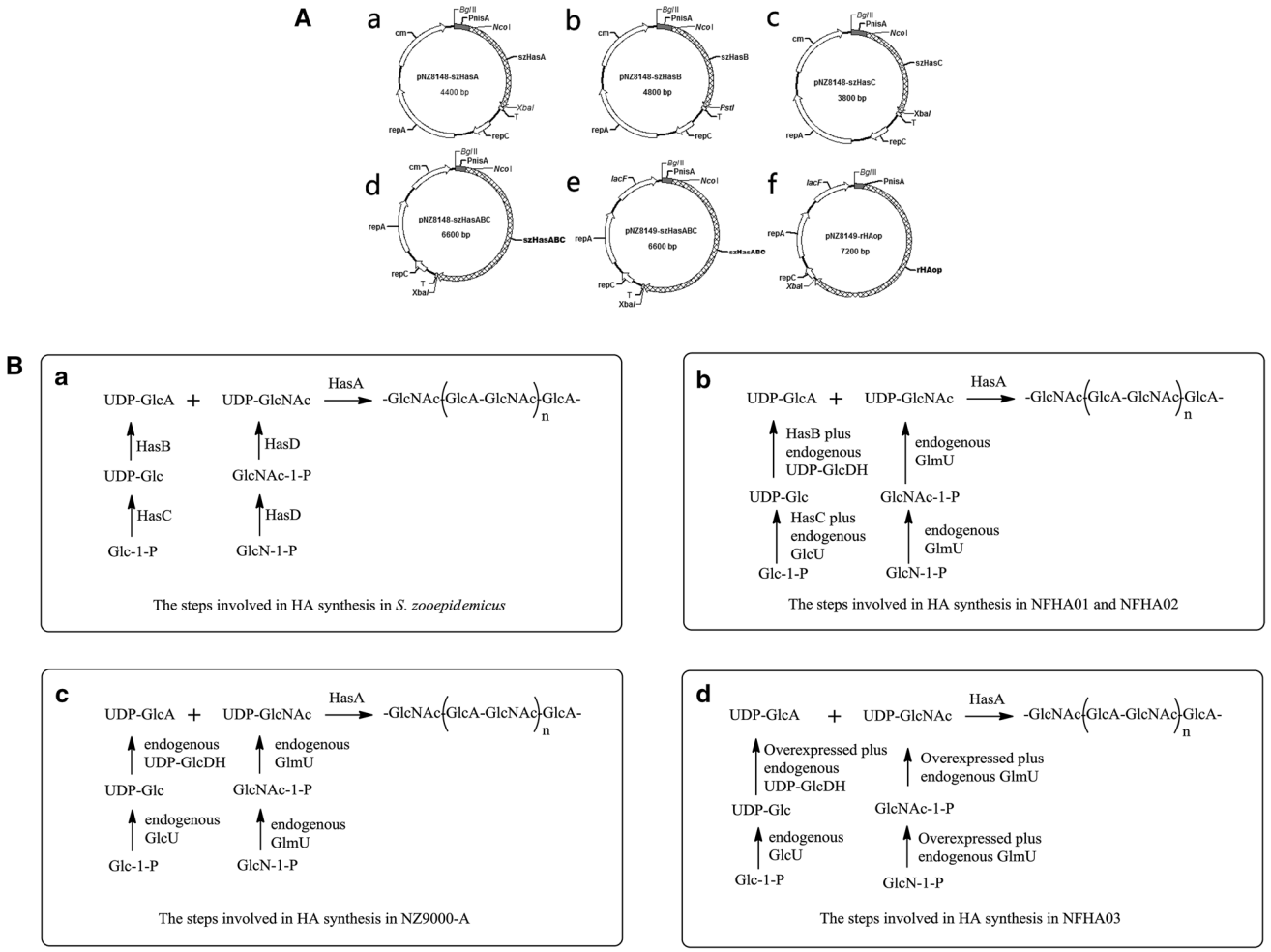

Fig. 2 Plasmid maps and the synthetic pathway of HA in *S. zooepidemicus* and recombinant *L. lactis*. A **a** pNZ8148-szHasA, containing the *szHasA* gene; **b** pNZ8148-szHasB, containing the *szHasB* gene; **c** pNZ8148-szHasC, containing the *szHasC* gene; **d** pNZ8148-szHasABC, containing the *szHasA* gene together with *szHasB* and *szHasC*; **e** pNZ8149-szHasABC, containing the *szHasA* gene together with *szHasB* and *szHasC*; **f** pNZ8149-rHAop, containing the recombinant operon fragment with *szHasA, ugd* and *glmU*. All the target genes are under the control of the nisA promoter. B HasA catalyzed the polymerization of GlcA and GlcNAc to produce HA in the wild-type *S. zooepidemicus* strain and in the recombinant strains. **a** In *S. zooepidemicus,* UDP-GlcA was synthesized by HasB and HasC, UDP-GlcNAc was synthesized by HasD. **b** In NFHA01 and NFHA02, endogenous GlmU catalyzed the synthesis of UDP-GlcNAc, while overexpressed HasB and HasC, as well as endogenous UDP-GlcDH and GlcU catalyzed the synthesis of UDP-GlcA. **c** In NZ9000-A, UDP-GlcNAc and UDP-GlcA were synthesized by endogenous enzymes UDP-GlcDH, GlcU and GlmU from *L. lactis*. **d** Besides the endogenous enzymes, overexpressed UDP-GlcDH and GlmU also were involved in the supply of UDP-GlcNAc and UDP-GlcA in NFHA03

detected in the total cell extracts of NZ9000-A, NZ9000-B and NZ9000-C, respectively, and a large amount of new proteins (of approximately 29 kDa and 43–48 kDa) were produced by strain NFHA01. This finding confirmed that recombinant strain NFHA01 successfully expressed HasA together with HasB and HasC.

Disaccharide analysis, electrospray ionization mass spectrometry and RIA were used to confirm that the polysaccharide produced by the recombinant *L. lactis* strains was HA. The polysaccharide was purified from the culture and degraded by hyaluronate lyase as previously described [12, 26]. It was then desalted on a Bio-Gel P-2 column and analyzed on a C18 reversed-phase column (Fig. 3b, c). Meanwhile, commercial HA was degraded by the same lyase and also analyzed by P-2 and C18 columns (Fig. 3b, c). As expected, analysis of the degradation product of the NFHA03 polysaccharide showed a disaccharide component with the same retention time as HA disaccharide. Then, using electrospray ionization mass spectrometry techniques, the molecular mass of this disaccharide was determined to be 379.1 ± 0.4 Da, almost identical to the calculated molecular mass for HA disaccharide (379.1 Da) (Fig. 3d), confirming that the polysaccharide recovered from the culture of NFHA03 was HA. In addition, the RIA

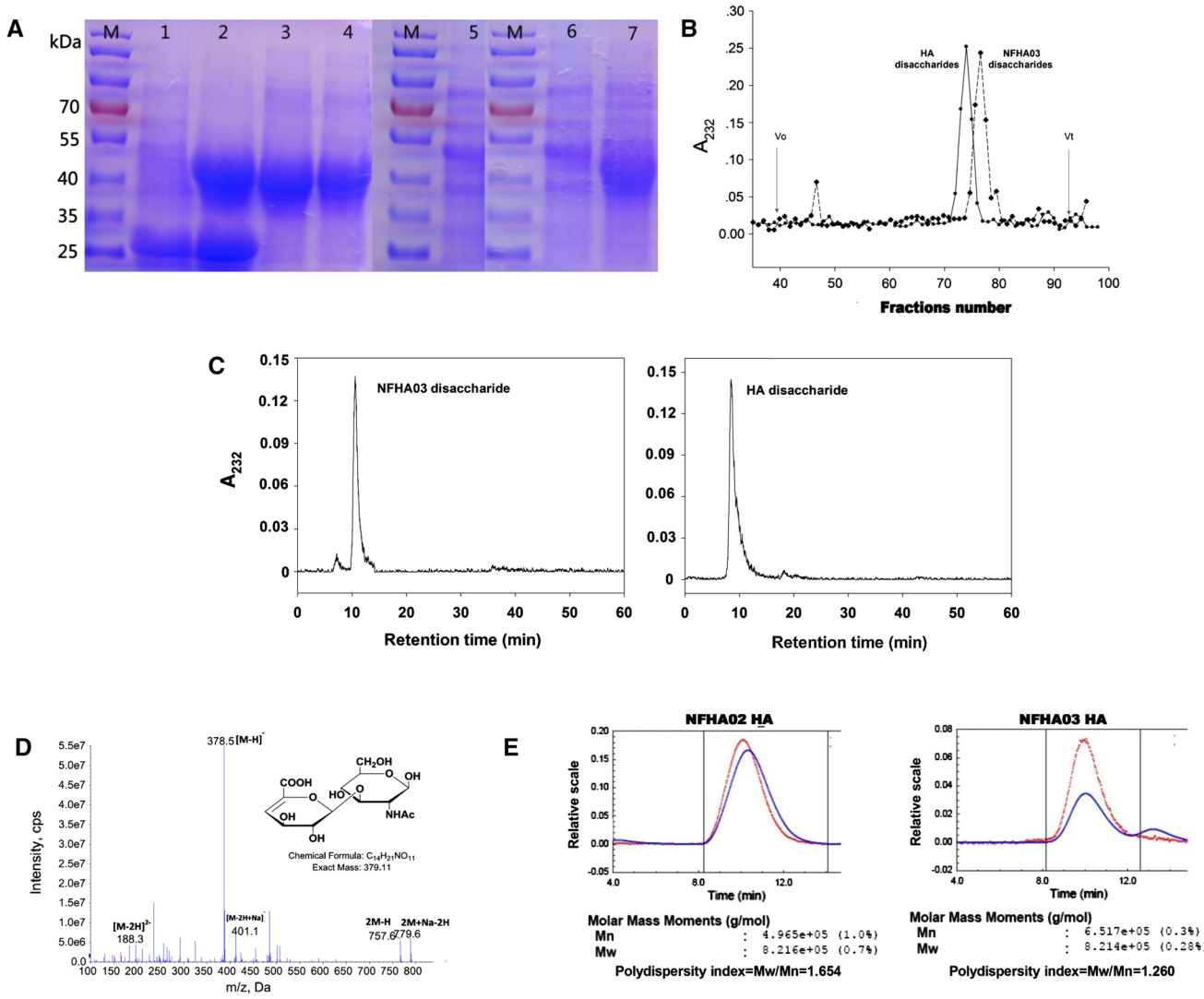

Fig. 3 Analysis of HA produced in the recombinant strains. **a** SDS-PAGE of the protein samples from recombinant strains after induction with 10 ng ml^{-1} nisin for 12 h. Proteins were separated by SDS-PAGE (9 %) and visualized by Coomassie brilliant blue G-250 staining. *Lane M* = molecular mass standards; *lane 1* lysate from NZ9000-C cells in GM17 medium; *lane 2* lysate from NFHA01 cells in GM17 medium; *lane 3* lysate from NZ9000-B cells in GM17 medium; *lane 4* lysate from NZ9000-A cells in GM17 medium; *lane 5* lysate from NZ9000-pNZ8148 cells in GM17 medium; *lane 6* lysate from NZ3900-pNZ8149 cells in LM17 medium; *lane 7* lysate from NFHA02 cells in LM17 medium. **b** Disaccharide analysis of HAase-treated HA. HA recovered from NFHA03 and commercial HA were digested with hyaluronate lyase at 37 °C and the reaction was carried out overnight to ensure completeness. The reaction was heat deactivated, centrifuged, and the supernatants (100 µl) were loaded onto a P-2 column. **c** RPIP-HPLC chromatograms of the disaccharide analysis of HA disaccharides. The digested disaccharides of the polysaccharides produced by NFHA03 were purified on a Bio-Gel P-2 and resolved by RPIP-HPLC. The disaccharide of HA was also resolved on the same column. **d** The MS spectrum of disaccharides from NFHA03. **e**, the Mn and $\overline{M}_w$ of HA recovered from the cultures of NFHA02 and NFHA03 were measured using SEC–MALLS analysis

data (Table 3) also demonstrated that recombinant *L. lactis* strains biosynthesized HA successfully.

The concentrations of HA in the cultures of these recombinant strains were analyzed by RIA (Table 3). Approximately 0.22 g l^{-1} HA was detected in the NZ9000-A culture while HA could not be detected in the culture of NZ9000, indicating that HA could be synthesized with the expression of HasA alone in *L. lactis* and that the required precursors, UDP-GlcA and UDP-GlcNAc, could also be synthesized by endogenous UDP-GlcDH and GlmU in the host strain. However, no HA was detected in the cultures of NZ9000-B and NZ9000-C, showing that HA could not be synthesized in the absence of the HasA enzyme. Furthermore, the final concentration of HA was approximately 0.6 g l^{-1} in the NFHA01 culture, around 170 % higher than that of NZ9000-A (0.22 g l^{-1}), showing that co-expression

Table 3 HA produced by recombinant strains of different genotypes

Carbon source (wt vol^{-1})	1 % glucose					1 % lactose		
Strain	NZ9000	NZ9000-A	NZ9000-B	NZ9000-C	NFHA01	NZ3900	NFHA02	NFHA03
Genotype	–	HasA	HasB	HasC	HasA HasB HasC	–	HasA HasB HasC	HasA ugd GlmU
HA yield (g L^{-1})	–	0.223 ± 0.041[a]	–	–	0.594 ± 0.029	–	0.380 ± 0.030	0.492 ± 0.032

The values are the average of five independent measurements. For ratio determination, cells were grown in GM17 medium or LM17 medium and induced for another 12 h at 30 °C, then 10 ng ml^{-1} nisin was added. The concentration of HA was measured using an RIA kit

[a] Deviation from the average

of the enzymes, HasA, HasB and HasC, is an effective way to increase HA yield. As expected, an undersupply of UDP-GlcA and UDP-GlcNAc decreased the yield. From these results, we can conclude that HasA is essential for HA production in *L. lactis* and improving the precursor sugar biosynthesis levels can increase the production of HA, confirming previous findings [4].

It was reported that the expression of *HasA* from *Streptococcus equisimilis* along with expression of *tuaD* (UDP-GlcDH) in *Bacillus subtilis* resulted in the production of HA [30]. Several studies also showed that expressing endogenous UDP-GlcDH, which directs a step in the synthesis pathway of UDP-GlcA, could improve HA production [4, 17, 26]. We hypothesized that the endogenous enzymes involved in the synthesis of the UDP sugars should work more efficiently than the heterologous enzymes from *S. zooepidemicus* in *L. lactis*. To confirm this, recombinant strains NFHA02 and NFHA03 were constructed and incubated in LM17 medium for 12 h at 30 °C with 10 ng ml^{-1} nisin. SDS-PAGE image analysis showed that the recombinant strain NFHA03 successfully expressed szHasA and the expression levels of Ugd (M_w: 43.4 kDa) and GlmU (M_w: 49.0 kDa) were clearly improved (Fig. 3a). The concentrations of HA in the cultures of NFHA02 and NFHA03 were also analyzed and compared (Table 3). The final concentration of HA was approximately 0.49 g l^{-1} in the NFHA02 culture, about 33 % higher than that in the NFHA03 culture (~0.38 g l^{-1}). As expected, the overexpression of endogenous Ugd and GlmU, which share the same catalysis pathway as HasB and HasD, together with HasA, improved the production of HA in recombinant *L. lactis*. Therefore, it can be concluded that overexpression of the endogenous enzymes directing the steps in the synthesis pathway of the precursor sugars is an effective way to increase HA production in recombinant cells. In addition, NFHA01 was cultured in the medium containing 1 % glucose, while 1 % lactose was the carbon source in the medium of NHFA02 and NHFA03. Glucose was better as carbon source than lactose, which should be the reason why the HA yield of NFHA01 was higher than those of NFHA02 and NFHA03.

Besides the yield, the M_w and $\overline{M}_w$ distribution were also important characteristics of HA. It is necessary to obtain specially designated $\overline{M}_w$ or uniform size-defined HA to extend the potential applications for this polysaccharide, in particular for producing HA-containing biomedical products. To determine the importance of the precursors, UDP-GlcA and UDP-GlcNAc, in the M_w and length distribution of the polysaccharide chains, M_n, $\overline{M}_w$ and the polydispersity index (PDI) of HA produced by NFHA02 and NFHA03 were measured by size-exclusion chromatography–multi-angle laser light scattering (SEC–MALLS) analysis (Fig. 3e). Although the $\overline{M}_w$ of HA recovered from these two recombinant strains were almost equal, the PDI of the $\overline{M}_w$ was 1.260 for the NFHA03 culture, approximately 24 % lower than that of NFHA02 (~1.654), suggesting that the HA synthesized by NFHA03 has narrow size distributions. Compared with NFHA02, the supply of UDP-GlcA and UDP-GlcNAc benefited from the overexpression of endogenous Ugd and GlmU in NFHA03, in which the availability of processor sugars to HasA molecules would be expected to be relatively abundant. This would mean that all HA polysaccharide chains would have a sufficient supply of precursor sugars for further synthesis and chain elongation catalyzed by HasA. This was confirmed by the fact that the HA polysaccharides recovered from the culture of NFHA03 had a lower PDI. As expected, increasing the supply of the UDP-activated monosaccharide donors was beneficial for the synthesis of monodisperse HA polysaccharides, which was consistent with previous data [26] and also put forward a guide to establish an efficacious way to control the size of HA in fermentation.

In addition, the host strain (*L. lactis*), the NICE system, the selected marker (*lacF* gene) and the fermentation medium of the recombinant strain NFHA03 all conformed to the required standards for the production of food-grade HA. HA produced by this *L. lactis* strain is, therefore, more suitable and applicable than HA produced by *Streptococcus*

for widespread use in the production of health foods, medicines and cosmetics.

Recently, HA is produced using the Gram-positive bacterium *S. zooepidemicus* in industry. However, *Streptococcus* is a less-than-ideal source because of its potential to produce exotoxins, the difficultly in fermentation control and the expensive medium required for growth [30]. *L. lactis*, one of GRAS microbes, is increasingly used in food fermentations and modern biotechnology [5, 6, 18, 22]. In 2007, CK Lee's group co-expressed heterologous UDP-GlcDH with HA synthase of Streptococcus in *L. lactis*, and the recombinant strain could produce HA with a concentration about 0.65 g l^{-1} [4]. 3 years later, KB Ramachandran's group reported that the recombinant *L. lactis* strains containing the full operon has produced 1.8 g l^{-1} HA in batch bioreactor with controlled pH and aeration [23]. In these papers, the recombinant strains were selected using chloramphenicol (10 μg ml^{-1}) or erythromycin (1 μg ml^{-1}) as the selection pressure. Here, besides GRAS host strain (*L. lactis*), NICE system and the fermentation medium, the selecting marker (*lacF* gene) of the recombinant strain is also food grade, which made NFHA03 to be a better source of HA appropriate for widespread use in the production of health foods, medicines and cosmetics.

Acknowledgments This work was supported by the National Natural Science Foundation of China (Project no. 31300673), the Specialized Research Fund for the Doctoral Program of Higher Education (Project no. 20130131120046) and the Independent Innovation Foundation of Shandong University (Project no. 2013TB009). We thank Shandong Freda Biochem Co. Ltd. for their excellent technical assistance. Furthermore, we sincerely thank NIZO Food Research for providing plasmids and bacterial strains.

Conflict of interest The authors declare no conflict of interest.

Ethical standards The authors declare that the experiments comply with the current laws of China.

Author contributions JS, LP and FW designed and coordinated the work. JS carried out the experiments. JS and FW wrote the manuscript. All authors have read and approved the final manuscript.

References

1. Blank LM, Hugenholtz P, Nielsen LK (2008) Evolution of the hyaluronic acid synthesis (has) operon in *Streptococcus zooepidemicus* and other pathogenic streptococci. J Mol Evol 67(1): 13–22
2. Boels IC, Kleerebezem M, de Vos WM (2003) Engineering of carbon distribution between glycolysis and sugar nucleotide biosynthesis in *Lactococcus lactis*. Appl Environ Microbiol 69(2):1129–1135
3. Casadaban MJ, Cohen SN (1980) Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J Mol Biol 138(2):179–207
4. Chien LJ, Lee CK (2007) Hyaluronic acid production by recombinant *Lactococcus lactis*. Appl Microbiol Biotechnol 77(2):339–346
5. de Ruyter PG, Kuipers OP, de Vos WM (1996) Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl Environ Microbiol 62(10):3662–3667
6. de Vos WM (1999) Gene expression systems for lactic acid bacteria. Curr Opin Microbiol 2(3):289–295
7. de Vos WM, Boerrigter I, van Rooyen RJ, Reiche B, Hengstenberg W (1990) Characterization of the lactose-specific enzymes of the phosphotransferase system in *Lactococcus lactis*. J Biol Chem 265(36):22554–22560
8. DeAngelis PL (1999) Hyaluronan synthases: fascinating glycosyltransferases from vertebrates, bacterial pathogens, and algal viruses. Cell Mol Life Sci 56(7–8):670–682
9. Dower WJ, Miller JF, Ragsdale CW (1988) High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res 16(13):6127–6145
10. Duncan MB, Liu M, Fox C, Liu J (2006) Characterization of the *N*-deacetylase domain from the heparan sulfate *N*-deacetylase/*N*-sulfotransferase 2. Biochem Biophys Res Commun 339(4):1232–1237. doi:10.1016/j.bbrc.2005.11.142
11. Eichenbaum Z, Federle MJ, Marra D, de Vos WM, Kuipers OP, Kleerebezem M, Scott JR (1998) Use of the lactococcal nisA promoter to regulate gene expression in gram-positive bacteria: comparison of induction level and promoter strength. Appl Environ Microbiol 64(8):2763–2769
12. Guo X, Shi Y, Sheng J, Wang F (2014) A novel hyaluronidase produced by *Bacillus* sp. A50. PLoS One 9(4):e94156. doi:10.1371/journal.pone.0094156
13. Huang SL, Ling PX, Zhang TM (2007) Oral absorption of hyaluronic acid and phospholipids complexes in rats. World J Gastroenterol 13(6):945–949
14. Jing W, DeAngelis PL (2004) Synchronized chemoenzymatic synthesis of monodisperse hyaluronan polymers. J Biol Chem 279(40):42345–42349
15. Kleerebezem M, Beerthuyzen MM, Vaughan EE, de Vos WM, Kuipers OP (1997) Controlled gene expression systems for lactic acid bacteria: transferable nisin-inducible expression cassettes for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp. Appl Environ Microbiol 63(11):4581–4584
16. Kuipers OP, de Ruyter PG, Kleerebezem M, de Vos WM (1997) Controlled overproduction of proteins by lactic acid bacteria. Trends Biotechnol 15(4):135–140
17. Liu L, Du G, Chen J, Wang M, Sun J (2008) Enhanced hyaluronic acid production by a two-stage culture strategy based on the modeling of batch and fed-batch cultivation of *Streptococcus zooepidemicus*. Bioresour Technol 99(17):8532–8536
18. Maguin E, Prevost H, Ehrlich SD, Gruss A (1996) Efficient insertional mutagenesis in lactococci and other Gram-positive bacteria. J Bacteriol 178(3):931–935
19. Markovitz A, Cifonelli JA, Dorfman A (1959) The biosynthesis of hyaluronic acid by group A *Streptococcus*. VI. Biosynthesis from uridine nucleotides in cell-free extracts. J Biol Chem 234:2343–2350
20. Markovitz A, Dorfman A (1962) Synthesis of capsular polysaccharide (hyaluronic acid) by protoplastmembrane preparations of group A *Streptococcus*. J Biol Chem 237:273–279
21. Mierau I, Kleerebezem M (2005) 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. Appl Microbiol Biotechnol 68(6):705–717
22. Mierau I, Leij P, van Swam I, Blommestein B, Floris E, Mond J, Smid EJ (2005) Industrial-scale production and purification of a heterologous protein in *Lactococcus lactis* using the nisin-controlled gene expression system NICE: the case of lysostaphin. Microb Cell Fact 4:15. doi:10.1186/1475-2859-4-15

23. Prasad SB, Jayaraman G, Ramachandran KB (2010) Hyaluronic acid production is enhanced by the additional co-expression of UDP-glucose pyrophosphorylase in *Lactococcus lactis*. Appl Microbiol Biotechnol 86(1):273–283. doi:10.1007/s00253-009-2293-0
24. Sambrook JFFF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
25. Sheng J, Xu Y, Dulaney SB, Huang X, Liu J (2012) Uncovering biphasic catalytic mode of C5-epimerase in heparan sulfate biosynthesis. J Biol Chem 287(25):20996–21002. doi:10.1074/jbc.M112.359885
26. Sheng JZ, Ling PX, Zhu XQ, Guo XP, Zhang TM, He YL, Wang FS (2009) Use of induction promoters to regulate hyaluronan synthase and UDP-glucose-6-dehydrogenase of *Streptococcus zooepidemicus* expression in *Lactococcus lactis*: a case study of the regulation mechanism of hyaluronic acid polymer. J Appl Microbiol 107(1):136–144. doi:10.1111/j.1365-2672.2009.04185.x
27. Tuomikoski P, Aittomaki K, Mikkola TS, Ropponen A, Ylikorkala O (2008) Effect of oral and transdermal hormone therapy on hyaluronic acid in women with and without a history of intrahepatic cholestasis of pregnancy. Am J Obstet Gynecol 198(4):375 e371–375
28. Weigel PH, Hascall VC, Tammi M (1997) Hyaluronan synthases. J Biol Chem 272(22):13997–14000
29. Wells JM, Wilson PW, Le Page RW (1993) Improved cloning vectors and transformation procedure for *Lactococcus lactis*. J Appl Bacteriol 74(6):629–636
30. Widner B, Behr R, Von Dollen S, Tang M, Heu T, Sloma A, Sternberg D, Deangelis PL, Weigel PH, Brown S (2005) Hyaluronic acid production in *Bacillus subtilis*. Appl Environ Microbiol 71(7):3747–3752
31. Yamada T, Kawasaki T (2005) Microbial synthesis of hyaluronan and chitin: new approaches. J Biosci Bioeng 99(6):521–528
32. Zhang ZZ, Chen XZ, Jia SF, Chen ML, Huan LD (2002) Food-grade gene expression systems for lactic acid bacteria. Sheng Wu Gong Cheng Xue Bao 18(4):516–520