



Short communication

Effect of eliminase gene (*elmA*) deletion on heparosan production and shedding in *Escherichia coli* K5

Anne Marie Hickey^a, Ujjwal Bhaskar^{a,b}, Robert J. Linhardt^{a,b,c,d},
Jonathan S. Dordick^{a,b,d,e,*}

^a Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

^b Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

^c Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

^d Department of Biology, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

^e Department of Material Sciences and Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

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ABSTRACT

Escherichia coli K5 produces heparosan and sheds it into the growth medium in a temperature dependent manner. The shedding is believed to be controlled, at least in part, by enzyme action on the cell-associated capsular polysaccharide, heparosan. One candidate enzyme in such shedding is eliminase. The eliminase gene (*elmA*) was deleted from the genome of *E. coli* K5 and its effect on secreted and cell-associated heparosan was investigated. Deletion of the eliminase gene resulted in a significant reduction in heparosan shedding into the medium and heparosan content in the capsule of the cells, indicating its pivotal role in heparosan synthesis and shedding by *E. coli* K5.

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N-Acetyl heparosan (heparosan), the capsular polysaccharide of *Escherichia coli* O10:K5:H4 (ATCC 23506) (*E. coli* K5), is similar to heparin, with a higher acetyl content. Since it lacks sulfation and iduronic acid residues, heparosan possesses no antithrombin III binding-site (Linhardt, 2003). Heparosan consists of repeating disaccharide units comprised of [→4] β-D-glucuronic acid (GlcA) (1→4) α-D-N-acetyl-glucosamine (GlcNAc) (1→)_n (Wang et al., 2010). Because of its polysaccharide similarity to heparin, heparosan is a promising starting material for the production of heparin by chemo-enzymatic synthesis (Masuko and Linhardt, 2012; Karst and Linhardt, 2003; Xu et al., 2012). Indeed, the synthesis and shedding of large amounts of heparosan by *E. coli* K5 is essential for the production of bioengineered heparin for potential use as a replacement of the animal-sourced drug (Wang et al., 2010).

The biosynthesis of heparosan in *E. coli* K5 involves formation of two key metabolic intermediates, UDP-glucose and UDP-N-acetyl-glucosamine, formed by *kfiD*. This is followed by a series of biotransformations catalyzed by enzymes encoded by two open reading frames, *kfiA* and *kfiC*, to synthesize heparosan (Hodson

et al., 2000). Heparosan is synthesized in the cytosol, transferred outside the cell by an ABC transporter-dependent mechanism (Whitfield and Roberts, 1999; Whitfield, 2006), and the reducing end is believed to be attached to a phosphatidic acid residue. Two enzymes that have been implicated in the shedding of heparosan by *E. coli* K5 are K5 lyase (KflA), from the K5A bacteriophage (Clarke et al., 2000) and eliminase (ElmA), from the *elmA* gene in the *E. coli* K5 genome (Legoux et al., 1996). These lyases display endo-β-eliminase activity, resulting in a 4,5-unsaturated uronic acid (ΔUA) moiety at the non-reducing end of the heparosan chain. In the present work *elmA* was deleted to obtain the deletion clone, Δ*elmA*. The effect of eliminase gene deletion on the production and secretion of heparosan was thus investigated and compared with that of the wild-type strain.

Legoux et al. (1996) previously investigated eliminase by focusing on cloning and overexpression of the enzyme in *E. coli* K-12 RR1; however, the specific role of eliminase on the heparosan producing strain, *E. coli* K5, remains unknown. Therefore, we endeavored to elucidate the role of the *elmA* gene product on heparosan production and shedding from *E. coli* K5.

Gene deletion was achieved using the Quick & Easy *E. coli* Gene Deletion Kit (B-Bridge International, Inc., Cupertino, CA), and *elmA* gene deletion was confirmed by PCR (Fig. 1). *E. coli* K5 and *E. coli* K5 (Δ*elmA*) were grown in LB medium and the undiluted supernatant was applied to a gradient PAGE gel (4–15%) (Fig. 1), which

* Corresponding author at: Rensselaer Polytechnic Institute, 4005 Center for Biotechnology and Interdisciplinary Studies, 110 8th Street, Troy, NY 12180, USA. Tel.: +1 518 276 2899.

E-mail address: dordick@rpi.edu (J.S. Dordick).

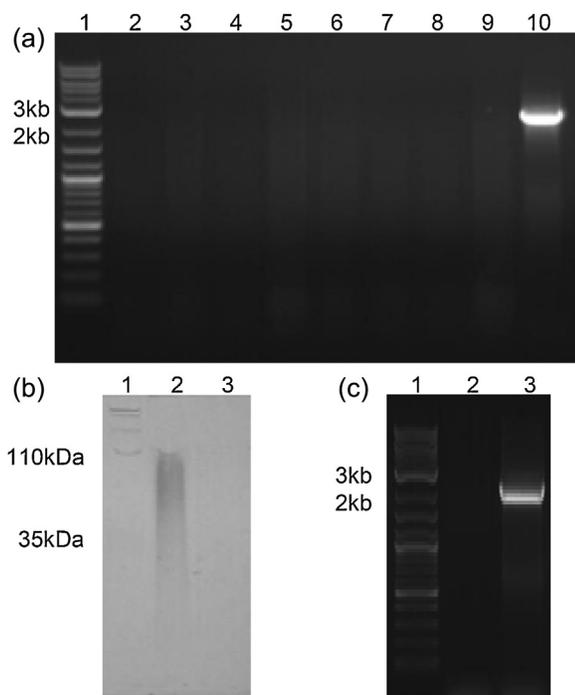


Fig. 1. (a) Gel electrophoresis of PCR products obtained from *E. coli* K5 ($\Delta elmA$) (lane 2–9) and wild-type *E. coli* K5 (lane 10) using PCR primers for the eliminase gene (*elmA*), with 2-log DNA ladder (lane 1); (b) PAGE gel of supernatant heparosan from *E. coli* K5 (lane 2) and *E. coli* K5 ($\Delta elmA$) (lane 3), with Hyalose loladder (lane 1); (c) Gel electrophoresis of PCR products obtained from *E. coli* Nissle 1917 (lane 2) and *E. coli* K5 (positive control) (lane 3), with 2-log DNA ladder (lane 1).

revealed significant reduction in levels of heparosan shedding by *E. coli* ($\Delta elmA$). Indeed, no supernatant heparosan was observed in gels stained with Alcian blue, suggesting that eliminase plays a key role in shedding of heparosan from the cells. All further analysis was done using size exclusion chromatography (SEC), since it was not dependent on charge density. Growth was also switched to M9 medium (Sambrook and Russell, 2001) to increase heparosan purity, as LB has been shown previously to generate more impurities (Wang et al., 2010).

Prior to SEC analysis, supernatant heparosan was concentrated 10-fold using a 3 kDa MWCO centrifugal concentrator, filtered through a 0.22- μ m filter, and applied to a TSKgel G4000-PWxl SEC column (Tosoh Biosciences, Grove City, OH, USA) for analysis. Cell-associated heparosan, concentrated 33-fold when extracted from cell pellets using Tris buffer containing Triton X-100 and sodium deoxycholate, was filtered prior to analysis. Samples were not purified further, since this could result in loss of heparosan. Using a calibration curve and a heparosan standard (10 mg/ml), SEC post-run analysis was performed to determine heparosan concentration, M_N , M_W , polydispersity (Table 1). SEC results confirmed a reduction in supernatant heparosan levels, with a 50% drop in concentration of heparosan detected in the medium from the *E. coli* K5 ($\Delta elmA$) strain. Deletion of *elmA* resulted in a reduction in supernatant

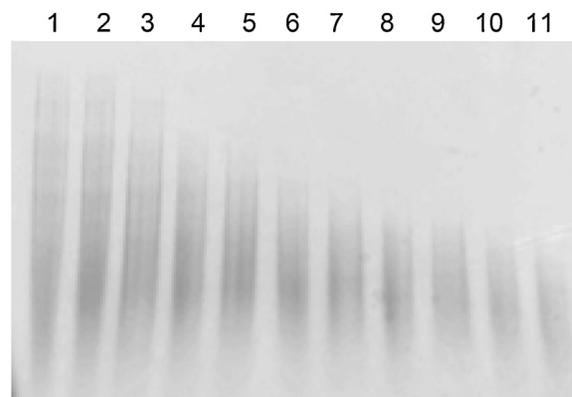


Fig. 2. PAGE gel of supernatant heparosan degradation by *E. coli* K5 cell lysate, samples taken after 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0 h incubation at 37 °C and 220 rpm.

heparosan M_N (56,450–46,480 Da), M_W (130,500–59,070 Da) and polydispersity (2.31–1.27) (Table 1). In the case of cell-associated heparosan, there was ca. 60% drop in heparosan concentration with the $\Delta elmA$ mutant. Deletion of *elmA* also caused a reduction in M_N (32,940–21,160 Da), and M_W (64,390–45,350 Da), and a slight increase in polydispersity (1.95–2.14) in the cell-associated heparosan when compared to wild-type *E. coli* K5 (Table 1).

These observations suggest that deletion of *elmA* may result in feedback inhibition in the heparosan synthetic pathway, thereby causing a reduction in heparosan production and a reduction in chain elongation for cell-associated and shed heparosan. Indeed, the total heparosan production by the deletion strain was ca. 45% of that of the wild-type *E. coli* K5. These findings are in agreement with the suggestion by Roberts and coworkers that eliminase may play a regulatory role in capsule synthesis (Clarke et al., 2000), and promoting the accumulation of heparosan.

Not all K5 strains contain eliminase, PCR analysis indicated that *E. coli* Nissle 1917 did not possess the gene for eliminase (Fig. 1) and, thus, this strain was used for comparison. Interestingly, SEC analysis indicated that supernatant heparosan produced by *E. coli* Nissle 1917 exhibited greater similarity to *E. coli* K5 ($\Delta elmA$) than wild-type *E. coli* K5. Thus, longer heparosan chains in the supernatant of wild-type *E. coli* K5 fermentation may indeed be due to eliminase, while the intermediate-length chains obtained with *E. coli* K5 ($\Delta elmA$) and *E. coli* Nissle 1917 may be due to one or more other, as yet unidentified, enzymes.

E. coli Nissle 1917 was found to generate higher levels of heparosan in the supernatant, producing 0.25 mg/ml heparosan (250 mg/g cells) as compared to 0.10 mg/ml (100 mg/g cells) generated by wild-type *E. coli* K5. It may be that eliminase further degrades heparosan once shed, leaving mainly unreacted high molecular weight heparosan and small oligosaccharides and disaccharides; a hypothesis supported by the ability of eliminase to degrade heparosan *in vitro* (Fig. 2). An alternative hypothesis is that *E. coli* Nissle 1917 possesses a more effective mechanism for heparosan shedding, generating heparosan of lower polydispersity, a

Table 1
Characteristics of heparosan from *E. coli* K5, *E. coli* K5 ($\Delta elmA$) and *E. coli* Nissle 1917.

Strain	Location	M_N	M_W	Polydispersity	Concentration (mg/ml)	Specific productivity (mg/g cells)
<i>E. coli</i> K5	Cell-associated	32,940	64,390	1.95	0.16	
	Supernatant	56,450	130,500	2.31	0.10	100
<i>E. coli</i> K5 ($\Delta elmA$)	Cell-associated	21,160	45,350	2.14	0.06	
	Supernatant	46,480	59,070	1.27	0.05	50
<i>E. coli</i> Nissle 1917	Cell-associated	46,620	77,930	1.67	0.08	
	Supernatant	46,020	75,420	1.64	0.25	250

mechanism that may be downregulated by eliminase in *E. coli* K5. The results do indicate that deletion of *elmA* resulted in the similarity between *E. coli* K5 ($\Delta elmA$) and *E. coli* Nissle 1917 supernatant heparosan. It should be noted that it is possible that the activity of eliminase caused an underestimation of heparosan levels in the supernatant of *E. coli* K5, since supernatant heparosan from *E. coli* Nissle 1917 had similar M_N and M_W to *E. coli* K5 ($\Delta elmA$).

Conversely, the profile of cell-associated heparosan extracted from *E. coli* Nissle 1917 resembled that of wild-type *E. coli* K5 (Table 1), although *E. coli* Nissle 1917 produced significantly less cell-associated heparosan (50%) than wild-type *E. coli* K5, suggesting that the presence of eliminase results in upregulation of capsular heparosan production to cope with the demands of eliminase. This could explain the relatively higher ratio of cell-associated heparosan to supernatant heparosan in *E. coli* K5 compared with *E. coli* K5 ($\Delta elmA$) and *E. coli* Nissle 1917. The variation in capsule thickness could also explain why *E. coli* K5 is an opportunistic pathogen and *E. coli* Nissle 1917 is a probiotic strain, since *E. coli* K5 would more likely be prone to biofilm formation and *E. coli* Nissle 1917 would likely be motile, as suggested by Zorraquino et al. (2013).

In conclusion, deletion of the gene encoding eliminase from the genome of *E. coli* K5 resulted in a dramatic change in the shedding and physical characteristics of *E. coli* K5 heparosan, with significant reduction in accumulation of heparosan in the cell capsule and in the culture supernatant.

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