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REVIEWS
AND THEORETICAL ARTICLES

Construction of Recombinant *Bacillus subtilis* Strains Producing Hyaluronic Acid

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Abstract—Hyaluronic acid finds expanding application in the pharmaceutical and cosmetic industries, resulting in an increasing need for the high-quality substance. The main production processes to obtain hyaluronic acid in commercial quantities are extraction from animal tissues and bacterial fermentation using opportunistic *Streptococcus* strains. The production by recombinant bacteria that are safe for humans seems to be an efficient and economically viable way to obtain hyaluronic acid. The recombinant producer strains constructed on the basis of the *Bacillus subtilis* platform make it possible to obtain the yield and quality of the product comparable to those of commercially developed *Streptococcus* strains. By varying genetic, biochemical, and biotechnological factors, it becomes possible to obtain products with different target molecular weights. Despite the results achieved, the potential of the *B. subtilis* platform for the construction of recombinant hyaluronic acid producer strains has not been exhausted.

Keywords: hyaluronic acid, *Bacillus subtilis*, recombinant producer strains
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INTRODUCTION

Hyaluronic acid (HA) is a high molecular weight linear non-sulfated glycosaminoglycan, consisting of repeating disaccharide units connected by β-1,4-glycosidic bonds. The disaccharide unit consists of D-glucuronic acid and *N*-acetyl-D-glucosamine fragments connected by a β-1,3-glycosidic bond [1, 2]. The presence of numerous sulfated groups in relative glycosaminoglycans is the reason for the existence of numerous isomers, which is not observed in hyaluronic acid, which is always chemically identical, regardless of the methods and sources of production. In aqueous solution, HA is stabilized into a secondary structure in the form of a single-strand left-handed helix. Helix duplexes form a tertiary structure in the form of an extensive network, the properties of which depend on the molecular weight (MW) and HA concentration [3]. Structural characteristics and polyelectrolyte nature determine the unique rheological properties of HA solutions [4, 5].

In the human body, HA is one of the main components of extracellular matrix. Considerable amounts of HA were found in the dermis and epidermis of the skin, synovial fluid, hyaline cartilage, and vitreous humor of the eye [6]. HA functions both as a structural and signaling molecule. Molecular weight is a key factor determining the biological functions of HA [7]. High molecular weight HA ($\geq 10^6$ Da) serves as lubricating factor in the synovial fluid, maintains water and electrolyte balance and the extracellular matrix structure [8], has an anti-angiogenic effect, and participates in the processes of inflammation and tissue injury and repair through interaction with fibrinogen and control of the immune cell activation, regulation of cytokines, and stem cell migration [9, 10]. In a number of pathological conditions, such as asthma, pulmonary fibrosis, and rheumatoid arthritis, low molecular weight HA (10^4–10^6 Da) is formed, which demonstrates proinflammatory and proangiogenic activity. Low molecular weight HA stimulates the production of proinflammatory cytokines [7] and also provokes the extracellular matrix rearrangement [11]. The HA fragments and oligosaccharides ($\leq 10^4$ Da), depending on the tissue type and physiological state, demonstrate both proinflammatory [12] and anti-inflammatory effects [13].

Hyaluronic acid of different MW can be used in the construction of delivery vehicles for therapeutic agents, in the treatment of cancer and diseases of the eyes, joints, lungs, upper respiratory tract, and urinary system, and in aesthetic medicine [2]. The HA-based medicines and products, including synovial fluid prostheses, agents for the treatment of injuries and skin burns, viscoelastic substances for cataract surgery

and eye drops, agents for the treatment of rhinitis, and dermal fillers are widely used in everyday practice [14]. The pronounced aesthetic and supportive effect warrants widespread use of HA in cosmetics and dietary supplements.

The review analyzes the data obtained during the development of recombinant HA producer strains based on the *Bacillus subtilis* platform.

METHODS FOR COMMERCIAL PRODUCTION OF HA

In commercial quantities, HA is obtained in two ways: by extraction from animal tissues and by fermentation using natural microbial producer strains. The method for obtaining HA from animal tissues is a proven technology that makes it possible to obtain a product with high MW and reasonable costs. Raw materials for large-scale production are rooster combs and bovine vitreous humor [15]. The disadvantage of this method is the low yield of the product with high variability in MW, which is associated with low concentration of the polymer in the tissue, uncontrolled degradation by endogenous hyaluronidases, and harsh extraction conditions. The product obtained by this method may contain infectious agents, i.e., viruses or prions, as well as trace amounts of proteins and nucleic acids that can cause allergic reactions [16, 17].

Fermentation of natural producer strains is the main commercial process for obtaining HA [18]. The main producers are selective strains of *Streptococcus equi* subsp. *zooepidemicus* and *S. equi* subsp. *equi*, which under optimal conditions can produce 6–7 g/L HA with the MW of 2.0–3.5 MDa [19]. The main disadvantage of this method is the use of strains constructed on the basis of streptococci pathogenic for farm animals and conditionally pathogenic for humans. The target product must go through many stages of purification to avoid contamination with endo- and exotoxins, which negatively affects the economic characteristics.

Identification of HA biosynthesis genes made it possible to carry out work on the construction of recombinant producers on various platforms devoid of disadvantages of using streptococci with the achievement of the same productivity and MW. HA producer strains have been constructed both on the basis of commercial platforms (*Escherichia coli*) and on the basis of platforms with GRAS status (*Corynebacterium glutamicum*, *Lactobacillus acidophilus*, *Lactococcus lactis*, *B. subtilis*) [18, 20]. A promising method is chemoenzymatic biosynthesis of HA, which makes it possible to obtain high purity monodisperse fractions [21]. However, despite the results achieved, there are currently no products on the market based on the HA substance obtained by fermentation of recombinant strains other than streptococci or by the chemoenzymatic method. Therefore, production of high-quality HA with high yield and low cost is an urgent problem in the field of molecular genetics and biotechnology.

HYALURONIC ACID BIOSYNTHETIC PATHWAY

Natural producers of HA are strains of gram-positive bacteria *Streptococcus pyogenes*, *S. uberis*, *S. equi* subsp. *zooepidemicus*, *S. equi* subsp. *equi*, *S. iniae*, *S. equisimilis*, and *Bacillus cereus* strain G9241 and gram-negative bacteria *Pasteurella multocida* [19, 22–25]. All natural producers of HA are pathogenic and opportunistic microorganisms that cause diseases in animals and humans. HA forms the basis of the cell capsule and acts as the virulence factor, making it possible for microorganisms to avoid recognition and counteraction of the immune system, and also promotes colonization of mucous surfaces [26]. Despite obvious benefits for enhancing virulence, only a few bacterial species have acquired the ability to synthesize capsular HA.

Genes involved in the HA biosynthesis are part of an operon in which the key gene is that for the hyaluronan synthase (EC 2.4.1.212), an enzyme that synthesizes HA from activated forms of the UDP-glucuronate and UDP-*N*-acetylglucosamine monomers. There are two classes of bacterial hyaluronan synthases that differ in molecular structure and amino acid sequence [27]. The most common is Class 1, which is responsible for HA biosynthesis in streptococci and vertebrates, and is a membrane enzyme [28]. Class 2 is represented only by the hyaluronan synthase encoded by the operon of *P. multocida* and which is a membrane-associated enzyme [25]. The streptococcal operon of HA biosynthesis also includes two to four genes participating in the biosynthesis of activated monomer precursors, and in the case of *P. multocida*, it also includes genes responsible for the translocation of growing HA chain to the cell exterior.

The pathway of HA biosynthesis was studied in detail in streptococci [29, 30]. UDP-glucuronate and UDP-*N*-acetylglucosamine are derivatives of glucose-6-phosphate and fructose-6-phosphate, respectively. The HA biosynthetic pathway is shown in Fig. 1. The first reaction in the biosynthesis of UDP-glucuronate is the reversible conversion of glucose-6-phosphate to glucose-1-phosphate by α-phosphoglucomutase (EC 5.4.2.2). Next, glucose-1-phosphate uridylyl transferase (EC 2.7.7.9) catalyzes the formation of UDP-glucose from UTP and glucose-1-phosphate. UDP-glucuronic acid is then formed in the reaction of oxidation of the primary alcohol group of UDP-glucose by UDP-glucose dehydrogenase (EC 1.1.1.22).

The first reaction of the UDP-*N*-acetylglucosamine biosynthesis is the amino group transfer from glutamine to fructose-6-phosphate by amidotransferase (EC 2.6.1.16) to form glucosamine-6-phosphate. The phosphate groups are then rearranged by mutase

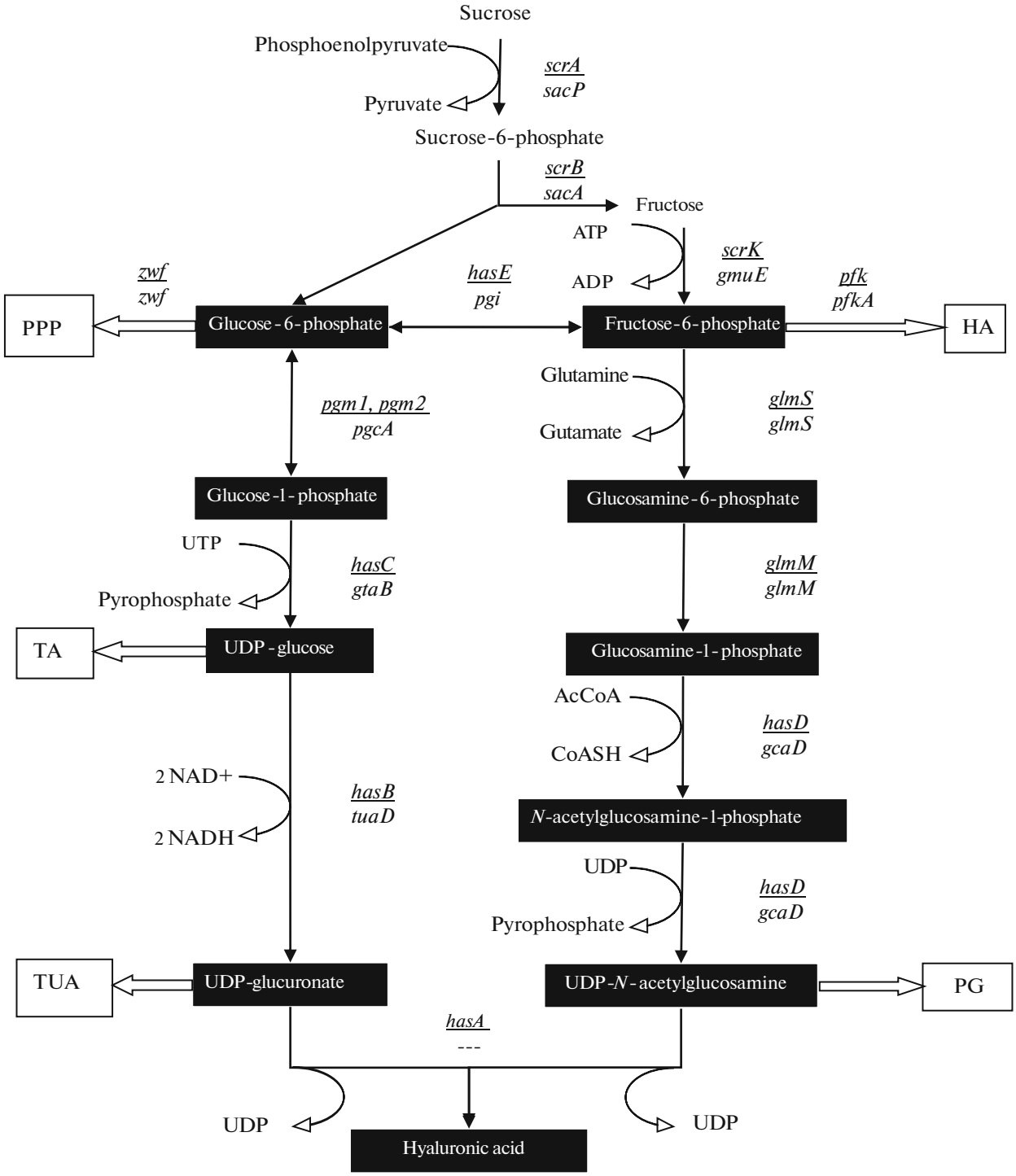

Fig. 1. Hyaluronic acid biosynthesis pathway and associated biochemical pathways. PPP, pentose phosphate pathway; TA, teichoic acids; TUA, teichuronic acids; GL, glycolysis; PG, peptidoglycan. Homologous genes are demonstrated: underlined superscripts, from the *Streptococcus* genome; subscripts, from the *B. subtilis* genome.

(EC 5.4.2.10) to form glucosamine-1-phosphate. Next, the acetyl group transfer by acetyltransferase (EC 2.3.1.157) occurs with the formation of *N*-acetylglucosamine-1-phosphate, and pyrophosphorylase (EC 2.7.7.23) in the reaction with UTP synthesizes UDP-*N*-acetylglucosamine.

UDP-*N*-acetylglucosamine, UDP-glucose, and glucose-1-phosphate are involved in the biosynthesis of peptidoglycan and other cell wall components, which provokes obvious competition for the HA biosynthetic pathway. To meet the metabolic needs of the cell in nucleotide sugars, the streptococcal genome

contains additional genes encoding glucose-1-phosphate uridylyltranspherase (*hasC2*), α-phosphoglucomutase (*pgm1*, *pgm2*), and acetyltransferase/pyrophosphorylase (*gcaD*). At the same time, the UDP-glucose dehydrogenase encoding gene (*hasB*) is represented by a single copy in the HA biosynthesis operon. Thus, streptococcal metabolism is able to support the synthesis of a large amount of HA in the extracellular capsule.

The pathways of UDP-*N*-acetylglucosamine and UDP-glucose biosynthesis in streptococci and *B. subtilis* are biochemically identical. The *B. subtilis* genome contains homologs of all genes for the biosynthesis of HA precursors. This makes it possible for the *B. subtilis* genes to be used to construct efficient artificial operons, since it is known that native genes are better expressed than homologous alien ones.

RECOMBINANT *B. SUBTILIS* STRAINS PRODUCING HYALURONIC ACID

The recombinant strains of *B. subtilis* producing hyaluronic acid obtained to date are represented in Tables 1 and 2.

The first study on heterologous production of HA in *B. subtilis* confirmed the high potential of the platform [31, 32]. The ability of recombinant strains to secrete HA into the cell exterior and accumulate the product in the culture medium was demonstrated. The characteristics of the obtained HA corresponded to those synthesized by natural microbial producers developed on the basis of *Streptococcus* strains. Heterologous expression of only *seHas* hyaluronan synthase, in contrast to *E. coli* strains, had no negative effect on the growth rate of *B. subtilis* and did not lead to HA biosynthesis without additional expression of UDP-glucuronate biosynthesis genes [33]. The combination of two precursor biosynthesis genes in the operon made it possible to identify the biosynthesis-limiting stage, which was the synthesis of UDP-glucuronate. The operon composed of *seHasA* and its own UDP-glucose dehydrogenase *tuaD* gene is sufficient for efficient HA production. Supplementation of the operon with the genes involved in the biosynthesis of UDP-*N*-acetylglucosamine (*gcaD*) and UDP-glucuronate (*gtaB*) increases the yield of HA by 10–20%. Placement of the *seHasA* and *tuaD-gtaB* genes into different operons of the *B. subtilis* chromosome does not lead to noticeable changes in the yield or MW of the product compared to their location in a single *seHasA–tuaD-gtaB* operon. The artificial operon composed of its own precursor synthesis genes in *B. subtilis* showed higher yield efficiency than that developed on the basis of HA biosynthesis genes from the operon of natural producer *S. equisimilis*. An unexpected effect was the deletion of the *cat* chloramphenicol resistance gene and that of the cytochrome C450 family oxidase, involved in the synthesis of red pigment (*cypX*), which was expressed as the increase in the yield of high molecular weight HA.

The HA biosynthesis by both streptococcal strains and recombinant *B. subtilis* producers is an energy-consuming process. The energy metabolism of a bacterial cell can be intensified by increasing oxygen availability with the help of bacterial hemoglobin [34]. In the recombinant *B. subtilis* strain producing HA, heterologous VHb expression had a positive effect on culture properties of the strain and the product yield [35]. The strain with *vhb* demonstrated a specific prolonged lag period and increased growth rate and reached 25% higher final cell density (7.5 versus 6.2 OD). The HA yield doubled from 0.9 to 1.8 g/L. In addition, experimental results supporting the effect of expression of the characteristic precursor biosynthesis genes on the product yield were obtained. Strains with operons consisting of the hyaluronan synthase gene in combination with the UDP-glucose dehydrogenase genes of different origin showed differences in productivity. The UDP-glucose dehydrogenase activity was 3 times higher in the strain with its own *tuaD* gene than in the strain with heterologous *hasB*. The difference in activity led to increase in the HA yield by 36%.

The use of an inducible promoter for the expression of the HA biosynthetic operon made it possible to obtain HA with different MW [36]. The fermentation conditions and cultivation time were critical for the MW and HA yield. For example, fermentation for 80–160 h resulted in a product with MW of 0.1–0.5 MDa; fermentation for 40–80 h resulted in a product of 0.5–1 MDa, and fermentation for 12–40 h resulted in a product of 1.0–2.0 MDa. However, effective and cell-safe operon expression required the transformant screening on the IPTG gradient and experimental determination of the optimal inductor concentration.

Two-step controlled expression with different inductors also made it possible to vary the MW of HA [37]. Expression of the integrated PmHAS hyaluronan synthase is controlled by the inducible Pxyl promoter, while the plasmid operon is controlled by the inducible Pspac promoter. In the case of simultaneous induction of the cassette and operon at the second hour from the beginning of the TPG223 strain cultivation, the HA production reaches 6.8 g/L at MW of 3.38–4.55 MDa. Induction of the PmHAS cassette at the eighth hour and of the *tuaD-gtaB* operon at the second hour demonstrates a decrease in the HA production to 3.1 g/L with a considerable decrease in MW, to 0.006–0.008 MDa.

In a systematic study, the effect of overexpression of genes involved in the biosynthesis of UDP-glucuronic acid and UDP-*N*-acetylglucosamine precursors on the HA yield and MW was examined [38]. The best performance with the yield of 2.7 g/L and MW of 1.61 MDa was demonstrated by the strain E168A/pP43-DU-PBMS, the operons of which contained the genes participating in the complete biosyn-

Table 1. Recombinant *B. subtilis* strains producing hyaluronic acid (flask culture, 37°C)

Strain	Genotype	Source of the hyaluronan synthase gene	Promoter of operon	Yield, g/L	MW, MDa	OD_{600}	Reference
RB-AB	1A751 *amyE::*(P_{VegII}-*seHasA-seHasB*)	*S. equi* subsp. *zooepidemicus*	Constitutive P_{VegII}	0.84	No data	6.5	[35]
RB-AD	1A751 *amyE::*(P_{VegII}-*seHasA-TuaD*)			1.14	No data	6.0	
RB-ADV	1A751 *amyE::*(P_{VegII}-*seHasA-TuaD*, P_{VegII}-*vhb*)			1.8	No data	7.5	
TPG223	168 *lacA::*(P_{xyl} -*PmHAS*), [*pHCMC05::*(P_{spac}-*tuaD-gtaB*)]	*Pasteurella multocida*	Inducible P_{xyl}, inducible P_{spac}	6.8	4.55	No data	[37]
PG6181	168 *lacA::*(P_{xyl} -*PmHAS*), [*pHCMC05::*(P_{spac} -*gcaD*)]			1.68	0.013	No data	
PP6502	168 *lacA::*(P_{xyl} -*PmHAS*), [*pHCMC05::*(P_{spac} -*pgi*)]			3	4.54	No data	
E168A	168 *lacA::*(P_{xyl} -*hasA*)	*S. zooepidemicus*	Inducible P_{xyl}	1	1.51	No data	[38]
E168A/pP43-D	168 *lacA::*(P_{xyl} -*hasA*), [*pP43NMK::*(P_{43}-*tuaD*)]		Inducible P_{xyl}, constitutive P_{43}	2	1.76	No data	
E168A/pP43-DB	168 *lacA::*(P_{xyl} -*hasA*), [*pP43NMK::*(P_{43}-*tuaD*, *gtaB*)]			2.5	1.6	No data	
E168A/pP43-DBA	168 *lacA::*(P_{xyl} -*hasA*), [*pP43NMK::*(P_{43}-*tuaD*, *gtaB*, *pgcA*)]			2.1	1.56	No data	
E168A/pP43-U	168 *lacA::*(P_{xyl} -*hasA*), [*pP43NMK::*(P_{43}-*glmU*)]			2.2	1.68	No data	
E168A/pP43-UMS	168 *lacA::*(P_{xyl} -*hasA*), [*pP43NMK::*(P_{43}-*glmU*, *glmM*, *glmS*)]			2.6	1.83	No data	
E168A/pP43-UMSI	168 *lacA::*(P_{xyl} -*hasA*), [*pP43NMK::*(P_{43}-*glmU*, *glmM*, *glmS*, *pgi*), *KanR*]			2.5	1.82	No data	

Table 1. (Contd.)

Strain	Genotype	Source of the hyaluronan synthase gene	Promoter of operon	Yield, g/L	MW, MDa	OD_{600}	Reference
E168A/pP43-DU	168 *lacA::*(P_{xyl} *-hasA*), [*pP43NMK::*(P_{43}*-tuaD, glmU*)]	*S. zooepidemicus*		2.2	1.4	No data	[38]
E168A/pP43-DU-PBMS	168 *lacA::*(P_{xyl} *-hasA*), [*pP43NMK::*(P_{43}*-tuaD-gluM*, P_{veg}*-gtaB-glmM-glmS*)]			2.7	1.61	No data	
E168T	168 *lacA::*(P_{xyl} *-hasA*), $pfkA^{(ATG->TTG)}$			1.2	1.57	No data	
E168G	168 *lacA::*(P_{xyl} *-hasA*), $pfkA^{(ATG->GTG)}$			1.18	1.59	No data	
E168T/pP43-DU-PBMS	168 *lacA::*(P_{xyl} *-hasA*), $pfkA^{(ATG->TTG)}$; [*pP43NMK::*(P_{43}*-tuaD-gluM*, P_{veg}*-gtaB-glmM-glmS*)]			3.2	1.69	No data	
E168TH/pP43-DU-PBMS	168 *lacA::*(P_{xyl} *-hasA*), $pfkA^{(ATG->TTG)}$; *nagA::*($P_{lepA-sp-R0}$*-H6LHyal*), [*pP43NMK::*(P_{43}*-tuaD-gluM*, P_{veg}*-gtaB-glmM-glmS*)]		Inducible P_{xyl}, constitutive P_{lepA}, P_{43}, P_{veg}	4.35	0.0022	No data	
E168THR1/pP43-DU-PBMS	168 *lacA::*(P_{xyl} *-hasA*), $pfkA^{(ATG->TTG)}$; *nagA::*$P_{lepA-sp-R1}$*-H6LHyal*), [*pP43NMK::*(P_{43}*-tuaD-gluM*, P_{veg}*-gtaB-glmM-glmS*)]			2.9	0.0026	No data	
E168THR2/pP43-DU-PBMS	168 *lacA::*(P_{xyl} *-hasA*), $pfkA^{(ATG->TTG)}$); *nagA::*$P_{lepA-sp-R2}$*-H6LHyal*), [*pP43NMK::*(P_{43}*-tuaD-gluM*, P_{veg}*-gtaB-glmM-glmS*)]			3.3	0.003	No data	

Table 1. (Contd.)

Strain	Genotype	Source of the hyaluronan synthase gene	Promoter of operon	Yield, g/L	MW, MDa	OD_{600}	Reference
AW008	1A751 *amyE::*(P_{grac}-*seHas-tuaD*)	*S. equisimilis*	Inducible P_{grac}	0.48	1.7	4	[45]
AW009	1A751 *amyE::*($P_{grac.\ UPmod}$-*seHas-tuaD*)		Inducible $P_{grac.UPmod}$	0.97	1.5	6	
AW005-3	1A751 *amyE::*($P_{grac.\ UPmod}$-*seHas-tuaD*), *wprA::*($P_{xylA.SphI+1}$-*pfkA-gRNA.P41NT*)			1.6	1.2	5	
AW006-3	1A751 *amyE::*($P_{grac.UPmod}$-*seHas-tuaD*), *wprA::*($P_{xylA.SphI+1}$-*pfkA-gRNA.P41NT*(*10C-A*))			1.7	1.3	5.5	
AW014-3	1A751 *amyE::*($P_{grac.UPmod}$-*seHas-tuaD*), *wprA::*($P_{xylA.SphI+1}$-*zwf-gRNA.P92NT*(*15C-A*))			1.3	1.6	9	
AW016-3	1A751 *amyE::*($P_{grac.UPmod}$-*seHas-tuaD*), *wprA::*($P_{xylA.SphI+1}$-*zwf-gRNA.P603NT*(*10U-C*))			1.4	1.4	4	
AW018-3	1A751 *amyE::*($P_{grac.UPmod}$-*seHas-tuaD*), *wprA::*($P_{xylA.SphI+1}$-*zwf-gRNA.P603NT*(*10U-C*), $P_{xylA.SphI+1}$-*pfkA-gRNA.P41NT*)			2.2	1.5	7.5	
AW019-3	1A751 *amyE::*($P_{grac.UPmod}$-*seHas-tuaD*), *wprA::*($P_{xylA.SphI+1}$-*zwf-gRNA.P603NT*(*10U-C*), $P_{xylA.SphI+1}$-*pfkA-gRNA.P41NT*(*10C-A*))			2.3	1.7	6.5	

Table 1. (Contd.)

Strain	Genotype	Source of the hyaluronan synthase gene	Promoter of operon	Yield, g/L	MW, MDa	OD_{600}	Reference
AW008	1A751 *amyE::*(P_{grac}-*seHas-tuaD*)	*S. equisimilis*	Inducible P_{grac}	1.48	1.7	4.2	[48]
AW001-4	1A751 *amyE::*(P_{grac}-*seHas-tuaD*), *thrC::*(P_{grac}-*pgsA-clsA*)			0.63	2.0	7.7	
AW004-4	1A751 *amyE::*(P_{grac}-*seHas-tuaD*), *wprA::*($P_{xylA.SphI+1}$-*ftsZ-gRNA.P79NT*(*15C-U*))			0.79	1.5	7.8	
AW005-4	1A751 *amyE::*(P_{grac}-*seHas-tuaD*), *wprA::*($P_{xylA.SphI+1}$-*ftsZ-gRNA.P79NT*(*15C-U*))			1.46	1.6	7	
AW007-4	1A751 *amyE::*(P_{grac}-*seHas-tuaD*), *thrC::*($P_{grac.UPmod}$-*pgsA-clsA*)		Inducible P_{grac}, $P_{grac.UPmod}$	0.8	1.4	7.5	
AW009-4	1A751 *amyE::*(P_{grac}-*seHas-tuaD*), *thrC::*($P_{grac.UPmod}$-*pgsA-clsA*), *wprA::*($P_{xylA.SphI+1}$-*ftsZ-gRNA.P244NT*)			0.95	2.0	8	
AW011-4	1A751 *amyE::*(P_{grac}-*seHas-tuaD*), *thrC::*($P_{grac.UPmod}$-*pgsA-clsA*), *wprA::*($P_{xylA.SphI+1}$-*ftsZ-gRNA.P533NT*)			1.05	2.1	7.5	
WA	WB600 Δ*upp*, [*pHY300plk::hasA*]	*S. ubris**	No data	0.9	No data	4.3	[41]
WmA	WB600 Δ*upp*, Δ*sigF*, [*pHY300plk::hasA*]			1.2	No data	4.8	
WmB	WB600 Δ*upp*, Δ*sigF*, [*pHY300plk::hasA,tuaD,gtaB*]			3.2**	No data	No data	
1B	168 [*pHY300plk::hasA,tuaD,gtaB*]			1.7**	No data	No data	

* *S. ubris* is not described in the literature; perhaps, the authors or editors made a mistake with the spelling.
** Data obtained by cultivation at 32°C.

Table 2. Recombinant *B. subtilis* strains producing hyaluronic acid (bioreactor culture, 37°C)

Strain	Genotype	Source of the hyaluronan synthase gene	Promoter of operon	Yield, g/L	MW, MDa	OD_{600}	Reference
RB163	A164Δ5 *amyE::*(P_{scBAN}-*seHasA-TuaD-gcaD*)	*S. equisimilis*	Constitutive P_{amyQ} (P_{scBAN})	No data	1.5	No data	[31] [32]
RB161	A164Δ5 *amyE::*(P_{scBAN}-*seHasA-TuaD-gtaB*)			No data	1.6	No data	
TH-1	A164Δ5 *amyE::*(P_{scBAN}-*seHasA-seHasB-seHasC-seHasD*)			No data	1.5	No data	
RB187	A164Δ5 *amyE::*(P_{scBAN}-*seHasA-TuaD-gtaB*), Δ*cat*			No data	3.5	No data	
RB194	A164Δ5 *amyE::*(P_{scBAN}-*seHasA-TuaD-gtaB*), Δ*cat*, Δ*cypX*			No data	3.4	No data	
RB200	A164Δ5 *amyE::*(P_{scBAN}-*seHasA-TuaD*), Δ*cat*, Δ*cypX*			No data	4.5	No data	
1012/pBS5	1012 [*pHT01::*(P_{grac}-*hasA-tuaD*)]	*S. zooepidemicus*	Inducible P_{grac}	7.5	0.2–0.4	No data	[36]
1012/pBS5	1012 [*pHT01::*(P_{grac}-*hasA-tuaD*)]			3.3*	1.5–2*	No data	
WB800N /pBS5	WB800N [*pHT01::*(P_{grac}-*hasA-tuaD*)]			4.0	0.5–0.8	No data	
E168T/pP43-DU-PBMS	168 *lacA::*(P_{xyl}-*hasA*), *pfkA*$^{(ATG->TTG)}$; [*pP43NMK::*(P_{43}-*tuaD-gluM*, P_{veg}-*gtaB-glmM-glmS*)]	*S. zooepidemicus*	Inducible P_{xyl}, constitutive P_{lepA}, P_{43}, P_{veg}	6	1.42	21	[38]
E168TH/pP43-DU-PBMS	168 *lacA::*(P_{xyl}-*hasA*), *pfkA*$^{(ATG->TTG)}$; *nagA::*($P_{lepA-sp-R0}$-*H6LHyal*), [*pP43NMK::*(P_{43}-*tuaD-gluM*, P_{veg}-*gtaB-glmM-glmS*)]			19.38	0.0066	28	
E168THR1/pP43-DU-PBMS	168 *lacA::*(P_{xyl}-*hasA*), *pfkA*$^{(ATG->TTG)}$; *nagA::*$P_{lepA-sp-R1}$-*H6LHyal*), [*pP43NMK::*(P_{43}-*tuaD-gluM*, P_{veg}-*gtaB-glmM-glmS*)]			9.18	0.018	23	

Table 2. (Contd.)

Strain	Genotype	Source of the hyaluronan synthase gene	Promoter of operon	Yield, g/L	MW, MDa	OD_{600}	Reference
E168THR2/pP43-DU-PBMS	168 *lacA::*(P_{xyl} *-hasA*), *pfkA*$^{(ATG->TTG)}$); *nagA::*$P_{lepA-sp-R2}$*-H6LHyal), [pP43NMK::*(P_{43}*-tuaD-gluM,* P_{veg}*-gtaB-glmM-glmS*)]	*S. zooepidemicus*	Inducible P_{xyl}, constitutive P_{lepA}, P_{43}, P_{veg}	7.13	0.0496	24	[38]
AM2640	168 *amyE::*($P_{rpsf-gsib}$*-seHasA-tuaD*)	*S. equisimilis*	Constitutive tandem $P_{rpsf-gsib}$	1.8	No data	No data	[43]
AM2681	168 *amyE::*($P_{rpsf-gsib}$*-seHasA-tuaD*), $P_{rpsf-gsib}$*-gtaB*			2.21	No data	No data	
AM2686	168 *amyE::*($P_{rpsf-gsib}$*-seHasA-tuaD*), $P_{rpsf-gsib}$*-gcaD-prs*			2.83	No data	No data	
AM2694	168 *amyE::*($P_{rpsf-gsib}$*- seHasA-tuaD*), $P_{rpsf-gsib}$*-gtaB,* $P_{rpsf-gsib}$ *-gcaD-prs*			3.21	No data	No data	
WmB	WB600 Δ*upp,* Δ*sigF,* [*pHY300plk::hasA-tuaD-gtaB*]	*S. ubris*****	No data	3.65**	0.392**	20	[41]
				3.4	0.45	17	
				1.6***	6.973***	7.5	
KCNHA10	3NA *amyE::*($P_{hyper-spank}$*-szHasA-tuaD-gtaB-gcaD*)	*S. zooepidermicus*	Inducible $P_{hyper-spank}$	7	0.953	No data	[53]

Data obtained by cultivation at * 30°C, ** 32°C, *** 47°C. **** *S. ubris* is not described in the literature; perhaps, the authors or editors made a mistake with the spelling.

thetic pathway of UDP-glucuronic acid (*tuaD-gluM*) and UDP-*N*-acetylglucosamine (*gtaB-glmM-glmS*). Positive correlation between the expression of the UDP-*N*-acetylglucosamine biosynthesis genes and increase in the product MW (which was not detected for the UDP-glucuronic acid biosynthesis genes) was revealed.

For the first time, the possibility of increasing the productivity of producer strains by reducing the consumption of UDP-*N*-acetylglucosamine precursor frutose-6-phosphate in the glycolysis pathway was demonstrated. Decrease in the expression of 6-phosphofructokinase gene *pfkA*, which is the first step of fructose-6-phosphate conversion in the glycolysis pathway, was reached through the replacement of the ATG start codon by TTG and GTG variants. The E168T/pP43-DU-PBMS strain, which differs from E168A/pP43-DU-PBMS only in the substitution of the ATG codon by the *pfkA* TTG codon, demonstrated increased HA yield (from 2.7 to 3.2 g/L) compared to the latter at a similar growth rate and MW of the product.

The possibility of obtaining low molecular weight HA using heterologous hyaluronidase expression in a HA producer strain was also demonstrated. Low molecular weight fractions and oligosaccharides of HA with a given weight were previously obtained by in vitro enzymatic hydrolysis by recombinant leech hyaluronidase LHyal [39]. The degree of HA depolymerization depends on the hyaluronidase concentration; therefore, to obtain fractions with a given weight, the level of LHyal hyaluronidase expressed from the constitutive Plepa promoter was varied using a library of ribosome binding sites [40]. Cassettes with LHyal were integrated into the genome of the E168T/pP43-DU-PBMS strain, which contained a modification of the *pfkA* start codon and complete precursor biosynthetic pathways. This resulted in the rise of the HA yield along with the corresponding decrease in MW, constituting 4.35, 2.9, and 3.3 g/L at 0.0022, 0.0026, and 0.003 MDa for different variants, respectively. The fermentation scaling supported the stepwise nature of the change in the molecular weights of the obtained fractions and the rise in the HA yield. Higher cell density of strains with hyaluronidase was observed, which was associated with better conditions for oxygen availability.

Expression of leech hyaluronidase from the temperature-sensitive plasmid pKSV7 in the WmB producer strain led to the production of HA with different molecular weight at different temperature [41, 42]. Cultivation at a permissive temperature of 32°C led to a decrease in the HA MW from 0.392 to 0.00861 MDa.

Increased HA yield was also observed in case of the promoter replacement in the genomic copies of precursor biosynthesis genes, which resulted in their increased expression and made it possible to dispense with the need to expand the recombinant operon [43, 44]. The replacement of the *gtaB* and *gcaD* promoters with the strong tandem Prpsf-gsib promoter made it possible to achieve productivity of 2.35 and 3.21 g/L without expanding the minimal *seHasA-tuaD* operon upon cultivation for 24 and 48 h, respectively. The use of the Prpsf-gsib tandem promoter makes it possible to provide a more efficient target gene expression profile and, accordingly, more efficient production of the target product. The PrpsF promoter is one of the strongest promoters in the *B. subtilis* genome and provides the highest level of transcription at the logarithmic growth phase. Transcription initiation of the *gsiB* gene occurs with the participation of the alternative σ^B sigma subunit and reaches its maximum at the stationary growth phase. Increased expression of precursor biosynthesis genes has a positive effect on the yield of the product, which supports and complements the conclusions of previous studies.

Further development of the approach with partial diversion of metabolic fluxes from the pentose phosphate pathway and glycolysis to the HA biosynthesis demonstrated a considerable potential of this approach for increasing the HA yield [45]. Glucose-6-phosphate is consumed in the pentose-phosphate pathway through conversion by glucose-6-phosphate-1-dehydrogenase, encoded by the *zwf* gene, to 6-phosphogluconolactone. Fructose-6-phosphate is consumed in the glycolysis pathway through conversion by 6-phosphofructokinase, encoded by the *pfkA* gene, to fructose-1,6-bisphosphate. Inactivation of the *zwf* gene leads to a considerable diversion of the metabolic flux to the glycolysis pathway, and inactivation of the *pfkA* gene is lethal for *B. subtilis* [46]. Regulated *zwf* and *pfkA* gene silencing was achieved using the CRISPR interference method, which makes it possible to vary the transcription efficiency over a wide range [47]. Silencing of the *pfkA* gene increased the HA yield to 50% compared to the original strain with slight decrease in the HA MW and cell density. Silencing of the *zwf* gene increased the HA yield by 44 and 74% only in the case of two variants of the guide RNA, AW014-3 and AW016-3, respectively, while the remaining guide RNAs reduced the yield and MW of the product.

Simultaneous silencing of the *pfkA* and *zwf* genes had a considerable effect on the *B. subtilis* strain productivity. Compared to the basic AW009 strain, the strains with simultaneous *pfkA* and *zwf* gene silencing, AW018-3 and AW019-3, demonstrated an increase in the HA yield by 98 and 108%, respectively, along with slight increase in the MW. The growth rate of strains AW018-3 and AW019-3 did not differ from that of AW009, but was higher than that of strains with either *pfkA* or *zwf* silencing. In addition, the *pfkA* and *zwf* silencing in strains AW018-3 and AW019-3 led to the decrease in acetoin accumulation in the culture medium compared to strain AW009 by 167 and 118%, respectively, which reflected the decrease in metabolic flux through the glycolysis pathway. The *pfkA* and *zwf*

silencing in strain AW009 was much more effective than heterologous expression of the precursor biosynthesis genes *pgcA* and *glmS*.

HA precursors are also consumed during bacterial cell wall synthesis. UDP-*N*-acetylglucosamine is involved in the synthesis of teichoic acids and peptidoglycan components with the help of enzymes encoded by the *tagO* and *murAA* genes. The silencing of the *tagO* and *murAA* genes by RNA interference led to a considerable decrease in the strain growth rates, genetic instability, and the loss of the characteristic mucoid phenotype.

Creating conditions for effective functioning of membrane hyaluronan synthases using membrane engineering makes it possible to raise the productivity of the HA producer strains [48]. The type 1 hyaluronan synthases require the presence of cardiolipin phospholipid in the membrane, with which the enzyme forms functional complex in vivo and in vitro [49]. Cardiolipin is a minor part of the *B. subtilis* cell membrane lipids. Cardiolipin biosynthesis occurs with the help of cardiolipin synthase (ClsA) from phosphatidylglycerol, which, in turn, is synthesized with the help of phosphatidylglycerol synthase (PgsA) [50]. The *B. subtilis* strain AW001-4 with constitutive expression of the *pgsA* and *clsA* genes demonstrated an increase in the HA yield by 32%, increase in MW to 2.06 MDa, and increase in the final cell density by 83% compared to the control strain AW008. Fluorescence microscopy showed an increase in the cardiolipin content at the cell pole and septal regions. Silencing of genes for the biosynthesis of other cell membrane lipids, phosphatidylethanolamine (*pssA*) and neutral glycolipids (*ugtP*), had no effect on the productivity and growth rate of the control strain AW008.

Localization of cardiolipin synthase ClsA occurs in septal region and depends on the tubulin homolog FtsZ [51]. Suppression of FtsZ expression can potentially lead to the distribution of ClsA and increase the cardiolipin concentration over the entire membrane [52]. The *ftsZ* silencing in strain AW001-4 with strong constitutive expression of *pgsA* and *clsA* led to genetic instability. Only strain AW004-4 retained the mucoid phenotype after recovery from glycerol stock and synthesized 25% more HA than AW001-4 with a slight decrease in MW. The *ftsZ* silencing in the control strain AW008 by the same guide RNA variant as in AW004-4 resulted in an increase in the HA yield by 204%, increase in final cell density, and decrease in MW to 1.67 MDa. Strain AW007-4 with weakened constitutive expression of *pgsA* and *clsA* was used for engineering of genetically stable strains with four guide RNA variants. Strains AW009-4 and AW011-4 demonstrated an increase in yield, MW, and final cell density by 10–15% compared to strain AW007-4.

Silencing of sporulation factor genes affects the HA yield and MW. Strain WmA with a deletion of the sigma factor gene *sigF*, which is involved in the activation of the early sporulation gene network, demonstrates 30% higher HA yield compared to strain WA without the deletion [41]. Producer strains constructed on the basis of strain WB600 demonstrated a higher HA yield compared to producer strains constructed on the basis of *B. subtilis* 168. Specifically, under optimal conditions, strain WmB with the *sigF* deletion constructed on the basis of strain WB600 accumulated 3.21 g/L of the product in the culture medium, while strain 1B constructed on the basis of strain 168 accumulated only 1.7 g/L. An interesting effect of cultivation temperature on the HA MW was found. Cultivation at increased temperature of 47°C led to a sharp increase in the HA MW to 6.973 MDa. Cultivation at 32°C, at which the largest amount of the product was observed, led to the synthesis of HA with MW up to 0.392 MDa.

Inactivation of the ability to sporulate in strains obtained by undirected mutagenesis has a positive effect on the HA production. For example, strain 3NA containing a mutation in the *spo0A* sporulation initiation gene is able to reach the cell density of 75 g/L under fed-batch fermentation conditions, which makes it an excellent candidate for industrial use [53]. Optimization of the cultivation process and nutrient medium made it possible for strain KCNHA10 containing the recombinant operon with the *szHasA*, *tuaD, gtaB*, and *gcaD* genes to reach the cell density of 29.4 g/L in 12 h and synthesize 7 g of HA with the MW of 1 MDa per liter of culture. Technical and economic analysis of this production process demonstrated higher economic efficiency compared to the process based on streptococcal strains.

FACTORS AFFECTING PRODUCER STRAIN AND PRODUCT CHARACTERISTICS AND THE WAYS OF FURTHER DEVELOPMENT OF PRODUCER STRAINS

The data obtained from the construction of bacterial HA producer strains made it possible to draw conclusions on the influence of different genetic factors on the yield and technical characteristics of the product.

Basic Strain and Its Modifications

Recombinant producer strains were constructed using different strains of *B. subtilis*, the most common of which was strain 168 and its derivatives (Table 3). The important advantages of strain 168 are the ability to grow on simple media, the studied biochemistry and genetics, ease of genetic manipulations, and high transformation efficiency. Strains WB600 and WB800N were constructed on the basis of strain 168 as a platform for efficient heterologous protein expression [54]. To increase the secreted protein stability, in strain WB800N, deletions in the genes of eight extracellular proteases, *nprE*, *aprE*, *epr*, *bpr*, *mpr*, *nprB*, *vpr*, and *wprA*, were introduced, and in strain WB600, the

Table 3. *B. subtilis* strains used to construct hyaluronic acid producing strains

Strain	Genotype	Strain number in collections	Reference
168	*trpC2*	BGSC 1A1	[37, 38]
1012	*trpC2, leuA8, metB5, hsdRM1*	DSM 8773	[36]
WB600	*trpC2, ΔnprE, ΔaprE, ΔnprB::EmR, Δbpr, Δmpr, Δepr*	–	[41]
WB800N	*trpC2, ΔnprE, ΔaprE, nprB::bsr, Δbpr, mpr::ble, Δepr, Δvpr, wprA::hyg, cm::NeoR*	–	[36]
3NA	*trpC2, spo0A*	BGSC 1S1	[53]
A164Δ5	ATCC 6051a *ΔamyE, ΔspoIIAC, ΔaprE, ΔnprE , ΔsrfC*	–	[31, 32]
BGSC 1A751	*His, ΔnprE, ΔaprE, ΔbglS, ΔbglC*	BCRC 51921	[35, 45, 48]

ATCC, American Type Culture Collection; BGSC, Bacillus Genetic Stock Center; BCRC, Bioresource Collection and Research Center; DSM, Deutsche Sammlung von Mikroorganismen.

genes of six proteases, *nprE*, *aprE*, *epr*, *bpr*, *mpr*, and *nprB*, were deleted. Such modifications make it possible to increase the secretion and stability of recombinant proteins, which is also relevant for membrane proteins like HasA. The only direct comparison of the efficiency of strains described in the literature showed the advantage of the deficiency in extracellular protease activity of strain WB600, carrying the *sigF* deletion, over strain 168 in the HA production [41]. Strain 3NA is a mutant version of strain 168 with a frameshift mutation in the *spo0A* gene and is characterized by the absence of sporulation, low protease expression, and the ability to achieve high cell density under fed-batch fermentation conditions [53]. Strain 168 and strains with mutations of sporulation sigma factors on a synthetic medium reach the cell density of 5–15 g/L, which is considerably lower than that of 3NA [55]. In comparison with strain 168, the genome of strain 3NA contains 425 genetic variations; therefore, it is impossible to unambiguously conclude that the *spo0A* mutation is the reason for acquiring improved technological properties.

At the same time, *B. subtilis* strain 168 is a model laboratory strain, and in the process of construction, it underwent numerous stages of undirected mutagenesis, which led to the accumulation of numerous mutations in its genome [56]. The genetic instability of strain 168 with the P_{grac}-*seHas-tuaD* operon integrated into the genome has been reported [48]. For some unknown reason, being restored from cryopreservation, this strain formed segregated populations with the majority of cells of the wild nonmucoid phenotype. The same genetic instability was demonstrated by the producer strain constructed on the basis of strain BGSC 1A786 (*amyE::cat, lacA::spec*, *leuC8*, *metA4*, *hsd(RI)R+M–*). Only the BGSC 1A751-based producers showed genetic stability of the characteristic mucoid phenotype. Strain 1A751, which is a derivative of strain DB104, contains mutations in the *nprE* and *aprE* protease genes, as well as deletions of the endo-β-1,3-1,4-glucanase *bglS* and endo-β-1,4-glucanase *bglC* genes [57].

Strains other than *B. subtilis* 168 may have properties that would suggest possible beneficial effect on the HA production. For example, commercial strain A164Δ5 used for recombinant protein production is characterized by improved growth characteristics and the high yield of secreted proteins [32]. The A164Δ5 strain is an improved modification of strain ATCC 6051a, which also is superior to *B. subtilis* 168 in the production of recombinant proteins [58]. Modifications that improved the technological properties of strain ATCC 6051a include the deletion of the following genes: surfactin biosynthesis *srfC* (reduced foaming), sigma factor F *SpoIIAC* (sporulation blockage), *nprE* and *aprE* proteases, and *amyE* alpha-amylase. The disadvantage of strain A164Δ5 was low transformation efficiency, which led to the need to use the intermediate strain *B. subtilis* 16844 for genetic engineering manipulations [32].

Hyaluronan Synthase

The choice of the hyaluronan synthase gene affects the HA production and MW. When creating strain-producers, the hyaluronan synthase gene was chosen on the basis of either the data from a few studies on the enzyme catalytic properties [31, 45, 48] or the productivity characteristics of natural producers [38, 53]. For instance, the recombinant seHAS synthase from *S. equisimilis* demonstrates a twofold higher elongation rate than the recombinant spHAS synthase from *S. pyogenes* [23, 59].

Direct comparison of the effectiveness of hyaluronan synthases from *Streptococcus* was carried out in vitro and in vivo using *L. lactis* as a platform [22]. Under the same conditions, hyaluronan synthases differ in the MW of synthesized HA. The maximum MW of HA synthesized by recombinant *L. lactis* strains in vivo correlates with the data obtained in vitro and constitutes about 2.2 MDa for suHAS from *S. uberis* and spaHAS from *S. parauberis*, about 1.4 MDa for szHAS from *S. zooepidemicus*, and about 0.4 MDa for spHAS. The HA MW value is in good agreement with the phy-

logenetic grouping of hyaluronan synthases according to the amino acid sequences. For instance, suHAS and spaHAS form one phylogenetic group, distinct from szHAS and spHAS. However, the authors failed to obtain results with the hyaluronan synthase genes from *S. equi* subsp. *equi* and *S. iniae* using the *Lactococcus* platform in vivo, while the strains of *S. equi* subsp. *equi* are known to be efficient HA producers.

Interesting data were obtained with type 2 hyaluronan synthase from *P. multocida* [37]. Unlike the synthases from streptococci, PmHAS hyaluronan synthase is not a membrane protein and it contains a C-terminal anchor domain that holds the enzyme at the inner face of plasma membrane. The *P. multocida* HA biosynthesis operon includes additional genes, *hexA*, *hexB*, *hexC*, and *hexD*, the products of which are homologous to membrane transport proteins [60]. In *P. multocida*, the functions of HA synthesis and transmembrane transport of the HA chain to the cell exterior are divided between PmHAS synthase and hexA, hexB, hexC, and hexD proteins [61, 62]. Type 1 hyaluronan synthases, which include streptococcal synthases, combine the functions of the HA synthesis and translocation of the HA chain to the cell exterior [63]. It was suggested that the ABC transporter complex is involved in the HA translocation to the cell exterior of *S. pyogenes* [64]. However, this hypothesis is disproved both by the data of in vitro studies [65, 66] and by successful heterologous production of HA by type 1 hyaluronan synthases using different microbial platforms [18]. PmHAS hyaluronan synthase was used to construct the HA producer strains using *E. coli* [67], *Agrobacterium* sp. [68], and *Synechococcus* sp. [69]. PmHAS expression in *E. coli* and *Agrobacterium* sp. led to an increase in the broth viscosity, which indicated extracellular accumulation of HA. The distribution of the synthesized HA between the extracellular, surface-absorbed, and intracellular fractions was elucidated by studying the producer strains constructed on the basis of the *Synechococcus* cyanobacterium. It was demonstrated that from 42 to 88% of total HA accumulated in the cell exterior of these strains. Intracellular accumulation of HA was one of the reasons for the decrease in the producer strain growth rates. The mechanism of the HA export to the cell exterior in recombinant strains expressing PmHAS remains obscure. PmHAS hyaluronan synthase has high biotechnological potential and is capable of synthesizing high molecular weight HA. Deletion of the anchor domain makes it possible to obtain PmHAS in soluble form, which makes it an apparent candidate for the cell-free HA biosynthesis, which makes it possible to obtain a monodisperse high molecular weight product [21]. In addition, the PmHAS enzyme is superior to streptococcal hyaluronan synthases in terms of kinetic characteristics. In particular, the K_M values are two times lower than the streptococcal ones and constitute 75 and 20 μM for UDP-*N*-acetylglucosamine and UDP-glucuronic acid, respectively [70]. To elucidate the role of transport systems in HA translocation to the cell exterior both in *P. multocida* strains and in PmHAS-based recombinant strains on various platforms, further studies are required.

The possibility of obtaining PmHAS in soluble form facilitates the task of improving the enzyme characteristics by evolutionary and rational engineering methods. For example, the combination of four amino acid substitutions found using the KnowVolution evolutionary method made it possible to obtain a PmHAS variant capable of synthesizing HA with molecular weight up to 4.7 MDa [71]. Rational engineering of improved variants of type 1 hyaluronan synthases is complicated by unavailability of the 3-D structure of the protein, although this gap is partially filled by numerical modeling methods [72]. To date, only one study on the improvement of szHAS hyaluronan synthase characteristics using evolutionary method is known [73]. Using in vivo selection, in *B. subtilis* strains, it was possible to identify the szHAS variant, which demonstrated the increase in the HA yield from 1.22 to 2.24 g/L with the MW increase from 1.20 to 1.36 MDa.

Precursor Biosynthesis Pathway

The genome of *B. subtilis* contains all genes for the biosynthesis of UDP-glucuronate and UDP-*N*-acetylglucosamine; however, heterologous expression of only hyaluronan synthase does not lead to the synthesis of HA. Efficient biosynthesis of HA by recombinant producers can be carried out only from a minimal operon, which includes, in addition to hyaluronan synthase gene, the UDP-glucose dehydrogenase gene, which catalyzes the last step of UDP-glucuronate biosynthesis. The addition of a minimal operon with the genes for UDP-*N*-acetylglucosamine biosynthesis leads to a considerably lower effect of increasing the yield of HA compared to the addition of the UDP-glucose dehydrogenase encoding gene [31]. The need for heterologous expression of UDP-glucose dehydrogenase is associated with the absence of the *tuaD* gene (a homolog of the *ugd* gene from *E. coli* and *hasB* from *Streptococcus*) expression, which is part of the teichuronic acid biosynthesis operon *tuaABCEDFGH*. Teichuronic acid is an anionic polymer the synthesis of which is activated under conditions of phosphate starvation to replace phosphorus-rich teichoic acid in the cell wall [74]. The fundamental importance of UDP-glucose dehydrogenase is evidenced by the obligatory presence of the *hasB* gene in the operons of HA biosynthesis in representatives of the genus *Streptococcus*, where the minimum operon consists of the *hasA* and *hasB* genes (as in *S. uberis*). It was hypothesized that the HA biosynthesis operon appeared in the ancestral strain as the *hasA/hasB* pair, to which the remaining precursor biosynthesis genes (*hasC*, *hasD*, *hasE*) were added in the course of evolution [75]. The *hasB* gene deletion in *S. zooepidemicus* leads to com-

plete inactivation of HA biosynthesis and has a small effect on the strain growth properties [30]. A number of studies demonstrated the HA accumulation by *B. subtilis* producer strains with recombinant operons that do not contain the *tuaD* gene or its homolog [37, 38]. Taking into account the role of UDP-glucose dehydrogenase and the nature of its expression, it seems necessary to conduct further studies on this issue.

Extension of the recombinant operon with precursor biosynthesis genes leads to an increase in the HA yield in recombinant strains developed on the basis of the *B. subtilis*, *C. glutamicum*, and *L. lactis* platforms [18]. The combination of different genes leads to an increase in the HA yield with different efficiency. The inclusion of the *gtaB*, *glmM*, and *gcaD* genes into the artificial operon containing s*zHas* and *tuaD* genes leads to an increase in the HA yield by 30% [38].

The extended HA biosynthesis operon has a considerable influence on the HA production from natural producers. For instance, the *S. equi* and *S. zooepidemicus* strains containing the *hasABCDE* operon in the genome produce considerably more HA than *S. uberis* and *S. pyogenes* containing *hasABC* and *hasAB*, respectively [75]. It seems likely that the inclusion of additional genes in the operons of the *S. equi* subspecies occurred under the selective pressure for HA production, which led to the ability to direct up to 10% of incoming sugars to HA biosynthesis [76].

An important aspect affecting the strain productivity and MW of the product is the expression level of hyaluronan synthase and the precursor biosynthesis genes. High expression level of *tuaD*, which is toxic to *E. coli* and complicates the construction of genetically engineered constructs [38], may also have a negative influence on the physiology of *B. subtilis* [36]. In particular, a decrease in the efficiency of the Pgrac promoter of the *seHas-tuaD* operon led to an increase in the HA yield by 100%, decrease in MW by 17%, and increase in the final cell density by 47% [45].

The expression level of hyaluronan synthase and precursor biosynthesis genes, which depends on the composition of recombinant operon and the promoter strength, determines the balance of UDP-glucuronate and UDP-*N*-acetylglucosamine in the cell and, finally, the MW of the product. Increased *hasA* expression relative to *hasB* leads to a decrease in the MW of HA synthesized by the recombinant *L. lactis* strain [77]. Overexpression of genes in the UDP-glucuronate biosynthetic pathway in *S. equi* reduces the MW of HA, while overexpression of genes in the UDP-*N*-acetylglucosamine biosynthetic pathway leads to an increase in MW from 1.8 to 3.4 MDa. A correlation between the MW of HA and the UDP-*N*-acetylglucosoamine concentration was revealed. The biosynthesis of high molecular weight HA is the result of the balance of precursor concentrations, which can be brought to the optimal value by genetic methods [78]. For recombinant producer strains constructed on the *L. lactis* platform, optimal conditions for the synthesis of high molecular weight HA were equimolar intracellular concentrations of UDP-*N*-acetylglucosoamine and UDP-glucuronate and increased expression of *hasB* relative to *hasA* [79]. The addition of *N*-acetylglucosamine to the culture medium has a similar effect of increasing the MW of HA, both in the case of natural producers and in the case of recombinant producer strains constructed on the basis of *B. subtilis* [45].

Energy and Basic Metabolism

The HA biosynthesis is an energy-consuming process. To obtain 1 mol of UDP-*N*-acetylglucosamine and 1 mol of UDP-glucuronate, the cell spends 2 mol of glucose, 3 mol of ATP, 2 mol of UTP, and 1 mol of acetyl-CoA. Promising approaches to increase the HA yield are the inactivation of pathways for the competitive utilization of precursors and increase in the level of ATP synthesis.

Homolactic fermentation, which is based on glycolysis, is the main source of energy for lactic acid bacteria such as *S. zooepidermicus* and *L. lactis*. The result of this process is the formation of two pyruvate molecules, two $NAD{\cdot}H_2$ molecules, and two ATP molecules per glucose molecule. The regeneration of NAD^+ is carried out by the transfer of two electrons from $NAD{\cdot}H_2$ to the pyruvate molecule, which leads to the lactate formation. However, under aerobic conditions, lactic acid bacteria, the cells of which are devoid of electron transport chain, regenerate NAD^+ using the $NAD{\cdot}H_2$ oxidase (NOX) enzyme, which leads to the diversion of the metabolic flux toward the formation of acetate and the formation of an additional ATP molecule per glucose molecule in reactions catalyzed by acetate kinase (AK) [80]. Synthesis of additional ATP in the acetate kinase reaction and increase in the NAD^+ regeneration rate due to NOX correlate with increase in the level of HA synthesis by producer strains constructed on the basis of lactic acid bacteria. Under aerobic conditions, *S. zoopidemicus* not only grew faster and accumulated biomass but also demonstrated a higher MW and level of HA biosynthesis compared to anaerobic conditions [81, 82]. However, direct overexpression of NOX in the *S. zooepidemicus* strain, despite the rise in the level of ATP synthesis by 33% and the biomass yield by 15%, did not lead to a rise in the HA yield or a change in MW. Probably, in this case, the main limiting factor was the activity of the pyruvate dehydrogenase complex, which converts pyruvate to acetyl-CoA [83]. At the same time, recombinant HA producers constructed on the basis of the *L. lactis* strain with the *ldh* deletion demonstrated a threefold increase in the HA production and MW [84].

Unlike streptococci, which regenerate NAD^+ with the help of NOX in the presence of oxygen and synthesize additional ATP with the help of the AK enzyme, *B. subtilis* uses the electron transport chain and the tricarboxylic acid cycle [85]. In the case of aerobic cultivation of recombinant HA producer strains constructed on the basis of *B. subtilis*, acetate and acetoin were byproducts of fermentation [45]. However, a decrease in the level of acetate synthesis by inactivation of the pyruvate dehydrogenase complex creates a deficiency of acetyl-CoA for the tricarboxylic acid cycle, which leads to a decrease in ATP production [86]. Inactivation of the 2-acetolactate decarboxylase *alsD* gene, which is involved in the synthesis of acetoin, negatively affects the growth of *B. subtilis* strains, although elucidation of the mechanism of this phenomenon requires further investigation [86].

Increasing oxygen availability for the cells is an effective strategy for activating HA biosynthesis, despite the fact that the mechanism of the effect differs for lactic acid bacteria and bacilli. The oxygen concentration in the culture medium is a limiting factor of the HA biosynthesis by *S. zooepidemicus* and *B. subtilis* strains owing to the low solubility of gaseous oxygen and the high viscosity of the HA solutions [87, 88]. The technological solution to this issue is to increase the oxygen capacity of the medium by optimizing the agitation rate and using oxygen vectors, i.e., hydrophobic liquids in which oxygen has higher solubility than in water. This approach increases the HA yield by *S. zooepidemicus* strains [89]. A substantial increase in the HA yield and increase in the cell density of the recombinant *B. subtilis* strain culture was observed with the use of n-heptane, n-hexadiene, perfluoromethyldecalin, and perfluoro-1,3-dimethylcyclohexane. Optimization of the fermentation conditions, i.e., the concentration of the oxygen vector, the time of its addition, and the agitation rate, made it possible to achieve the HA concentration of 4.5 g/L in just 10 h of cultivation [88].

In the construction of recombinant producer strains, bacterial hemoglobin VHb from the Gram-negative bacterium *Vitreoscilla* is actively used [34]. Hemoglobin VHb enhances the oxygen flux to terminal oxidases under hypoxic conditions; therefore, VHb overexpression leads to an increase in the cell density and increase in the oxidative metabolism and the yield of the target product, especially under conditions of limited oxygen availability. Expression of VHb in *B. subtilis* led to an increase in protein secretion and increase in the yield of alpha-amylase and neutral protease [90]. Heterologous VHb expression by the HA producer *S. zooepidemicus* strain ATCC 39920 increased the HA yield from 1.61 to 2.16 g/L upon the decrease in MW from 1.8 to 1.6 MDa [91]. At the same time, there was a decrease in the lactic acid production by 35% against the background of reduced *ldh* lactate dehydrogenase activity by 41% and a rise in the activity of acetate kinase and NOX by 9 and 106%, respectively. More impressive results were obtained using the producer strain constructed on the basis of *B. subtilis*; however, the effect of VHb coexpression on the MW of HA was not examined in this study [35].

UDP-glucuronate and UDP-*N*-acetylglucosamine are used by the cell in the biosynthesis of cell wall components, and their precursors glucose-6-phosphate and fructose-6-phosphate are consumed in the pentose phosphate pathway and glycolysis. Inactivation of the pathways for the competitive utilization of UDP-glucuronate and UDP-*N*-acetylglucosamine had a greater effect on the growth characteristics of *B. subtilis* strains and the yield of HA than overexpression of the genes of the precursor biosynthesis pathway [38, 45]. In addition, strains with decreased *pfkA* and *zwf* activity demonstrated a lower level of acetate and acetoin production, which indicated an effective diversion of the metabolic flux from biosynthetic byproducts to HA [45].

Sugar Utilization

The way to improve producer strains is to increase the transport and metabolism of energy sources. The function of sucrose metabolism genes was studied and the stages restricting the HA yield in the *S. zooepidemicus* strain were identified [92]. The first stages of sucrose utilization are membrane transport with phosphorylation via the phosphoenolpyruvate-dependent phosphotransferase system (encoded by the *scrA* gene) followed by hydrolysis of sucrose-6-phosphate by sucrose-6-phosphate hydrolase (encoded by the *scrB* gene) to fructose and glucose-6-phosphate. Overexpression of *scrB*, in contrast to the reverse effect of *scrA* overexpression, increases the biomass by 26% and the HA yield by 30%. Shifting the metabolic flux to fructose-6-phosphate through the deletion of the *fruA* fructose transport gene or the *fruK* phosphofructokinase gene increased the HA yield by 22 and 27%, respectively, without the effect on cell growth. Overexpression of *scrB* in strains with either *fruA* or *fruK* deletion increased the HA yield by 44 and 55%, respectively.

The main system of sucrose transport and utilization in *B. subtilis* is identical to that in *S. zooepidemicus* and consists of an operon encoding SacP phosphotransferase and SacA sucrose-6-phosphate hydrolase [93]. Replacement of the *B. subtilis* own sucrose transport and metabolism system with an energetically more favorable heterologous system was demonstrated using the polyglutamic acid and 2,3-butanediol producer strains. The combination of the sucrose permease *cscB* gene from *E. coli* and sucrose phosphorylase *sucP* gene from *Bifidobacterium adolescentis* demonstrated the rise in sucrose consumption by 49.4% and polyglutamic acid production by 38.5% compared with the unchanged strain [94]. The combination of the sucrose permease *cscB* gene from *E. coli* and the sucrose phosphorylase *gtfA* gene from the

Streptococcus mutans demonstrated a 36% increase in product yield compared to unmodified strains [95].

CONCLUSIONS

At present, on the basis of the *B. subtilis* platform, a panel of recombinant HA producer strains was constructed. Important advantages of these strains are GRAS (Generally Recognized as Safe) status, low cost of industrial fermentation, ease of genetic manipulation, and the absence of endo- and exotoxins. Recombinant HA producer strains constructed on the basis of *B. subtilis* make it possible to obtain a yield and MW of the product comparable to that of commercially developed streptococcal strains. At the same time, the studies carried out revealed platform limitations that can be overcome with the involvement of the experience of constructing HA producer strains on the basis of other microbial platforms. Analysis of the published data suggests that the conditions for the construction of an industrial technology for the HA production on the basis of *B. subtilis* recombinant producer strains have been formed.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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