Appl Microbiol Biotechnol (2016) 100:7877–7885
DOI 10.1007/s00253-016-7511-y

Recombinant *Escherichia coli* K5 strain with the deletion of *waaR* gene decreases the molecular weight of the heparosan capsular polysaccharide

Haichan Huang[1] · Xiaobo Liu[1] · Shencong Lv[1] · Weihong Zhong[1] · Fuming Zhang[2] · Robert J. Linhardt[2,3]

Received: 29 February 2016 / Revised: 23 March 2016 / Accepted: 25 March 2016 / Published online: 14 April 2016

Abstract Heparosan, the capsular polysaccharide of *Escherichia coli* K5 having a carbohydrate backbone similar to that of heparin, has become a potential precursor for bioengineering heparin. In the heparosan biosynthesis pathway, the gene *waaR* encoding α-1-, 2-glycosyltransferase catalyzes the third glucosyl residues linking to the oligosaccharide chain. In the present study, a *waaR* deletion mutant of *E. coli* K5 was constructed. The mutant showed improvement of capsule polysaccharide yield. It is interesting that the heparosan molecular weight of the mutant is reduced and may become more suitable as a precursor for the production of low molecular weight heparin derived from the wild-type K5 capsular polysaccharide.

Keywords *Escherichia coli* K5 · Gene *waaR* · Capsular polysaccharide · Low molecular weight heparin

Electronic supplementary material The online version of this article (doi:10.1007/s00253-016-7511-y) contains supplementary material, which is available to authorized users.

✉ Weihong Zhong
whzhong@zjut.edu.cn

[1] College of Biological Engineering, Zhejiang University of Technology, Hangzhou 310032, China

[2] Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

[3] Department of Biological Science, Departments of Chemistry and Chemical Biology and Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

Introduction

Heparin, one of the most widely used anticoagulant drugs for decades, is a complex natural mucopolysaccharide comprised of sulfated repeating disaccharide units of (1 → 4)-linked glucosamine and uronic acid residues. Heparin is a heterogeneous polydispersed mixture and is categorized into three types of drugs corresponding to their average molecular weights: unfractionated heparin (UFH, average molecular weight (MW_{avg}) ~20 kDa), low-molecular-weight heparin (LMWH, MW_{avg} ~ 6 kDa), and ultra-low-molecular-weight heparin (ULMWH, MW_{avg} <2 kDa) (Linhardt and Gunay 1999; Bhaskar et al. 2012; Su et al. 2015). UFH is currently extracted from animal tissues such as porcine intestine and bovine lung and intestine, while LMWH is obtained by the controlled chemical or enzymatic depolymerization of UFH. LMWH has gained increasing levels of use due to its improved pharmacokinetic characteristics, longer half-life, better bioavailability, reduced the risk of hemorrhage, reduction in heparin-induced thrombocytopenia (HIT), and decreased osteoporosis (Higashi et al. 2011, 2012). LMWH drugs are widely used in the treatment and prophylaxis of venous thromboembolism, acute myocardial infarction, and unstable angina. Chemical depolymerization methods used to prepare LMWHs from UFH rely on strong reaction conditions using harsh chemical reagents that result in decomposition and modification of saccharide units within the polysaccharide backbone (Linhardt and Gunay 1999; Linhardt 2003). Milder conditions for the preparation of the LWMH, tinzaparin, using enzymatic degradation of UFH, still result in structural modification of its saccharide units (Linhardt and Gunay 1999). Three types of heparin lyases have been prepared from *Flavobacterium heparinum*. Heparin lyase I acts primarily on heparin, heparin lyase II cleaves both heparin and heparan sulfate, while heparin lyase III acts only on heparan sulfate

(Lohse and Linhardt 1992; Huang et al. 2013b, 2014). Compared to chemical methods of LMWH, the enzymatic method shows the advantage of using mild reaction conditions, has higher specificity, and is more environmental friendly. ULMWH can be prepared through total chemical synthesis, and fondaparinux (MW 1728 Da) is an antithrombin III-binding pentasaccharide that is currently in clinical use (Bauer et al. 2002; Petitou and van Boeckel 2004). The synthesis of fondaparinux is laborious, involving over 60 steps, and cannot replace UFH or LMWH (Linhardt and Liu 2012).

Heparosan is generated as a bacterial capsular polysaccharide (Cress et al. 2014) in organisms including *Escherichia coli* K5 and *Pasteurella multocida* (DeAngelis et al. 2002). Heparosan has a similar structure to unmodified UFH and has been used as a precursor for heparin and heparan sulfate in chemoenzymatic synthesis (Suflita et al. 2015). Heparosan can be easily extracted from bacteria fermentation supernatant (Wang et al. 2010; Wang et al. 2011a) and then can be converted to anticoagulant heparin by *N*-deacetylation using NaOH, *N*-sulfonation with $(CH_3)_3N{\cdot}SO_3$ (Wang et al. 2011b) and subsequently modified using enzymes (Chen et al. 2005; Wang et al. 2010). These enzymatic steps include treatment with (1) C_5-epimerase/2-*O*-sulfotransferase, (2) 6-*O*-sulfotransferases, and (3) 3-*O*-sulfotransferase, to afford anticoagulant heparin (Zhang et al. 2008; Wang et al. 2010). Today, heparosan serves as the critical precursor in heparin biosynthesis and chemoenzymatic synthesis of bioengineered heparin (Linhardt and Liu 2012; Li et al. 2013; Suflita et al. 2015). Heparosan separated from the culture has a higher molecular weight (MW_{avg} > 20 kDa) than heparin and its size is subsequently reduced through chemical processing (Wang et al. 2011b). It is possible to metabolically engineer *E. coli* to change the production levels and structure of its capsular polysaccharides (Cress et al. 2014; He et al. 2015; Cress et al. 2015). Our laboratory has focused on the metabolic engineering of *E. coli* K5 and produce low molecular weight heparosan to produce a LMWH from chemoenzymatic synthesis.

The cell surface in *E. coli* is decorated with lipopolysaccharide, enterobacterial common antigen, and capsular polysaccharides or K antigens (Whitfield and Roberts 1999; Cress et al. 2014). There is a variation in the genetic organization between the five different *waa* gene clusters encoding the different outer core types. The *waaR* gene encodes α-1, 2-glycosyltransferase, which can add the third glucose residues (GLcIII) to the outer core. A deficiency of *waaR* gene will cause the deletion of GLc and Hep IV terminal residues in the mutant (Heinrichs et al. 1998; Amor et al. 2000; Raetz and Whitfield 2002; Frirdich et al. 2003). The MS101 strain was mutagenized using the EZ::TN < *R6Kγori/KAN-2* > Tnp Transposome kit, which identified that the *waaR* mutant affected retention of the K5 polysaccharide on the cell surface and increased capsular polysaccharide accumulating in the culture supernatant (Taylor et al. 2006). However, the specific information of polysaccharides produced by the mutant was not established.

This work reports new information in a recombinant *E. coli* K5 strain, which *waaR* gene was knocked out, including both its yield and the molecular weight. We demonstrate that the yield of polysaccharide in the mutant was not affected but the molecular weight of heparosan was considerably reduced as determined by gel permeation chromatograph (GPC) and PAGE determination. The results of our study suggest that it may be possible to produce a low molecular weight heparosan from engineered *E. coli*.

Materials and methods

Strain and media

E. coli K5 (ATCC 23506) was used for the research as the wild-type strain. Luria-Bertani (LB) solid medium (2 % agar) and liquid medium with or without kanamycin (50 μg/mL) were used for cell growth and transformation screening.

Defined glucose medium (DGM) was used for shake-flake experiment which contains in every liter of water: 22.60 g $Na_2HPO_4{\cdot}12H_2O$, 0.45 g KH_2PO_4, 1.50 g NH_4Cl, 20 g glucose, 10 mg thiamin-HCl, and 10.0 mL trace element solution. Trace element solution contains 10.0 g $FeSO_4{\cdot}7H_2O$, 2.0 g $CaCl_2$, 2.2 g $ZnSO_4{\cdot}7H_2O$, 0.5 g $MnSO_4{\cdot}4H_2O$, 1.0 g $CuSO_4{\cdot}5H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, and 0.02 g $Na_2B_4O_7{\cdot}10H_2O$ in per liter of 5 M HCl. The pH was adjusted to 7.0–7.2 before autoclaving.

Chemicals

Ampicillin sodium, kanamycin sulfate, L-arabinose, and thiamin-HCl were purchased from Sangon Biotech, Shanghai, China. PrimeSTAR HS DNA polymerase and TaqDNA polymerase were purchased from Takara, Shanghai, China. The peptone and yeast extract used were from Angel (China). Alcian Blue was purchased from Solarbio (Beijing, China).

Construction of *E. coli* K5^Δ*waaR* strain

The DNA of *E. coli* K5 was extracted and purified according to the Manual of the EZNA™ Bacterial DNA Kit (OMEGA, Shanghai, China). As listed in Table 1, the primer WY1 and primer WY2 were designed according to the gene *waaR* in the strain *E. coli* F632 (GenBank:AF019375.1), begun with 65 bp upstream from the 5′ region sequence and 65 bp downstream from the 3′ region sequence of the gene respectively to profile the gene *waa*R in *E. coli* K5 with several extra base pairs for sequencing. The primer P1 and the primer P2 were designed for amplifying the gene *kan* with 56 bp homologous DNA

Table 1 Primers used in this study

Primer	Primer sequence (5′ → 3′)
WY1	ttcatcggtaaaaacaaatct
WY2	cagcacatgcaacttgaagtataac
P_1	ttatttacggtaatattttcggcaaagataacacactgcaatgagtcctgagaatgggaattagccatggtcc
P_2	gtggactcatttcctgccatagagatagataaagtaaagcctgggatttaggctgtgtaggctggagctgcttc

fragment with gene *waaR* in the two ends. All primers were synthesized by Sangon Biotech Company (Shanghai, China).

The plasmids pKD46 and pKD4 were extracted and purified according to the instruction of EZNA™ Plasmid Mini Kit (OMEGA, Shanghai, China). The plasmid pKD46 was transmitted into *E. coli* K5 by electroporation (2500 V, 25 μF, 200 Ω, Gene Pulser Xcell™, Bio-Rad, USA). The transformant *E. coli* K5/pKD46 could be screened on the LB plates containing 100 μg/mL ampicillin sodium salt. Meanwhile, the knockout DNA fragment was prepared by PCR using the plasmid pKD4 as the template and P1 and P2 as the primer pair. Then, the knockout DNA fragment was transformed into *E. coli* K5/pKD46 cell via electroporation (2500 V, 25 μF, 200 Ω). The mutant *E. coli* K5/△*waa*R was screened on the LB plate containing kanamycin. All the products were characterized by agarose gel electrophoresis with the DNA sequencing in each process. The strain morphology of the wild type and the mutant was observed via electron microscope (H-7650, HITACHI, Japan) after negative staining with 1–2 % phosphotungstic acid (PTA) at pH 6.5–7.0 for 5–10 s.

Strain culture and heparosan separation

The strains were cultured in the DGM medium for 24 h at 37 °C on the shaker with 180 rpm. The optical density at 600 nm was determined by a spectrophotometer (UV-1800 spectrophotometer, Mapada, Shanghai, China) after an appropriate dilution.

The supernatant of the fermentation broth was harvested by centrifugation for 30 min at 4 °C, 4000 rpm (Sorvall Biofuge Stratos centrifuge, Thermo Scientific, Germany). After being bleached with 1.5 % hydrogen peroxide solution over night under 4 °C, the supernatant was harvested again via centrifugation under the above condition for 5 min and then precipitated with ammonium sulfate or anhydrous ethanol overnight at 4 °C. The heparosan in sediment was collected via centrifugation for 10 min at 10,000 rpm and 4 °C and then dissolved in the deionized water for dialysis. The dialysate was condensed to proper volume for lyophilization.

Quantification of heparosan and protein

The lyophilized heparosan sample was dissolved in the deionized water (<100 mg/L) and quantified by carbazole assay (Bitter and Muir 1962) which determines the uronic acid content in the sample. The concentration of heparosan was calculated according to the formula $y = 0.01738\times + 0.0052$ ($r^2 = 0.9999$), in which *x*-axis was the concentration of D-glucuronic acid (Amresco Co.) and *y*-axis was its absorbance at 530 nm.

The quantification of protein in the polysaccharide sample solution was determined by following the instruction of the Bradford assay kit (Sangon Biotech, Shanghai, China).

Molecular weight analysis using PAGE and GPC

One-dimensional polyacrylamide gel electrophoresis (PAGE) was chosen for molecular weight analysis of heparosan using a standard method of the Bio-Rad Mini-Protean II system (7 cm × 10 cm mini-gels). Two cross-linker acrylamide gels, 22 % and with 0.5 % with 1 mm thickness, were used for separating and condensing respectively in the same gel. The loading buffer contains 0.02 % phenol red and 40 % sucrose in distilled water. The PAGE was run under a constant voltage of 200 V within the Tris-glycine buffer that contained 0.2 M Tris and 1.25 M glycine in distilled water. The gel was stained with 0.5 % Alcian Blue in water containing 1.5 % acetic acid for 0.5 h and distained overnight with distilled water before image collecting by the molecular imager (Gel DocTMXR+ Imaging System with Image Lab™ Software, Bio-Rad, USA).

All samples were dissolved in distilled water to proper concentrations in the range from 0.01 to 1 mg/L before analysis by GPC (Agilent 1100, USA) with a PL aquagel-OH column, which uses pure water as mobile phase with a flow rate of 1 min/mL and collected the signal by refractive index detector.

Results

Construction of *E. coli* K5△*waaR* strain

PCR and sequencing results shown that the DNA sequence length of *E. coli* K5 *waaR* was 1017 bp (Fig. S1 in the supplementary material). The similarity between the gene *waaR* in *E. coli* K5 and that in *E. coli* strain F632 (GeneBank:AF019375.1) was >99 % with only two base pairs different (Fig. S1 and Fig. S2 in the supplementary material). The bases located at +4 and +191 of gene *waaR* in *E. coli* K5 were G and C, while that in *E. coli* F632 were both A. Based

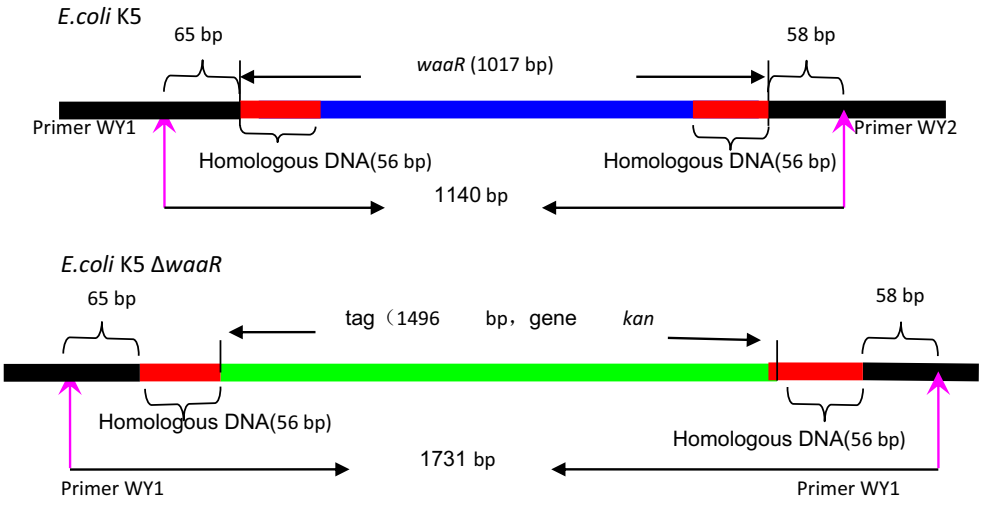

Fig. 1 Structure of the *waaR* region in wild type and mutant

on the above results, the knockout strategy was designed as in Fig. 1. Within the 1017 base pairs of gene *waaR*, 56 base pairs that are highlighted in red are homologous DNA fragments in Primer P1 and P2 respectively. The sequence indicated with a pink arrow is homologous DNA in primer WY1 and WY2, which were 65 and 58 bp away from the *waaR* gene, respectively. Those base pairs in green were the screening tag with gene *kan* that amplified from the plasmid pKD4.

As shown in Fig. 1, the length between primer WY1 and WY2 was 1140 bp in wild type while 1731 bp in the mutant. The agarose gel electrophoresis results (Fig. 2) showed that the DNA amplified from the mutant (PCR product) was in the right position between 1500 to 2000 bp and longer than that from the wild type, suggesting that the knockout was successful. The PCR product of the mutant was sequenced and the result was shown in Fig. 3. In Fig 3, the base pairs highlighted in blue were the homologous DNA, while the 5′-upstream and 3′-downstream of which was the sequence of outer space gene of *waaR*. The sequences in yellow are the primers of the screening tag copied from the plasmid pKD4 with the antibiotic gene *kan* and FRT locus. All the homologous DNA fragments were complete and the gene *waaR* in the *E. coli* K5 was replaced with the screening tag. However, there was one base omitted within the replaced DNA sequence (shown in red, Fig. 3). It is assumed that the base deletion occurred in template plasmid pKD4. In summary, the sequence length of mutant PCR product was 1607 bp and consistent to the assumption in Fig. 1. The gene *waaR* was successfully knocked out from *E. coli* K5, and the mutant was named as *E. coli* K5Δ*waaR* for use in subsequent experiments.

Morphology of *E. coli* K5 Δ*waaR*

Wild type and the mutant of *E. coli* K5 were examined by transmission electron microscopy (TEM) (Fig. 4). The cell surface of the wild type shows a thicker, rougher capsular polysaccharide than the mutant. The cell surface of the mutant looked smoother with less capsular polysaccharide, the result of the deletion of the *waaR* gene. The mutant could not express α-1,2-glycosyltransferase for LPS synthesis resulting in the defect of the biosynthesis of LPS outer coat and the link of the capsular polysaccharide on the cell surface.

Heparosan production by *E. coli* K5 Δ*waaR*

The wild type and the mutant of *E. coli* K5 were inoculated into the same DGM medium and incubated under the same culture conditions to evaluate the effect of deletion of gene *waaR* on the heparosan production by *E. coli* K5. The results are shown in Fig. 5. It was found that the mutant showed higher productivity of heparosan than the wild type, but this increase was not significant.

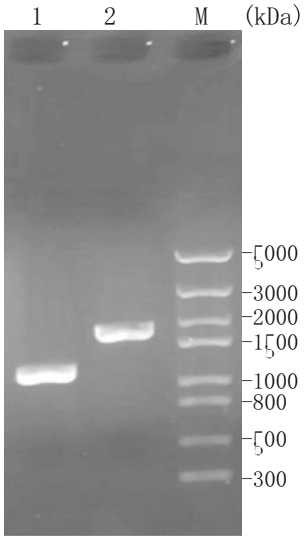

Fig. 2 PCR identification of *waaR* mutant: *lane 1* PCR products of *E. coli* K5 wild type; *lane 2* PCR products of *E. coli* K5Δ*waaR* strain; *lane M* DNA molecular marker (Trans5k DNA marker)

Fig. 3 *E. coli* K5 Δ*waaR* recombination region sequence

CTGAATCATGTTAACCAGTTAAATGTTATTTACGGTAATATTTTCGGCAAAGATAACACACTCCTGCAATGAGT
CCTGAGAATGGGAATTAGCCATGGTCCATATGAATATCCTCCTTAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAG
TATAGGAACTTCAGAGCGCTTTTGAAGCTGGGGTGGGCGAAGAACTCCAGCATGAGATCCCCGCGCTGGAGGAT
CATCCAGCCGGCGTCCCGGAAAACGATTCCGAAGCCCAACCTTTCATAGAAGGCGGCGGTGGAATCGAAATCTC
GTGATGGCAGGTTGGGCGTCGCTTGGTCGGTCATTTCGAACCCCAGAGTCCCGCTCAGAAGAACTCGTCAAGAA
GGCGATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTC
GCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGGCC
ACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGCATCGCCATGGGTCACGAC
GAGATCCTCGCCGTCGGGCATGCGCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCTGATGCTCTT
CGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTG
GTCGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGC
AGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGT
GACAACGTCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGTCCTGCA
GTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAAAAAGAACCGGGCGCCCCTGCGCTGACAGCCGGAACAC
GGCGGCATCAGAGCAGCCGATTGTCTGTTGTGCCCAGTCATAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGA
ACCTGCGTGCAATCCATCTTGTTCAATCATGCGAAACGATCCTCATCCTGTCTCTTGATCAGATCTTGATCCCCTGCG
CCATCAGATCCTTGGCGGCAAGAAAGCCATCCAGTTTACTTTGCAGGGCTTCCCAACCTTACCAGAGGGCGCCCC
AGCTGGCAATTCCGGTTCGCTTGCTGTCCATAAAACCGCCCAGTCTAGCTATCGCCATGTAAGCCCACTGCAAGCT
ACCTGCTTTCTCTTTGCGCTTGCGTTTTCCCTTGTCCAGATAGCCCAGTAGCTGACATTCATCCGGGGTCAGCACCG
TTTCTGCGGACTGGCTTTCTACGTGTTCCGCTTCCTTTAGCAGCCCTTGCGCCCTGAGTGCTTGCGGCAGCGTGAG
GGGATCTTGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCGAAGCAGCTCCAGCCTACAC
AGCCTAAAATCCCAGGCTTTAACTTTATCTATCTCTATGGCAGGAAATGAGTCCACAAGGCTATCCTTATATCA

PAGE was run to analyze the molecular weight distribution of heparosan of *E. coli* K5 Δ*waaR*. Using heparin as control, 35 μL heparosan and heparin samples were loaded to measure the MW distribution. The results (Fig. 6) showed that the polysaccharides of the mutant distributed and was concentrated in lower molecular weight regions of the gel while that of the wild type distributed and concentrated in the high molecular weight region on the gel. These results suggest that the molecular weight of heparosan in the mutant had decreased as the result of deletion of *waaR*.

The polysaccharide within the entire lane of the separating gel was extracted from the gel and used for GPC after desalting and lyophilization. The GPC results of the mutant heparosan showed that the main peak appeared at 5.17 min and covered 62.50 % of the total scale (Fig. 7a). Meanwhile, the GPC results of the wild-type heparosan showed that the main peak appeared at 6.86 min. These results further indicated that the molecular weight of heparosan in the mutant decreased resulting from the deletion of *waaR*.

Discussion

The knockout primers were designed based on the gene *waaR* in *E. coli* F632. The successful construction of the △*waa*R strains proved that the gene *waaR* in *E. coli* K5 is highly

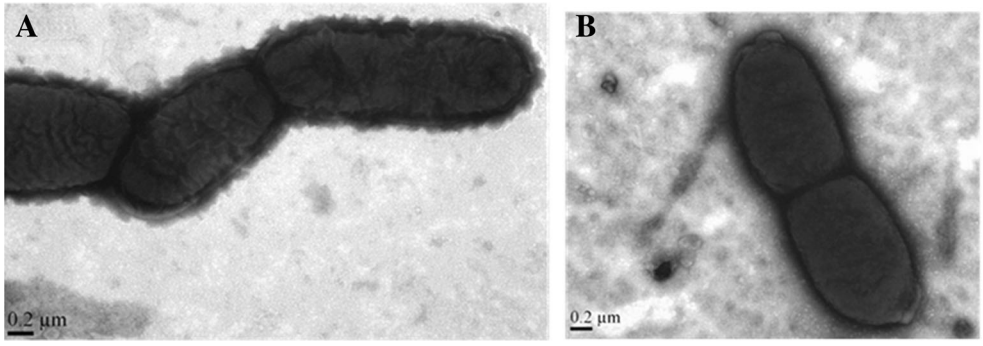

Fig. 4 Electron micrographs of *E. coli* K5 and *E. coli* K5 Δ*waaR* by TEM. **a** *E. coli* K5 cultured on agar plate, ×40,000. **b** *E. coli* K5Δ*waaR* cultured on agar plate, ×40,000

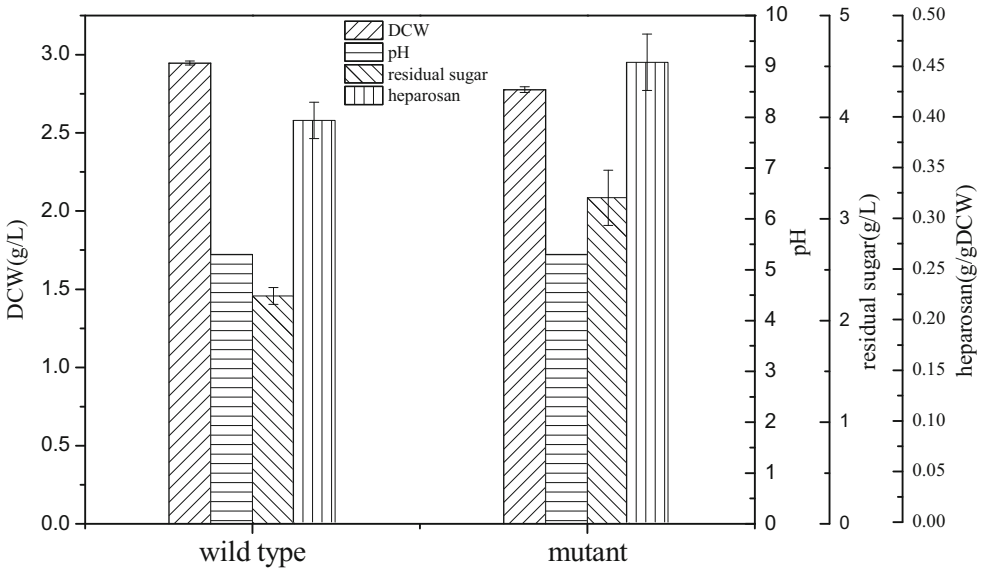

Fig. 5 Fermentation profiles in DGM medium for *E. coli* K5 wild type and mutant *E. coli* K5 Δ*waaR*

homogenous to that in *E. coli* F632 (Amor et al. 2000). The electron micrographs of *E. coli* K5 and *E. coli* K5Δ*waaR* by transmission electron microscope showed that the capsule of mutant was smoother than the capsule of the wild-type organism (Fig. 4a, b). This observation agrees with the assumption that the deletion of gene *waaR* in *E. coli* K5 is helpful in the shedding of the capsule polysaccharide (Amor et al. 2000).

The growth of the mutant was slightly weaker than that of the growth of the wild-type organism under the same conditions (Fig. 5). The reason for this might be that the deletion of the gene *waaR* from *E. coli* K5 resulted in a defect in the synthesis capsule polysaccharide, which reduced the resistance of osmosis (Huang et al. 2013a). The high glucose concentration in DGM would result in a too high osmotic pressure for the mutant to grow well. However, the mutant produced more polysaccharide on the shake flask scale.

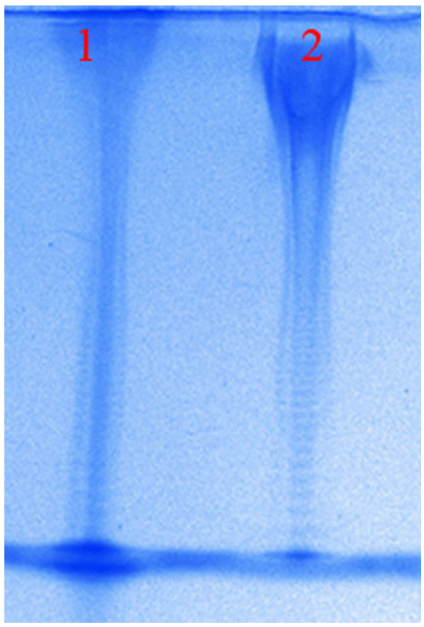

Fig. 6 PAGE analysis on the capsule polysaccharides from *E. coli* K5 wild type and mutant. *Lane 1* the capsule polysaccharide of mutant (0.6 mg/L). *Lane 2* the capsule polysaccharide of wild type (0.5 mg/mL)

In addition, the deletion of gene *waaR* also might result in the metabolic pathway change under the same conditions with the wild type. Therefore, in the future, proper fermentation medium and conditions will require optimization (Huang et al. 2013a). Meanwhile, the separation technology and the conditions of heparosan expression from the mutant also require optimization, as these might be different from that of the wild type. The ammonium sulfate precipitation of heparosan is more easily affected by the ions in the supernatant than ethanol precipitation (data not shown). An interesting finding of this study is that the molecule of the capsular polysaccharide produced by the mutant was smaller and this impacted the separation process. Ethanol was better than the ammonium sulfate for the precipitation of the capsular polysaccharide. The capsular polysaccharide of mutant distributed and concentrated in the low molecular weight region of the gel lane, while the wild-type capsular polysaccharide distributed and concentrated in the high molecular weight region (Fig. 6). This result is especially interesting and suggests that the defect of gene *waaR* result in the capsule polysaccharide results in a molecular change making it smaller than the wild type. GPC shows a peak for the capsular polysaccharide from the mutant that elutes after the capsular polysaccharide from the wild-type organism (Fig. 7a, b), consistent with the results of PAGE analysis. The *waaR* gene in *E. coli* K5 encodes the α-1, 2-glycosyltransferase that is involved in the synthesis of

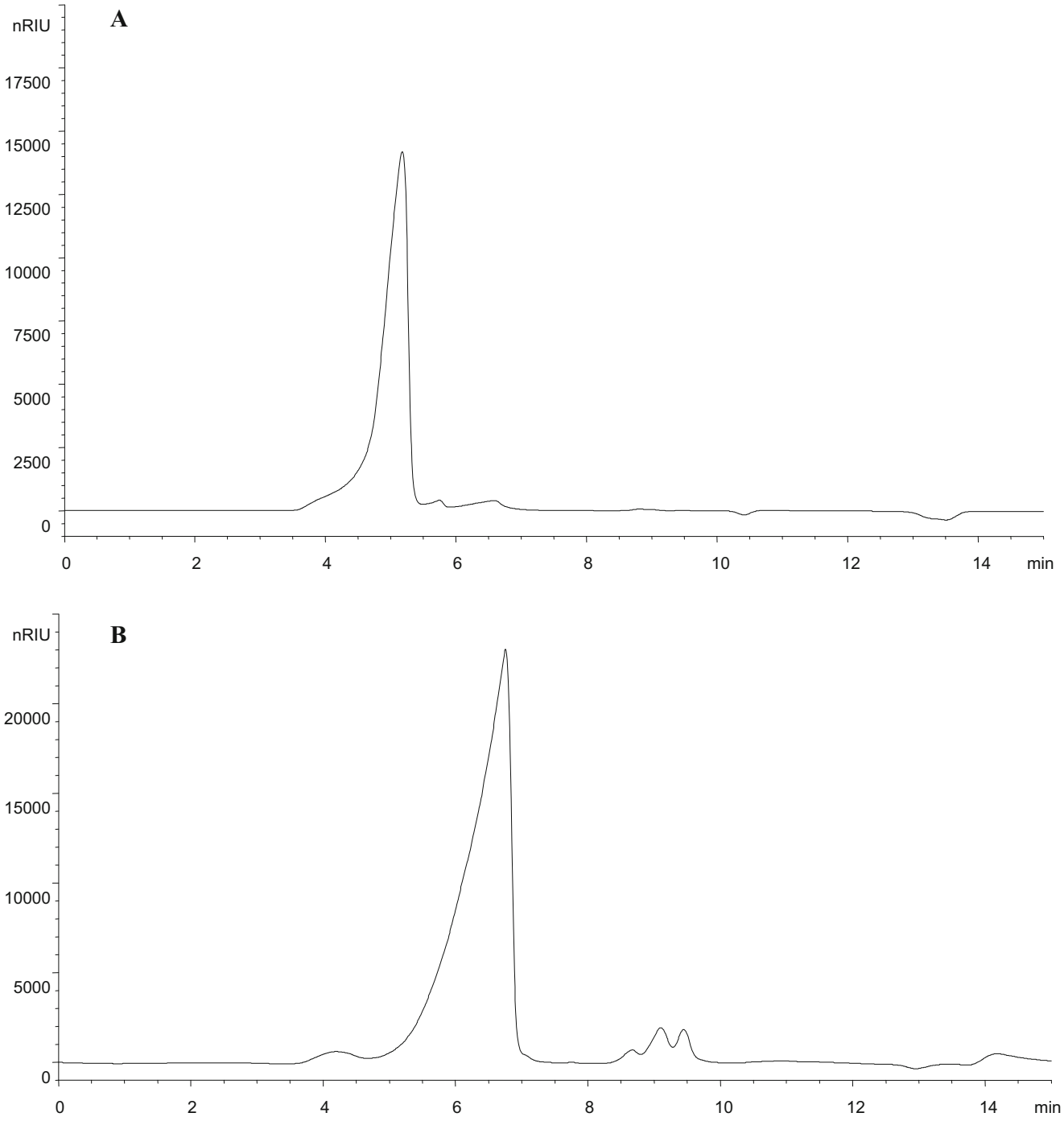

Fig. 7 GPC chromatograms of capsule polysaccharide from *E. coli* K5 wild type (**a**) and mutant (**b**)

lipopolysaccharide (Amor et al. 2000). Deletion of *waaR* gene results in the chain of the capsular polysaccharide becoming shorter. It suggests that there is the potential for preparing LMWH derivatives derived from a low molecular weight heparosan without the requirement of a depolymerization step.

The market of the LMWH has become larger, and the common path of the preparation of LMWH requires depolymerization, such as photochemical depolymerization (Higashi et al. 2011, 2012) or enzymatic depolymerization (Wu et al. 2014; Brown and Kuberan 2015). *E. coli* K5 was chosen to produce the precursor of heparin because of the basic structure of capsule polysaccharide (heparosan) produced by *E. coli* K5 similar to the heparin prepared from mammals. However, the molecular weight of heparosan from *E. coli* K5 is usually is too high and not conducive to the preparation of LMWH. With the knockout of the *waaR* gene from *E. coli* K5, the productivity of the heparosan increased and the molecular

weight of the capsular polysaccharide became lower. While the improvement of the productivity of the heparosan was not significant compared to the wild type, this is a potential strategy for the improved preparation of LMWH derived from heparosan directly without the required enzymatic depolymerization by deleting the *waaR* gene from *E. coli* K5. This is an easier method to meet the needed molecular weight requirements without the use of enzymatic depolymerization.

Acknowledgments This study was funded by the Natural Science Foundation of China (Grant No. 21076195), the Science Technology Department of Zhejiang Province (Grant No. 2011C24007), the Specialized Research Fund for the Doctoral Program of Higher Education (Grant No. 20113317110002), and the Open Research Fund of Zhejiang Province Top Key Discipline of Biological Engineering (2015).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

Amor K, Heinrichs DE, Frirdich E, Ziebell K, Johnson RP, Whitfield C (2000) Distribution of core oligosaccharide types in lipopolysaccharides from *Escherichia coli*. Infect Immun 68(3):1116–1124

Bhaskar U, Sterner E, Hickey AM, Onishi A, Zhang F, Dordick JS, Linhardt RJ (2012) Engineering of routes to heparin and related polysaccharides. Appl Microbiol Biotechnol 93(1):1–16

Bauer KA, Hawkins DW, Peters PC, Petitou M, Herbert JM, van Boeckel CAA, Meuleman DG (2002) Fondaparinux, a synthetic pentasaccharide: the first in a new class of antithrombotic agents–the selective factor Xa inhibitors. Cardiovasc Drug Rev 20(1):37–52

Bitter T, Muir HM (1962) A modified uronic acid carbazole reaction. Anal Biochem 4(4):330–334

Brown S, Kuberan B (2015) Production of size-defined heparosan, heparan sulfate, and heparin oligosaccharides by enzymatic depolymerization. Methods Mol Biol 1229:21–29

Chen JH, Avci FY, Munoz EM, McDowell LM, Chen M, Pedersen LC, Zhang LJ, Linhardt RJ, Liu J (2005) Enzymatic redesigning of biologically active heparin sulfate. J Biol Chem 280(52):42817–42825

Cress BF, Englaender JA, He W, Kasper D, Linhardt RJ, Koffas MAG (2014) Masquerading microbial pathogens: capsular polysaccharides mimic the host-tissue molecules. FEMS Microbiol Rev 38: 660–697

Cress BF, Toparlak ÖD, Guleria S, Lebovich M, Stieglitz JT, Englaender JA, Jones JA, Linhardt RJ, Koffas MAG (2015) CRISPathBrick: modular combinatorial assembly of type II-A CRISPR arrays for dCas9-mediated multiplex transcriptional repression in *E. coli*. ACS Synth Biol 4(9):987–1000

DeAngelis PL, Gunay NS, Toida T, Mao WJ, Linhardt RJ (2002) Identification of the capsular polysaccharides of type D and F *Pasteurell amultocida* as unmodified heparin and chondroitin, respectively. Carbohydr Res 337:1547–1552

Frirdich E, Lindner B, Holst O, Whitifield C (2003) Overexpression of the *waaZ* gene leads to modification of the structure of the inner core region of *Escherichia coli* lipopolysaccharide, truncation of the outer core, and reduction of the amount of O polysaccharide on the cell surface. J Bacteriol 185(5):1659–1671

He WQ, Fu L, Li G, Jones JA, Linhardt RJ, Koffas M (2015) Production of chondroitin in metabolically engineered *E. coli*. Metab Eng 27: 92–100

Heinrichs DE, Yethon JA, Whitfield C (1998) Molecular basis for structural diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and *Salmonella enterica*. Mol Microbiol 30(2): 221–232

Higashi K, Hosoyama S, Ohno A, Masuko S, Yang B, Sterner E, Wang ZY, Linhardt RJ, Toida T (2012) Photochemical preparation of a novel low molecular weight heparin. Carbohydr Polym 87(2): 1737–1743

Higashi K, Ly M, Wang ZY, Masuko S, Bhaskar U, Sterner E, Zhang FM, Toida T, Dordick JS, Linhardt RJ (2011) Controlled photochemical depolymerization of K5 heparosan, a bioengineered heparin precursor. Carbohydr Polym 86(3):1365–1370

Huang HC, Zhao YY, Lv SC, Zhong WH, Zhang FM, Linhardt RJ (2014) Quantitation of heparosan with heparin lyase III and spectrophotometry. Anal Biochem 447:46–48

Huang HC, Li XL, Li N, Zhong WH (2013a) Effect of enviromental stresses on heparosan biosynthesis by *Escherichia coli* K5. J Pure Appl Microbiol 7(4):3025–3029

Huang HC, Lv SC, Zhao L, Yan ZQ, Zhong WH, Zhang FM (2013b) Capsule treatment by soluble expressed recombinant heparinase II enhance the foreign gene transformation to *Escherichia coli* K5. J Pure Appl Microbiol 7(4):3009–3015

Linhardt RJ (2003) 2003 Claude S. Hudson Award address in carbohydrate chemistry. Heparin: structure and activity. J Med Chem 46(13): 2551–2554

Linhardt RJ, Gunay NS (1999) Production and chemical processing of low molecular weight heparins. Sem Thromb Hemostas 25(3):5–16

Linhardt RJ, Liu J (2012) Synthetic heparin. Curr Opin Pharmacol 12(2): 217–219

Li PL, Sheng JZ, Liu YH, Li J, Liu J, Wang FS (2013) Heparosan-derived heparan sulfate/heparin-like compounds: one kind of potential therapeutic agents. Medicinal Res Rev 33(3):665–692

Lohse DL, Linhardt RJ (1992) Purification and characterization of heparin lyases from *Flavobacterium heparinum*. J Biol Chem 267(34): 24347–24355

Petitou M, van Boeckel CAA (2004) A synthetic antithrombin III binding pentasaccharide is now a drug! What comes next? Angew Chemie-Int Ed 43(24):3118–3133

Raetz CRH, Whitfield C (2002) Lipopolysaccharide endotoxins. Ann Rev Biochem 71:635–700

Suflita M, Fu L, He W, Koffas M, Linhardt RJ (2015) Heparin and related polysaccharides: synthesis using recombinant enzymes and metabolic engineering. Appl Microbiol Biotechnol 99:7465–7479

Su GW, Li LH, Huang HC, Zhong WH, Yu P, Zhang FM, Linhardt RJ (2015) Production of a low molecular weight heparin production using recombinant glycuronidase. Carbohydr Polym 134:151–157

Taylor CM, Goldrick M, Lord L, Roberts IS (2006) Mutations in the *waaR* gene of *Escherichia coli* which disrupt lipopolysaccharide outer core biosynthesis affect cell surface retention of group 2 capsular polysaccharides. J Bacteriol 188(3):1165–1168

Wang Z, Ly M, Zhang F, Zhong W, Suen A, Hickey AM, Dordick JS, Linhardt RJ (2010) *E. coli* K5 fermentation and the preparation of heparosan, a bioengineered heparin precursor. Biotechnol Bioengineer 107(6):964–973

Wang ZY, Dordick JS, Linhardt RJ (2011a) *Escherichia coli* K5 heparosan fermentation and improvement by genetic engineering. Bioengineer Bugs 2(1):63–67

Wang ZY, Yang B, Zhang ZQ, Ly M, Takieddin M, Mousa S, Liu J, Dordick JS, Linhardt RJ (2011b) Control of the heparosan N-deacetylation leads to an improved bioengineered heparin. Appl Microbiol Biotechnol 91(1):91–99

Whitfield C, Roberts IS (1999) Structure, assembly and regulation of expression of capsules in *Escherichia coli*. Mol Microbiol 31(5): 1307–1319

Wu JJ, Zhang C, Mei X, Li Y, Xing XH (2014) Controllable production of low molecular weight heparins by combinations of heparinase I/II/III. Carbohydr Polym 101:484–492

Zhang ZQ, McCallum SA, Xie J, Nieto L, Corzana F, Jimenez-Barbero J, Chen M, Liu J, Linhardt RJ (2008) Solution structures of chemoenzymatically synthesized heparin and its precursors. J Am Chem Soc 130(39):12998–13007