JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2013. Vol. 7(4), p. 3025-3029

Effect of Environmental Stresses on Heparosan Biosynthesis by *Escherichia coli* K5

Haichan Huang, Xueliang Li, Ning Li and Weihong Zhong*

College of Biological and Environmental Engineering,
Zhejiang University of Technology, Hangzhou, 310032, PR China.

(Received: 23 September 2013; accepted: 06 November 2013)

Heparosan, the capsular polysaccharide (CPS) of some pathogenic microorganisms, play an important role in protecting bacteria from the effects of the enviromental stresses. It is almost identical to haprin and can be used as the key precursor for the preparation of bioengineered heparin and heparan sulfate. In this study, the heparosan production and growth of *Escherichia coli* K5 under the stresses, of osmotic, $SnCl_2$, organic solvents (ethanol, formamide, and hexane), and H_2O_2, were evaluated. The results indicated that the growth of *E. coli* K5 was not affected, while the production of heparosan was influenced under these stresses. The addition of appropriate amount of methinine, glycine, $SnCl_2$, and organic solvents, is helpful to heparosan production. High concentration of NaCl and H_2O_2 are unfavourable for heparosan production. Moreover, *E. coli* KT (with *waaR* gene deletion) showed the similar response to the above stress.

Key words: *Escherichia coli*, CPS, Heparosan, Stress, *waaR*.

Heparin, an anticoagulant and antithrombotic drug, has been used widely and has aroused increasing interest in anticancer and anti- inflammation therapies. Until now commercial heparin has been extracted from animal tissues resulting in complex purification procedures because of viral and prionic contamination risks and high costs. Therefore, it is becoming more attractive to produce bioengineered heparin by microbial fermentation.

Heparosan, a capsular polysaccharide (CPS) obtained from the fermentation of *Escherichia coli* K5 or *Pasteurella multicida*[1,2], is constituted of alternating α-N-acetyl-α-glucosamine [GlcNAc] and β-D-glucuronic acid [GlcA], which is identical to animal-derived heparin in backbone structure. Thus, heparosan can be used as the key precursor for the preparation of bioengineered heparin and heparan sulfate.

Many strains of *Escherichia coli* exhibit favorable tolerance to adverse environmental stresses. When exposed to the environmental stresses, the property of microorganisms will change, including production of stress-responding metabolites, harbor of resistance plasmid, and synthesis of protective surface appendages. CPSs are important surface structures necessary for bacterial survival, and protect bacterial cells from adverse environments associated with desiccation, osmotic or oxidative stresses, confer resistance to antimicrobial compounds, and aid in the evasion of the host immune response[3,4]. Clearly, CPSs are definitely among the most important defense mechanisms for microorganism under the stresses and capsular production is a protective means of cells to survive under stress. The expression of CPS has been shown to be influenced by culture conditions and nutrient composition of the growth medium[5]. The presence of sugar and salt in media stimulates exopolysaccharide colanic acid production in *Aeromonas salmonicida*[6]. This

* To whom all correspondence should be addressed.
TeleFax: +86 571 88320739;
E-mail: whzhong@zjut.edu.cn

study aims to evaluate the effect of environmental stresses, including organic reagent stress, osmotic stress, heavy metal stress, and oxidative stress, on heparosan biosynthesis by *E. coli* K5.

MATERIALS AND METHODS

Microorganism and heparosan production

E. coli K5 was kindly supplied by Professor Jian Liu (The University of North Carolina at Chapel Hill) and *E. coli* KT was the engineered strain which had *waaR* gene deletion in *E. coli* K5. The cultivation steps used in this study were followed by the culture method of Wang using *E. coli* K5 [7].

Determination method of biomass

E. coli K5 cells were harvested from broth by centrifuge at 12,000 × g for 30 min (Sorvall biofuge Stratos, Thermo SCIENTIFIC, USA). The cells was washed by physiological saline twice, and dried to constant weight under 60°C. Finally, it was weighed.

Crude extract of heparosan polysaccharide

Five milliliter of the supernatant of *E. coli* K5 fermentation broth was sampled and mixed with three-time volumes of ethanol, and then stored at 4! overnight in an explosion-proof freezer for completely precipitation of heparosan. The precipitate was harvested by centrifuge at 12,000 × g for 15 min and dissolved in 5mL distilled water after the ethanol was volatilized completely. Then the heparosan concentration was determined by the sulfuric acid-carbazole method.

Determination of heparosan concentration

Heparosan is composed of equimolar amounts of N-acetyl-α-glucosamineand and D-glucuronic acid, which D-glucuronic acid can be determined by the sulfuric acid-carbazole method[8]. A standard curve between the concentration of D-glucuronic acid and OD_{530} was first established with a detection range of 10-50 μg/mL of D-glucuronic acid. From the standard curve, a linear fitting equation was obtained as Y=0.0173X+0.0052 (R^2=0.9999), in which X axis was the concentration of D-glucuronic acid and Y axis was its absorbance at 530 nm.

Organic solvents stress

Organic solvents stress method setting and the range of concentration setting were used following the method as described by Gupta *et al*.,[9]. *E. coli* K5 and KT were inoculated in 250 mL flasks containing 30 mL LB medium and incubated for 18h at 37 °C and 200 rpm. Then, ethanol, formamide, and hexane with a concentration of 2, 3, and 4% (v/v), were added respectively. The flasks were sealed with butyl rubber stoppers to prevent organic solvents from evaporation and incubate at 37 °C and 200 rpm for 3 h. Meanwhile, 5 mL broth were withdrawn and centrifuged at 10,000 × g for 5 min. The precipitate was washed by sterile saline twice, and then suspended in 5 mL sterile saline as inoculum for fermentation in the glucose-defined medium. The experiments were in triplicate with a control that without organic solvents not be treated.

RESULTS AND DISCUSSION

Effect of exogenous amino acids on heparosan production of *E. coli* K5

Generally, amino acids are necessary to microorganisms in a concentration range of 0.01 g/L-0.1 g/L. Methionine and Glycine were first selected and added to glucose-defined medium at concentrations of 0.0 g/L, 0.1g/L, 0.2 g/L, 0.3 g/L, 0.4 g/L, 0.5 g/L, and 0.6 g/L, respectively. Then, *E. coli* K5 was inoculated and incubated at 37 °C for 24 h before the biomass and heparosan yield were determined. As shown in Fig 1A and B, heparosan biosynthesis of *E. coli* K5 was sensitive to Met and Gly, while Met and Gly showed a small effect on *E. coli* K5 growth. A suitable concentration of Met was beneficial to the synthesis of heparosan by *E. coli* K5. When 0.3 g/L Met was added, the maximum heparosan yield reached 270.25 μg/mL, which increased by 53.2% compared to that of the control. While the additional concentration of Met was above 0.3g/L, heparosan yield restrained. The addition of Gly exhibited the similar results. When 0.3 g/L Gly was added, the maximum heparosan yield increased by 56.5%. These results suggested that the low concentration of exogenous amino acids were favorable to *E. coli* K5, which were the essential components of enzymes in heparosan synthesis.

$SnCl_2$ stress

Based on our previous studies, $SnCl_2$ was chosen and added to glucose-defined medium with concentrations of 0.005 g/L, 0.010 g/L, 0.015 g/L, 0.020 g/L, 0.030 g/L, 0.040 g/L, 0.050 g/L, 0.080 g/L,

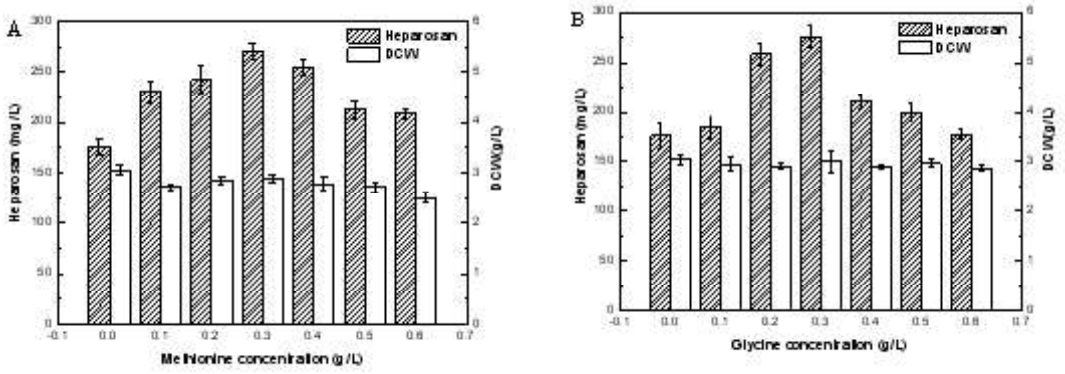

Fig. 1. Effect of methionine (A) and glycine (B) on the growth and heparosan yield of *E. coli* K5

and 0.100 g/L, respectively. The glucose-defined medium without $SnCl_2$ was as the control. Afterwards, *E. coli* K5 was inoculated and cultured at 37 °C and 200 rpm for 24 h. Then, heparosan production of *E. coli* K5 was determined (Fig. 2). The cells' survival was not significantly influenced by the presence of $SnCl_2$ in the fermentation media, while heparosan production of *E. coli* K5 was improved. The yield of heparosan increased by 12.0% compared tp the control, when 0.020 g/L $SnCl_2$ was added. This also indicates that CPS plays a role in protecting *E. coli* K5 from metal stress.

Salt stress

To obtain further insight into the effect of the stresses on heparosan biosynthesis by *E.coli* K5, the fermentation experiments of *E. coli* KT exposed to the salt stress were performed, which was proved to be not influenced on heparosan biosynthesis[10]. *E. coli* K5 and *E. coli* KT were incubated with NaCl at concentrations of 0, 4, 8, 12, 16, and 20 g/L to mesure the growth and production of heparosan (Fig 3A and B). It was observed that the growth of *E. coli* K5 and KT was not significantly influenced and the heparosan yield decreased due to the salt perturbations. *E. coli* showed good tolerance in the long-term exposure to salt stress, this is similar to that already reported[11-14]. However, heparosan yield decreased significantly. *E. coli* KT showed steadier than *E. coli* K5, which might be due *to waaR* gene deletion.

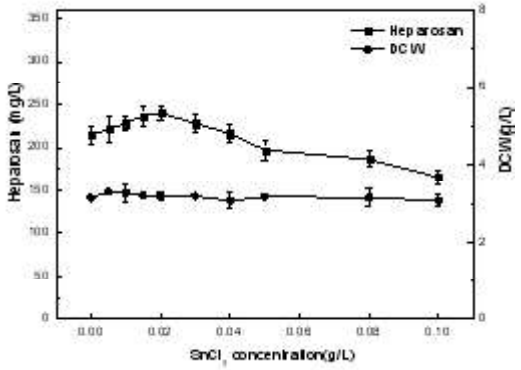

Fig. 2. Effects of $SnCl_2$ on the growth and heparosan yield of *E. coli* K5

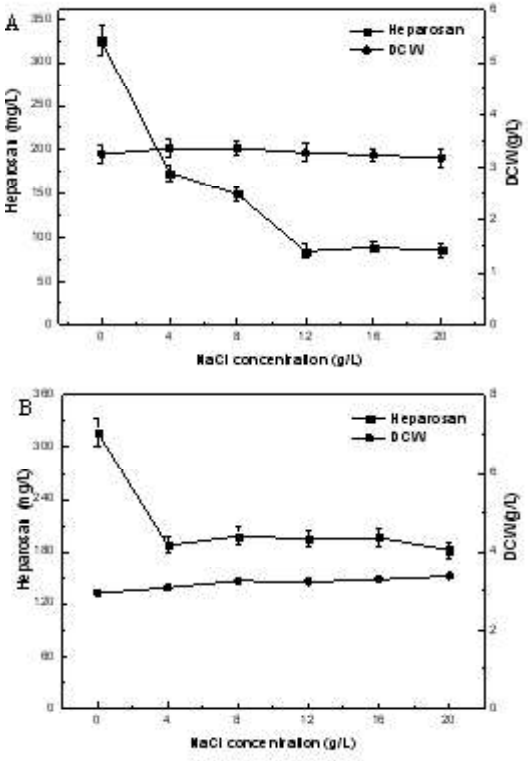

Fig. 3. Effects of NaCl on the growth and heparosan yield of *E. coli* K5 (A) and *E. coli* KT (B)

Organic solvents stress

Organic solvents can destroy microbial cell membrane structure and affect its function, which result in restraining or killing microorganisms. However, it is reported that microorganisms can enhance extracellular polysaccharides secretion to improve strain resistance to organic solvents[15]. The effect of organic solvents on *E. coli* K5 and KT were studied. Addition of different concentrations of ethanol, formamide, and hexane showed (Fig. 4A) different effect on the growth of *E. coli* K5. K5 exhibited a high tolerance to ethanol and formamide stresses and heparosan production was enhanced. While, hexane restrained strongly the cell growth and heparosan synthesis (data was not shown). The similar results were found in *E. coli* KT. The heparosan production of *E. coli* KT was restrained under the treatment of hexane (Fig. 4B). The yield of heparosan increased by 29.38% in *E. coli* K5 under 2% ethanol stress, while increased by 51.96% on *E. coli* KT under 3% ethanol stress. Both *E. coli* K5 and KT were inhibited under hexane stress.

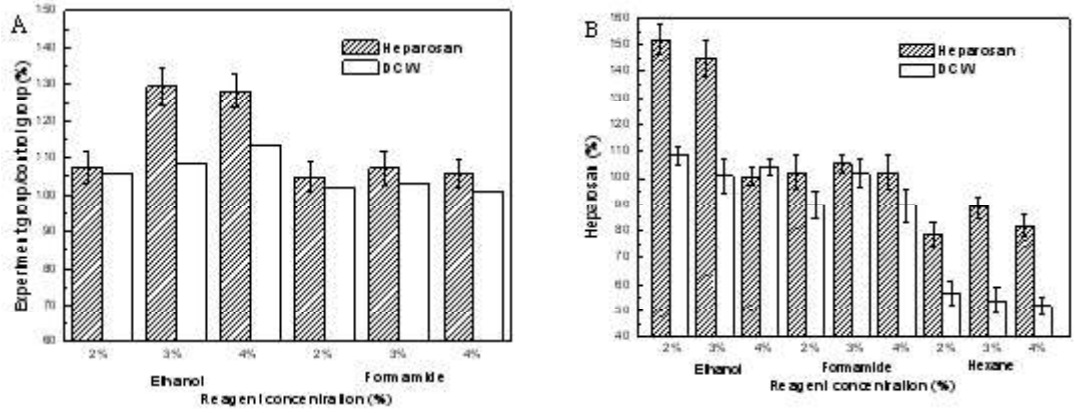

Fig. 4. Effects of organic solvents on the growth and heparosan yield of *E. coli* K5 (A) and *E. coli* KT (B)

Oxidative stress

Cells under oxidative stress display various dysfunctions due to the damages caused by reactive oxygen species to lipid, protein, and DNA[16]. Under oxidative stress, *E. coli* O157:H7 W6-13 showed the high tolerance to H_2O_2 stress[17]. *E. coli* K5 and KT was incubated in the presence of H_2O_2 at concentrations of 0 µmol/L, 10 µmol/L, 20 µmol/L, 30 µmol/L, 40 µmol/L, and 50 µmol/L, at 37 °C and 200 rpm for 24 h to detecte the growth and heparosan production of *E. coli*. No significantly change of the growth of *E. coli* K5 and KT were measured under H_2O_2 stress (Fig. 5A and B). Exposed to H_2O_2, heparosan biosynthesis of *E. coli* K5 was affected less than that of *E. coli* KT. The yield of heparosan of K5 decreased by

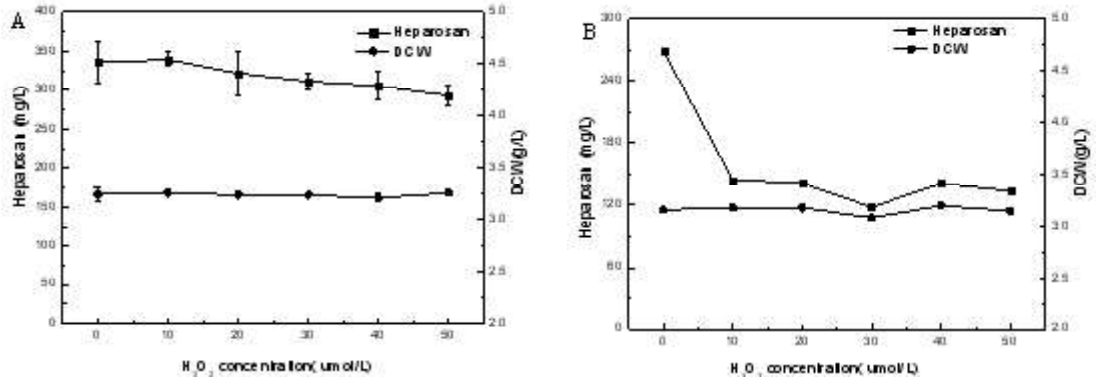

Fig.5. Effects of H_2O_2 on the production of heparosan by *E. coli* K5 (A) and *E. coli* KT (B)

12.64% under 50 µmol/L of H_2O_2 stress. While the yield of heparosan of KT decreased by 46.15% under 10 µmol/L of H_2O_2 stress. It also indicated that *waaR* gene was favorable to heparosan synthesis of *E. coli* K5 under oxidative stress.

CONCLUSIONS

As a result of long-term exposure to environmental stresses, *E. coli* K5 and KT showed high tolerant to some environmental stresses. These results are attributed to the presence of CPS which significantly reduced their susceptibility and heparosan production was stimulated under some stresses. However, iIt was not clear how heparosan assists the cells of *E. coli* K5 and KT to survive and growth under these stresses.

ACKNOWLEDGMENTS

This study was financially supported by the Natural Science Foundation of China (Grant No. 21076195), the Science Technology Department of Zhejiang Province (Grant No. 2011C24007), and the Specialized Research Fund for the Doctoral Program of Higher Education (Grant No. 20113317110002).

REFERENCES

1. DeAngelis, P.L., Gunay, N.S., Toida, T., Mao, W.J., Linhardt, R.J. Identification of the capsular polysaccharides of Type D and F *Pasteurella multocida* as unmodified heparin and chondroitin, respectively. *Carbohydr Res*, 2002; **337**(17): 1547-1552.
2. Chavaroche, A.A.E., Van Den Broek, L.A.M., Eggink, G. Production methods for heparosan, a precursor of heparin and heparan sulfate. *Carbohydr Poly*, 2013; **93**(1):38-47.
3. Campos, M.A., Vargas, M.A., Regueiro, V., Llompart, C.M., Alberti, S., Bengoechea, J.A. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. *Infect immun*, 2004; **72**: 7107-7114.
4. Roberts, I.S. The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annu Rev Microbio*l, 1996; **50**: 285-315.
5. Seungwook, S., Karl, R.M. Exposure of *Escherichia coli* O157: H7 to soil, manure, or water influences its survival on plants and initiation of plant defense response. *Int J Food Microbiol*, 2014; **38**: 87-92.
6. Bonet, R., Simon-Pujo, M.D., Congregado, F. Effects of nutritions on exopolysaccharide production and surface properties of *Aeromonas salmon*icida. *Appl. Environ. Microbiol*,1993; **59**: 437-2441.
7. Wang, Z.Y., Ly, M., Zhang, F.M., Zhong, W.H., Suen, A., Hickey, A.M., Dordick, J.S., Linhardt, R.J. *E.col*i K5 fermentation and the preparation of heparosan, a bioengineered heparin precursor. *Biotechnol Bioeng*, 2010; **107**: 964-973.
8. Bitter, T., Muir, H.M. A modified uronic acid carbazole reaction. *Anal Biochem*, 1962; **4**(4): 330-334.
9. Gupta, A., Khare, S.K. A protease stable in organic solvents from solvent tolerant strain of *Pseudomonas aeruginosa*. *Bioresour Technol*, 2007; **97**: 1788-1793.
10. Taylor, C.M., Goldrick, M., Lord, L., Roberts I.S. 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*, 2006; **188**: 1165-1168.
11. Csonka, L.N., Hanson, A.D. Prokaryotic osmoregulation-genetics and physiology. *Annu Rev of Microbiol*, 1991; **45**: 569-606.
12. Verheul, A., Wouters, J.A., Rombouts, F.M., Abee ,T. A possible role of ProP, ProU and CaiT in osmoprotection of *Escherichia coli* by carnitine. *J Appl Microbiol*, 1998; **85**: 1036-1046.
13. Canovas, M., Bernal, V., Sevilla, A., Iborra, J.L. Salt stress effects on the central and carnitine metabolisms of *Escherichia coli*. *Biotechnol Bioeng*, 2007; **96**: 722-737.
14. Paula, A., Vicente, B., José, L.I., Manuel, C. Metabolic adaptation of *Escherichia coli* to long-term exposure to salt stress. *Process Biochem*istry. 2010; **45**: 1459-1467.
15. Onbasli, D., Aslim, B. Effects of some organic pollutants on the exopolysaccharides (EPSs) produced by some *Pseudomonas spp* strains. *J Hazard Mater*, 2009; **168**: 64-67.
16. Ercal, N., Gurer-Orhan, H., Aykin-Burns, N. Toxic metals and oxidative stress: Part I. Mechanisms involved in metal-induced oxidative damage. *Curr Top Med Chem*, 2001; **1**: 529-539.
17. Chen, J.R., Lee, S.M., Mao, Y. Protective effect of exopolysaccharide colanic acid of *Escherichia coli* O157:H7 to osmotic and oxidative stress. *Int J Food Microbiol*, 2004; **93**: 281-286.