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Production of specific-molecular-weight hyaluronan by metabolically engineered *Bacillus subtilis* 168

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ABSTRACT

Low-molecular-weight hyaluronan (LMW-HA) has attracted much attention because of its many potential applications. Here, we efficiently produced specific LMW-HAs from sucrose in *Bacillus subtilis*. By coexpressing the identified committed genes (*tuaD*, *gtaB*, *glmU*, *glmM*, and *glmS*) and downregulating the glycolytic pathway, HA production was significantly increased from 1.01 g L^{-1} to 3.16 g L^{-1}, with a molecular weight range of 1.40×10^6–1.83×10^6 Da. When leech hyaluronidase was actively expressed after N-terminal engineering (1.62×10^6 U mL^{-1}), the production of HA was substantially increased from 5.96 g L^{-1} to 19.38 g L^{-1}. The level of hyaluronidase was rationally regulated with a ribosome-binding site engineering strategy, allowing the production of LMW-HAs with a molecular weight range of 2.20×10^3–1.42×10^6 Da. Our results confirm that this strategy for the controllable expression of hyaluronidase, together with the optimization of the HA synthetic pathway, effectively produces specific LMW-HAs, and could also be used to produce other LMW polysaccharides.

1. Introduction

Hyaluronic acid (HA or hyaluronan) is a linear unbranched high-molecular-weight glycosaminoglycan composed of repeating disaccharide D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) units with β-1,4 and β-1,3 linkages (Weissmann and Meyer, 1954). Its unique rheological, viscoelastic, and hygroscopic properties, together with its biocompatibility and non-immunogenicity, have allowed HA to be widely used in cosmetic and pharmaceutical applications (Kogan et al., 2007). Interestingly, the biological functions of HA are strongly dependent on its molecular weight (M_W) (Stern et al., 2006). Many studies have shown that HAs with low M_W (less than 10,000 Da) are mainly involved in wound healing, angiogenesis, cell differentiation and proliferation, tumor cell migration, and apoptosis (Stern et al., 2006; Toole et al., 2008), and HAs (70,000–120,000 Da) are considered to have especially important applications in chronic wound healing and the development of novel HA conjugates. Low-molecular-weight HAs have unique biological activities, including the stimulation of fibroblast proliferation and collagen synthesis, and can also selectively kill many types of cancer cells by disrupting the receptor–hyaluronan interaction (Ghatak et al., 2002; Seeberger and Werz, 2007; Toole et al., 2008). Compared with high-molecular-weight HAs, LMW-HAs can be readily absorbed by the human body (Boltje et al., 2009). Consequently, the efficient production of specific LMW-HAs is very important for related in-depth studies and broad applications (Yuan et al., 2015).

Traditional HA extraction methods (from rooster comb) have many disadvantages, such as the risk of cross-species viral infection. As such, commercialized HA production mainly relies on the fermentation of certain attenuated strains of group C *Streptococcus*. The production costs are lower for this process, and results in less environmental pollution (Chong et al., 2005; Liu et al., 2011). *Streptococcus zooepidemicus*, in particular, is a strain that has been intensively studied for HA production (Blank et al., 2005; Prasad et al., 2012). However, the high risk of pathogenicity and the few DNA manipulation techniques available have restricted the use of *Streptococcus* species. Therefore, the construction of heterologous HA-producing strains with known genetic backgrounds (Chien and Lee, 2007; Widner et al., 2005) provides an attractive alternative, especially with the rapid development of metabolic engineering techniques (Chong et al., 2005; Coussement et al., 2014; Kogan et al., 2007; Nielsen et al., 2014; Schmid et al., 2015; Wells et al., 2011). In the past, many hosts, including *Escherichia coli* (Yu and

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Table 1
Strains and plasmids in this study.

Strain or plasmid	Relevant characteristic	References
Strain		
E. coli DH5α	F-φ80 *dlacZ* ΔM15 Δ*(lacZYA-argF)U*169 *endA*1 *recA*1 *hsdR*17 (rK$^-$ mK$^+$) *deoR thi*-1 *phoA supE*44*λ*- *gyrA*96 *relA*1	Stratagene
B. subtilis 168	trpC2	BGSC
E168A	*B. subtilis* 168 derivate, Δ*lacA*::P_{xylA}-*hasA*, Em^r	This study
E168A/pP43-D	E168A derivate, overexpression of *tuaD* gene	This study
E168A/pP43-DB	E168A derivate, co-overexpression of *tuaD* and *gtaB* genes	This study
E168A/pP43-DBA	E168A derivate, co-overexpression of *tuaD gtaB* and *pgcA*	This study
E168A/pP43-U	E168A derivate, overexpression of *glmU* gene	This study
E168A/pP43-UMS	E168A derivate, co-overexpression of *glmU, glmM* and *glmS*	This study
E168A/pP43-UMSI	E168A derivate, co-overexpression of *glmU, glmM, glmS and pgi genes*	This study
E168A/pP43-DU	E168A derivate, co-overexpression of *tuaD* and *glmU* genes	This study
E168A/pP43-DU-PBMS	E168A derivate, co-overexpression of *tuaD, glmU* and P_{veg}-*gtaB-glmM-glmS* genes	This study
E168T	E168A derivate, replaced the Initiation codon ATG of *pfk*A by TTG	This study
E168G	E168A derivate, replaced the Initiation codon ATG of *pfk*A by GTG	This study
E168T/pP43-DU-PBMS	E168T derivate, co-overexpression of *tuaD, glmU* and P_{veg}-*gtaB-glmM-glmS* genes	This study
E168TH	E168T derivate, Δ*nagA*::P_{lepA}-*sp-H6LHyal*	This study
E168TH/pP43-DU-PBMS	E168TH derivate, co-overexpression of *tuaD, glmU* and P_{veg}-*gtaB-glmM-glmS* genes	This study
E168THR1/pP43-DU-PBMS	E168TH/pP43-DU-PBMS derivate, RBS variant of LHAase.	This study
E168THR2/pP43-DU-PBMS	E168TH/pP43-DU-PBMS derivate, RBS variant of LHAase.	This study
E168THR3/pP43-DU-PBMS	E168TH/pP43-DU-PBMS derivate, RBS variant of LHAase.	This study
E168THR4/pP43-DU-PBMS	E168TH/pP43-DU-PBMS derivate, RBS variant of LHAase.	This study
E168THR5/pP43-DU-PBMS	E168TH/pP43-DU-PBMS derivate, RBS variant of LHAase.	This study
Plasmid		
pAX01	Amp^r, Em^r, *E. coli–B. subtilis* shuttle integration vector, P_{xylA}-MCS	BGSC
pBluescript SK(+)	Amp^r	Stratagene
pAX01-*hasA*	Amp^r, Em^r, P_{xylA}-*hasA*	This study
pSKIZH	Amp^r, Zeo^r, pBluescript SK(+) containing the P_{lepA}-*sp-H6LHyal* integration cassette	This study
p7Z6	pMD18-T containing lox71-zeo-lox66 cassette	(Yan et al., 2008)
pDG148	Kan^r. Amp^r; temperature sensitive in *B. subtilis, Cre* recombinase	(Stragier et al.,1988)
pP43NMK	Amp^r, Km^r, *E. coli–B. subtilis* shuttle vector, P_{43}-MCS	(Zhang et al., 2005)
pP43-D	pP43NMK derivate, P_{43}-*tuaD*	This study
pP43-DB	pP43NMK derivate, P_{43}-*tuaD-gtaB*	This study
pP43-DBA	pP43NMK derivate, P_{43}-*tuaD-gtaB-pgcA*	This study
pP43-U	pP43NMK derivate, P_{43}-*glmU*	This study
pP43-UM	pP43NMK derivate, P_{43}-*glmU-glmM*	This study
pP43-UMS	pP43NMK derivate, P_{43}-*glmU-glmM-glmS*	This study
pP43-UMSI	pP43NMK derivate, P_{43}-*glmU-glmM-glmS-pgi*	This study
pP43-DU	pP43NMK derivate, P_{43}-*tuaD-glmU*	This study
pP43-DU-PBMS	pP43NMK derivate, P_{43}-*tuaD-glmU*-P_{veg}-*gtaB-glmM-glmS*	This study

Stephanopoulos, 2008), *Agrobacterium* sp. (Mao and Chen, 2007), *Lactococcos lactis* (Chien and Lee, 2007; Hmar et al., 2014; Prasad et al., 2010; Sheng et al., 2009), and *Pichia pastoris* (Jeong et al., 2014), have been evaluated and engineered for the production of HA. In particular, *Bacillus subtilis*, generally recognized as a safe strain, has been regarded as an ideal cell factory (Commichau et al., 2015; Widner et al., 2005) and has been intensively studied for HA production. Although the titer of HA produced by *B. subtilis* 168 has been significantly increased, the pathway genes involved in the biosynthesis of the two HA precursors, GlcUA and GlcNAc, have not been systematically studied and engineered to improve the production of HA. Furthermore, the reduced dissolved oxygen (DO) caused by the viscoelastic properties of HA is always a primary obstacle in maintaining normal cell metabolism and improving HA production. According to previous studies and our observations (Huang et al., 2006; Jeong et al., 2014; Mao et al., 2009; Widner et al., 2005), it is difficult in practice to increase the production of high-molecular-weight HA (about 2.0×10^6 Da, 3.0 g L^{-1}) any further in *B. subtilis* 168 flask cultures because of its viscoelastic properties and the extremely low levels of DO. It is difficult to maintain high DO levels throughout the whole fermentation, even in fermenters equipped with aeration and stirring devices. Consequently, increasing the DO levels and improving the production of HA remains a challenge.

Although measures to optimize the fermentation process have recently been used to regulate HA M_W values, the production of specific LMW-HAs is still impractical because of the extremely narrow control scope of M_W values (Armstrong and Johns, 1997; Jagannath and Ramachandran, 2010). Therefore, much attention has been directed towards chemical and enzymatic methods (Jin et al., 2014; Mahoney et al., 2001; Stern et al., 2007). Recently, we expressed high levels of leech hyaluronidase (LHAase) in *P. pastoris* (Jin et al., 2014; Kang et al., 2015) and successfully used it for the enzymatic production of specific LMW-HAs (Yuan et al., 2015). Even so, the separate processes of producing LHAase in *P. pastoris* and the subsequent enzymatic hydrolysis are time-consuming and comparatively tedious. In contrast, the construction of efficient *B. subtilis* cell factories for the one-step production of specific HAs from inexpensive carbon sources (such as sucrose or glucose) is a much more promising and attractive option.

2. Materials and methods

2.1. Media and culture conditions

Luria-Bertani (LB) medium (g L^{-1}: tryptone 10, yeast extract 5, and NaCl 10) was used for the cloning experiments. Modified medium (g L^{-1}: yeast extract 20, tryptone 2, $MgSO_4$ 1.5, in 50 mM potassium phosphate buffer, pH 7.0) was used for fermentation. Sucrose was added as the carbon source as indicated. Xylose (20 g L^{-1}) was added 2 h after inoculation to induce the expression of the *hasA* gene. The appropriate antibiotics (100 μg mL^{-1} ampicillin, 50 μg mL^{-1} kanamycin, 20 μg mL^{-1} zeocin, or 1 μg mL^{-1} erythromycin) were used for the selection of plasmids when necessary. All engineered *B. subtilis* 168 strains carrying HA