Cyclic AMP (cAMP) Receptor Protein-cAMP Complex Regulates Heparosan Production in *Escherichia coli* Strain Nissle 1917

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Heparosan serves as the starting carbon backbone for the chemoenzymatic synthesis of heparin, a widely used clinical anticoagulant drug. The availability of heparosan is a significant concern for the cost-effective synthesis of bioengineered heparin. The carbon source is known as the pivotal factor affecting heparosan production. However, the mechanism by which carbon sources control the biosynthesis of heparosan is unclear. In this study, we found that the biosynthesis of heparosan was influenced by different carbon sources. Glucose inhibits the biosynthesis of heparosan, while the addition of either fructose or mannose increases the yield of heparosan. Further study demonstrated that the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex binds to the upstream region of the region 3 promoter and stimulates the transcription of the gene cluster for heparosan biosynthesis. Site-directed mutagenesis of the CRP binding site abolished its capability of binding CRP and eliminated the stimulative effect on transcription. ¹H nuclear magnetic resonance (NMR) analysis was further performed to determine the *Escherichia coli* strain Nissle 1917 (EcN) heparosan structure and quantify extracellular heparosan production. Our results add to the understanding of the regulation of heparosan biosynthesis and may contribute to the study of other exopolysaccharide-producing strains.

Heparosan is the starting carbon backbone for the chemoenzymatic synthesis of heparin. Heparin is a widely used clinical anticoagulant drug with a worldwide level of production exceeding 100 tons/year (1). Pharmaceutical heparin is currently produced from porcine intestinal mucosa through a long supply chain that poses a potential risk of contamination and adulteration (2). The worldwide heparin contamination crisis in 2008 underscores the vulnerability of the heparin supply chain. *In vitro* chemoenzymatic synthesis of bioengineered heparin-like polysaccharide has shown promise as an alternative approach to the production of heparin from a nonanimal source (3–5). The chemoenzymatic synthesis of heparin-like polysaccharide starts from heparosan, comprising a [(→4)β-D-glucuronic acid (GlcA)(1→4)*N*-acetyl-α-D-glucosamine (GlcNAc)(1→)]_n repeating disaccharide unit (Fig. 1A). The backbone is then further modified by enzymes including *N*-deacetylase/*N*-sulfotransferase, C₅-epimerase, 2-*O*-sulfotransferase, 6-*O*-sulfotransferase, and 3-*O*-sulfotransferase to produce the fully elaborated heparin.

As the starting carbon backbone for the cost-effective synthesis of bioengineered heparin, the availability of heparosan is a big concern (6–9). Heparosan is extracted from the capsular polysaccharide (CPS) of *Escherichia coli* K5, *Pasteurella multocida*, and *E. coli* strain Nissle 1917 (EcN) (8, 10, 11). The proteins encoded by the *kps* locus govern the biosynthesis and export of heparosan (Fig. 1B). The *kps* locus comprises serotype-specific region 2 (*kfiABCD*) flanked by two conserved regions (regions 1 and 3) (12, 13). Region 1 includes the genes of the *kpsFEDUCS* cluster, and region 3 includes *kpsMT*. The gene products encoded by *kfiABCD* are responsible for the biosynthesis of heparosan (14). Translocation across the cytoplasmic membrane is mediated by the products of *kpsC*, *kpsS*, and *kpsMT*, while translocation across the periplasm and outer membrane involves the KpsD and KpsE proteins (15). The *kpsU* gene encodes a functional CMP–3-deoxy-D-manno-octulosonic acid (Kdo) synthetase, and the specific activity levels of CMP-Kdo synthetase are elevated at capsule-

permissive temperatures (16, 17). KpsF catalyzes the conversion of the pentose pathway intermediate Ru5P (D-ribulose 5-phosphate) into A5P (D-arabinose 5-phosphate), the precursor of Kdo (18).

A complex regulatory network controls the expression of the *kps* locus (Fig. 1C). The *kps* locus is temperature regulated, being expressed at 37°C but not at 20°C (19). The integration host factor (IHF) is required for maximum transcription from the region 1 promoter at 37°C and binds to a single site located 130 bp 3' to the transcription start point (19, 20). Three additional regulators, SlyA, BipA, and H-NS, play a crucial role in the temperature regulation of the region 1 promoter (19, 21). The region 2 promoters are weak and generate low levels of expression, which, in the absence of RfaH-mediated readthrough transcription from the region 3 promoter, are insufficient for the synthesis of detectable heparosan (22). The region 3 promoter is located 741 bp 5' to the *kpsM* gene (23). The transcription of the region 3 promoter proceeds through region 2 with the aid of the transcription antitermination factor RfaH (23). RfaH function is dependent on a short sequence present in the region 3 mRNA known as the JUMPstart (just upstream of many polysaccharide-associated gene starts) element (24). Besides the region 1 promoter, the region 3 promoter is also temperature regulated via both SlyA and H-NS (25).

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FIG 1 Structure of heparosan and transcriptional organization of the *kps* locus. (A) Structure of a native chain of heparosan. Heparosan is comprised of a $[(\rightarrow 4)\text{GlcA}(1\rightarrow 4)\text{GlcNAc}(1\rightarrow)]_n$ repeating disaccharide unit. The average value of n is ~ 70 . (B) Transcriptional organization of the *kps* locus. Both the biosynthesis and export of heparosan are carried out by the *kps* locus-encoded proteins. The transcription start points are indicated by broken arrows. The horizontal arrows show the primary transcripts from region 1 and region 3. The region 2 promoters are weak and insufficient for the synthesis of detectable heparosan. (C) DNA sequence of the region 3 promoter. The bent arrow marks the transcriptional start site (23). The stop codon of the upstream *gspM* gene, the -10 sequence, the Shine-Dalgarno (SD) sequence, and the *kpsM* start codon are denoted by thick underlining. The JUMPstart sequence is indicated by double underlining, and the *ops* sequence (shaded), which is essential for the action of RfaH, is contained within the JUMPstart sequence (23). The H-NS binding regions are underlined, and the SlyA binding region is indicated by shading (25). The putative CRP binding motif is shown in an unshaded box.

As a nonpathogenic probiotic strain without known toxins, EcN can be used as a safe source of heparosan preparations. EcN carries all the genes for heparosan biosynthesis and export. Although the above-mentioned studies have not been performed on the *kps* locus of EcN, it is possible to investigate heparosan production in EcN based on knowledge of the studied strains. Recent studies have indicated that carbon sources have differential impacts on heparosan production. Glycerol-defined medium allows a 3-fold increase in the level of heparosan production compared to that in Luria-Bertani (LB) medium (26). Another report demonstrated that glucose-defined medium compares favorably to glycerol-defined medium in heparosan yield (27). However, the mechanism by which carbon sources control the biosynthesis of heparosan is unclear.

In this study, we have found that glucose, fructose, and mannose have discriminative impacts on the biosynthesis of heparosan. Further studies demonstrated that the expression of the region 3 promoter is regulated by the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex. Gel shift assays show that the cAMP-CRP complex binds to a CRP binding motif in the region 3 promoter. The deletion of *crp*, the deletion of *cya*, and base substitutions in the CRP binding site dramatically decrease expression from the region 3 promoter. Furthermore, the yield of heparosan is apparently lower when glucose is used as the sole carbon source than when fructose and mannose are used.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in LB medium or on LB plates containing 1.5% agar. PCRs were performed on the Arktik thermal cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA). Minimal medium (MM) contained 2% carbon source (glucose, fructose, or mannose), 0.24 g/liter MgSO_4 , 0.01 g/liter CaCl_2 , 6 g/liter Na_2HPO_4 , 3 g/liter KH_2PO_4 , 0.5 g/liter NaCl , and 1 g/liter NH_4Cl . MM was used to culture EcN for heparosan preparation. ^1H nuclear magnetic resonance (NMR) analysis was performed on a Varian 600-MHz NMR spectrometer (Agilent Technologies Inc., USA). Unless otherwise stated, glucose and cAMP were utilized at 0.8% and 10 mM, respectively. Ampicillin (Ap), kanamycin (Kan), and chloramphenicol (Cm) were added at 50 $\mu\text{g}/\text{ml}$, 50 $\mu\text{g}/\text{ml}$, and 25 $\mu\text{g}/\text{ml}$, respectively, when necessary. The chemicals were provided by Sangon Co. Ltd. (Shanghai, China).

Plasmid construction. The low-copy-number vector pFZY1 was used to construct the promoter fusion plasmids in this study (28). The region 3 promoter was amplified by using primer pair 0011/0012 and inserted into pAH125 at the KpnI-EcoRI site to create pAH125-KpsMP. pAH125-KpsMP was then cleaved with BamHI. The purified DNA fragment carrying the region 3 promoter was cloned into plasmid pFZY1 to create pFZY1-KpsMP. Site-directed mutagenesis was used to create pFZY1-KpsMPm that carried a mutated CRP binding site. Briefly, primers 0060 and 0061 were used to introduce the mutant base pairs using pFZY1-KpsMP as the template. The PCR products were then digested with the enzyme DpnI, purified, and transformed into competent cells. The *crp* gene of EcN was cloned by using primers 0009 and 0010 to create pET28a-

TABLE 1 Strains and plasmids used in this study

<i>E. coli</i> strain or plasmid	Characteristic(s)	Source or reference
<i>E. coli</i> strains		
BL21(DE3)	Expression host	TaKaRa
Nissle 1917	Wild type	DSMZ
K5	Wild type	ATCC
ZK126	W3110 $\Delta lac tna-2$	41
YHH1301	EcN $\Delta lacZ::Kan$	This work
YHH1302	EcN $\Delta lacZ$	This work
YHH1303	EcN $\Delta lacZ \Delta crp::Cm$	This work
YHH1304	ZK126 $\Delta crp::Cm$	This work
YHH1305	ZK126 $\Delta cya::Cm$	This work
YHH1306	ZK126 $\Delta crp(comp)$	This work
YHH1307	ZK126 $\Delta cya(comp)$	This work
Plasmids		
pFZY1	<i>galK'-lacZYA</i> transcriptional fusion vector; Ap	28
pFZY1-KpsMP	pFZY1 carrying the region 3 promoter with a CRP binding motif; Ap	This work
pFZY1-KpsMPm	pFZY1 carrying the region 3 promoter with the mutated CRP binding motif; Ap	This work
pET28a-crp	pET28a(+) derivative for CRP expression; Kan	This work

crp. The purified PCR product was digested and inserted into pET28a(+) at the NdeI-XhoI site. The constructed plasmids were sequenced to verify their integrity.

Gene disruption and complementation. The Red-mediated homologous recombination system was used to construct in-frame deletions (29). A kanamycin resistance cassette was amplified by using pKD4 as the template and primer pair 0007/0008 and used to delete the *lacZ* gene in EcN. The PCR products were treated with the enzyme DpnI and introduced by electroporation into EcN containing the pKD46-expressed Red recombinase. Transformants were selected on LB plates supplemented with kanamycin. The helper plasmid pKD46 was later cured by incubation at 42°C. In order to construct strain YHH1302 (EcN $\Delta lacZ$), the kanamycin resistance cassette of strain YHH1301 (EcN $\Delta lacZ::Kan$) was eliminated by

using pCP20, as previously described (29). To construct strain YHH1303 (EcN $\Delta lacZ \Delta crp::Cm$), a chloramphenicol cassette was amplified from pKD3 by using primer pair 0013/0014. The chloramphenicol cassette was then introduced into YHH1302 (EcN $\Delta lacZ$) containing plasmid pKD46. Strains YHH1304 (ZK126 $\Delta crp::Cm$) and YHH1305 (ZK126 $\Delta cya::Cm$) were created similarly by using the chloramphenicol cassette amplified from pKD3 with primer pairs 0013/0014 and 0056/0072, respectively. To create the complementary strains, the DNA fragments carrying the *crp* and *cya* genes were amplified with primer pairs 0017/0018 and 0058/0059. The DNA products were purified and introduced into YHH1304/pKD46 and YHH1305/pKD46. The complemented strains, which grew faster than the isogenic *crp* and *cya* mutants, were selected on the LB plates without any antibiotics. All the deletions and complementations were verified by PCR.

Expression and purification of CRP. *E. coli* BL21(DE3) carrying plasmid pET28a-crp was grown in LB medium at 37°C to an optical density at 600 nm (OD_{600}) of 0.6 and then induced with 0.2 mM isopropyl- β -D-thiogalactopyranoside (IPTG) overnight at 22°C. All subsequent procedures were performed at 4°C. The cells were harvested and resuspended in 30 ml of solution I (20 mM Tris-HCl [pH 7.6], 200 mM NaCl). After the addition of 100 μ M phenylmethylsulfonyl fluoride (PMSF), the cells were lysed by sonication. The lysate was centrifuged at 12,000 rpm for 20 min, and the supernatant was applied to a nickel-nitrilotriacetic acid (NTA) column. The column was washed with 30 ml solution II (20 mM Tris-HCl [pH 7.6], 200 mM NaCl, 50 mM imidazole). CRP was eluted with a gradient of 50 to 250 mM imidazole in solution I. The His-tagged CRPs were dialyzed against solution I and stored at -80°C until use. The purity of CRP was analyzed by SDS-PAGE.

β -Galactosidase assay. *E. coli* ZK126 was used as the wild-type (WT) strain. ZK126 and its derivatives were used in β -galactosidase activity assays. Cultures of *E. coli* grown overnight were diluted into fresh LB medium to an OD_{600} below 0.03. The cultures were incubated at 37°C with shaking at 250 rpm. At different time points during cell growth, aliquots were removed for the determination of OD_{600} values and β -galactosidase activity, as previously described (30). β -Galactosidase activity

TABLE 2 Oligonucleotide primers used in this study

Oligonucleotide	Sequence ^a
0007	ATGAGCGGATAACAATTTACACAGGATACAGCTATGACGTGTAGGCTGGAGCTGCTTC
0008	TACGCGAAATACGGGCAGACATAGCTGCCCGTTATTAATGGGAATTAGCCATGGTCC
0009	GGAATTCATATGGTGTGGCAAACCGCAAAC
0010	TCCGCTCGAGTTAACGAGTGCCGTAAGCA
0011	ACGGGGTACCGTATTGCCATTTCTTAACCCCA
0012	ATCGGAATCTTGATGATGTGATCCTAATCTCTTC
0013	ATGGTGCTTGCAAACCGCAAACAGACCCGACTCTCGAACTGTCAAACATGAGAATTAA
0014	TTAACGAGTGCCGTAACGACGATGGTTTTACCGTGTGCGTGTAGGCTGGAGCTGCTTC
0017	AGGAGACACAAAGCGAAAGC
0018	CGTTTATGAGGCGTATCAAGG
0060	TTTAAAcGatcTATAAAATtgcAATATGGCTGTAAAGAGGGGGC
0061	TTACAGCCATATTgcaATTTATAgatCgTTTAAATGGTGTATTATTAAGTCGCA
0056	TTGTACCTCTATATTGAGACTCTGAAACAGAGACTGGATATGGGAATTAGCCATGGTCC
0072	TCACGAAAAATATTGCTGTAATAGCGGCGTATCGTGATCGTGTAGGCTGGAGCTGCTTC
0058	ATAAACGGTGCTACACTTGTATGTA
0059	GCAAAATCATTATCAACCGC
0080	ATAACACCATTAAAAATGTGATATAAAATCAAAATATGGCTGT
0081	CTTTACAGCCATATTGTGATTTATATCACATTTAAATGGTG
0082	ATAACACCATTAAAAATGatcTATAAAATtgcAATATGGCTGT
0083	CTTTACAGCCATATTgcaATTTATAgatCgTTTAAATGGTG
0084	TCTTCATCTCCGGTTCTGCTGGCGGAGGTGGATCTGGCGGAGGTGGATCGG
0004	CCGATCCACCTCCGCCAGATCCACCTCCGCCAGCAGAACCGGAGATGAAGA

^a Underlined residues in sequences anneal to the template plasmids, while the remaining residues of the sequences correspond to the ends of the deleted genes. Residues in italic type represent the CRP binding motif, while the base substitutions are shown in lowercase type.

FIG 2 Glucose has a negative effect on heparosan expression. (A) PAGE analysis of the capsular polysaccharides extracted from EcN grown on MM plates with different carbon sources. Lane 1, glucose; lane 2, fructose; lane 3, mannose. (B) Effects of glucose, cAMP, and CRP on transcription from the region 3 promoter. Both *E. coli* ZK126 (wild type) and the isogenic *crp* mutant carry the promoter fusion plasmid pFZY1-KpsMP. The strains were grown in LB medium, LB medium plus 0.8% glucose, or LB medium plus 0.8% glucose and 10 mM cAMP. At different time points during cell growth, aliquots were collected for measurement of the OD₆₀₀ (squares and triangles) and β -galactosidase activity (bars).

was expressed in Miller units. All assays were performed in triplicate. The error bars in the graphs indicate the standard deviations.

Gel shift assay. The double-stranded region 3 promoter fragments containing the WT/mutated CRP binding motif were produced by boiling and slowly cooling the synthetic DNA oligonucleotide pairs 0080/0081 and 0082/0083 (Table 2). The digoxigenin (DIG) gel shift kit (Roche Ltd., Mannheim, Germany) was used for DNA labeling and signal detection. A DNA fragment without the CRP binding motif was used as the competitive probe (primer pair 0004/0084). The labeled DNA fragments (1.6 nM) were incubated with various amounts of purified CRP at 37°C for 10 min in CRP binding buffer (10 mM Tris-HCl [pH 8.0], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], 50 μ g/ml bovine serum albumin [BSA], 100 μ M cAMP, and 160 nM the competitive DNA probe). The formed DNA-protein complexes were separated by 8% PAGE in 1 $\times$ Tris-borate-EDTA (TBE) buffer containing 100 μ M cAMP.

Preparation of heparosan from liquid cultures. Cultures of EcN and *E. coli* K5 grown overnight were expanded into fresh medium and shaken at 250 rpm at 37°C for variable times. The supernatant of the bacterial cultures was recovered by centrifugation, filtered through a 0.45- μ m

membrane, and concentrated by using a rotary vacuum evaporator. Heparosan in the supernatant was precipitated with 3 volumes of ethanol at -20°C overnight and pelleted by centrifugation at 12,000 rpm for 15 min at 4°C. After washing with 75% ethanol, heparosan was resuspended in deionized water. Phenol-chloroform extraction was performed to remove proteins. The heparosan sample was then dialyzed by using the Spectra/Por dialysis membrane (molecular weight cutoff [MWCO] of 10,000) against buffer A (20 mM sodium acetate, 50 mM NaCl [pH 4]) and applied to a DEAE-Sepharose column (1.6 by 50 cm) for purification. The sample was loaded onto the column at a rate of 1 ml/min. After loading of heparosan, the column was washed with 10 column volumes of buffer A at a rate of 3 ml/min. Heparosan was then eluted at a rate of 3 ml/min with buffer B (20 mM sodium acetate, 1 M NaCl [pH 4]). Fractions containing heparosan were pooled, dialyzed against water, and freeze-dried. Purified heparosan was stored at -80°C for later use.

Preparation of heparosan from bacteria grown on MM plates. The extracellular capsular polysaccharide from EcN was purified according to a method described previously, with modifications (31). Cultures of EcN grown overnight were diluted with fresh medium, and 100- μ l aliquots

FIG 3 Complementation of the in-frame deletion of *crp*. (A) The growth curves of ZK126 (WT), the *crp* mutant, and the Δ crp(comp) strain. (B) Doubling times of ZK126 (WT), the *crp* mutant, and the Δ crp(comp) strain. (C) PCR verification of disruption and complementation of the *crp* gene. Lane 1, DNA ladder; lane 2, ZK126 (WT); lane 3, Δ crp::Cm strain; lane 4, Δ crp(comp) strain.

FIG 4 Complementation of the in-frame deletion of *cya*. (A) Growth curves of ZK126 (WT), the *cya* mutant, and the $\Delta cya(comp)$ strain. (B) Doubling times of ZK126 (WT), the *cya* mutant, and the $\Delta cya(comp)$ strain. (C) PCR verification of disruption and complementation of the *cya* gene. Lane 1, DNA ladder; lane 2, ZK126 (WT); lane 3, $\Delta cya::Cm$ strain; lane 4, $\Delta cya(comp)$ strain.

were plated onto MM plates supplemented with the specific carbon source. The plates were incubated at 37°C for 24 h for the bacteria to biosynthesize and translocate heparosan. The cells were then harvested and resuspended in phosphate-buffered saline (PBS). To restrain cell breakage and the release of large amounts of chemical compounds, we gently extracted heparosan by shaking the cell resuspension solution overnight at 100 rpm at 4°C. The cell suspension was then centrifuged at 12,000 rpm for 10 min at 4°C. Heparosan in the supernatant was precipitated with 3 volumes of ethanol at −20°C overnight and pelleted by centrifugation at 12,000 rpm for 15 min at 4°C. After washing with 75% ethanol, heparosan was dried and resuspended in deionized water. DNA in the heparosan suspension was degraded by the addition of 20 U/ml DpnI. Proteinase K was then added at a final concentration of 400 μg/ml, and the suspension was incubated at 37°C overnight. Following phenol-chloroform extractions, the CPS preparation was dialyzed by using the Spectra/Por dialysis membrane (MWCO of 10,000) against deionized water. The sample was then freeze-dried and stored at −80°C for later use.

PAGE analysis of the capsular polysaccharide. An isocratic large-slab PAGE gel was cast as previously described (6). A total of 45 μl of the sample plus 5 μl of loading buffer were loaded into each well. Gel electrophoresis was performed at 120 V for 5 h. Following electrophoresis, the gel was gently shaken at room temperature in washing buffer (10% acetic acid and 25% ethanol) for 30 min. After staining with a solution containing

alcian blue (0.5%) in acetic acid (2%) for 30 min, the gel was destained in washing buffer until the gel background was transparent.

¹H NMR analysis. ¹H NMR analysis was performed as described previously, with sodium terephthalate as an internal standard for heparosan quantification (7). The CPS preparations were lyophilized and dissolved in 0.5 ml D₂O, and this step was repeated two times. The lyophilized CPS was then redissolved in 0.5 ml of D₂O containing 71 μg of sodium terephthalate before being transferred to a 5-mm NMR tube. ¹H NMR was performed on a Varian 600-MHz NMR spectrometer (Agilent Technologies Inc., USA), and acquisition of the spectra was carried out by using VnmrJ Rev. 3.2A software. The ¹H NMR spectra were processed with MestReNova software. Integration of the peaks was performed by using the “integration” function, with the peak area being selected manually.

RESULTS

Biosynthesis of heparosan is affected by catabolite repression.

To evaluate the impact of carbon sources on heparosan production, we extracted heparosan from EcN cells grown on MM plates. The medium was supplemented with 2% glucose, fructose, or mannose as the sole carbon source. Bacterial lawns grown overnight were scraped off, and heparosan was extracted as described

FIG 5 The complemented *crp* (A) and *cya* (B) strains recover expression from the region 3 promoter. Cells carrying pFZY1-KpsMP were grown in LB medium at 37°C with shaking at 250 rpm. At different time intervals, aliquots were taken for the determination of growth curves (squares, triangles, and circles) and β-galactosidase activity (bars).

in Materials and Methods. The amount of heparosan was checked by PAGE. As shown in Fig. 2A, the yields of heparosan from bacteria using fructose and mannose as the carbon sources were obviously higher than those with glucose.

Glucose is well known to affect gene expression through the cAMP-CRP complex (32, 33). Thus, we set out to check the sequence of the *kps* locus and find a potential CRP binding site located in the region 3 promoter (Fig. 1C). To study whether the cAMP-CRP complex is involved in the regulation of heparosan biosynthesis, we constructed the $\Delta crp::Cm$ strain and a region 3 promoter-*lacZ* fusion plasmid. The deletion of the *crp* gene dramatically decreased expression from the region 3 promoter (Fig. 2B). On the other hand, the addition of 0.8% glucose to LB medium significantly inhibited expression from the region 3 promoter, and the corresponding β -galactosidase activity was halved. However, the addition of 0.8% glucose plus 10 mM cAMP offset the decreased β -galactosidase activity. These results suggest that the expression of the region 3 promoter is downregulated by the addition of glucose and stimulated by both cAMP and CRP.

The complementary *crp* and *cya* genes restore expression from the region 3 promoter. To further study the regulatory relationship between cAMP-CRP and the region 3 promoter, $\Delta crp::Cm$, $\Delta cya::Cm$, and corresponding complementary strains were constructed. All the in-frame deletions and the complementary manipulations were carried out by using the λ Red homologous recombination system (29). The in-frame deletion of the *crp* and *cya* genes results in a clearly decreased growth ability (Fig. 3A and B and 4A and B). The *crp* and *cya* genes were amplified with the EcN chromosome as the template and were utilized to complement the in-frame deletions of both the $\Delta crp::Cm$ and $\Delta cya::Cm$ constructs. The complemented strains with restored doubling times exhibited larger colonies on the LB plate than other ones in the bacterial lawns. The complemented strains were selected and repurified on an LB plate without any antibiotics. The in-frame deletion and complementary strains were verified via colony PCR analysis (Fig. 3C and 4C).

Complementary experiments were conducted to further verify the stimulation of expression from the region 3 promoter by the cAMP-CRP complex. The region 3 promoter-*lacZ* fusion plasmid was transformed into WT, $\Delta crp::Cm$, $\Delta cya::Cm$, and complementary strains, including the complemented $\Delta crp::Cm$ [Δcrp (comp)] and Δcya (comp) strains. β -Galactosidase activity, expressed in Miller units, was determined to evaluate the impacts of different genotypes on expression from the region 3 promoter. As shown in Fig. 5A, expression from the region 3 promoter was significantly inhibited in the $\Delta crp::Cm$ strain, and that of the *crp* complementary strain was recovered to the level of the WT strain. The in-frame deletion of *cya* eliminates intracellular cAMP, leading to the loss of function of the cAMP-CRP complex (33). Similar to the phenotype of the $\Delta crp::Cm$ strain, the deletion of the *cya* gene eliminated transcription from the region 3 promoter (Fig. 5B). The β -galactosidase activity of the *cya* complementary strain was restored to the level in the WT strain.

The cAMP-CRP complex binds directly to a CRP binding motif in the region 3 promoter and stimulates the expression of this operon. The cAMP-CRP complex controls gene transcription via binding to the consensus CRP binding site (34, 35). The region 3 promoter is located 741 bp 5' to the *kpsM* gene (23). In the region 3 promoter, there is a potential CRP binding site (TGTGA tataaaTCACA) located 487 bp 5' to the *kpsM* gene (Fig. 6A). (The

FIG 6 The cAMP-CRP complex binds to a CRP binding motif and stimulates transcription from the region 3 promoter. (A) The DNA fragment of the region 3 promoter carries a CRP binding motif located 487 bp 5' to the *kpsM* gene. (B) The DNA fragment carries a mutant CRP binding motif with base substitutions shown in lowercase type. The conserved base pairs of the CRP binding motif were randomly mutated. (C) Gel shift assays of the DNA fragments containing the WT/mutant CRP binding motif. Various amounts of the purified CRP protein (0 to 80 nM) were utilized to bind to the digoxigenin-labeled DNA fragments in the presence of 100 μ M cAMP. The arrowhead denotes the cAMP-CRP-DNA complex. (D) β -Galactosidase activity assays of the region 3 promoter carrying a WT/mutant CRP binding motif. The squares and triangles represent the growth curves; the bars indicate the β -galactosidase activity.

italic lowercase sequence refers to a 6-bp spacer that separates two conserved motifs of the CRP binding site.) To determine whether the cAMP-CRP complex directly modulates the expression of the region 3 promoter via the potential CRP binding site, we performed gel shift assays. Our results reveal that cAMP-CRP directly binds to the DNA fragment of the region 3 promoter in a dose-dependent manner. However, the DNA fragment with a mutant CRP binding site (cGatctataaaTtgcg) loses the ability to form a complex with cAMP-CRP (Fig. 6B and C).

Further studies were performed to verify the essentiality of the CRP binding site for expression from the region 3 promoter. The CRP binding motif of the region 3 promoter was base substituted,

FIG 7 Heparosan is not detectable in the isogenic *crp* mutant of EcN. (A) PAGE analysis of heparosan extracted from EcN (lane 1) and its isogenic *crp* mutant (lane 2). The bacteria were grown on glucose-defined MM plates at 37°C for 24 h. The cells were then harvested from plates, and heparosan was extracted. (B) ^1H NMR analysis of EcN heparosan. Heparosan was extracted from EcN cultured on glucose-defined MM plates.

and the β -galactosidase activity was determined. As shown in Fig. 6D, the region 3 promoter carrying a mutated CRP binding site failed to express LacZ and exhibited no substantial β -galactosidase activity compared to that of the WT region 3 promoter. This result is consistent with the deficiency of β -galactosidase activities in the $\Delta\text{crp}::\text{Cm}$ and $\Delta\text{cya}::\text{Cm}$ strains that carry a WT region 3 promoter-*lacZ* fusion plasmid. These results indicate a requirement for the CRP binding site in cAMP-CRP complex-mediated activation.

Substitution of selective hexoses for glucose increases the yield of heparosan. As mentioned above, our data clearly demonstrate that both the consensus CRP binding site and the cAMP-CRP complex are essential for the expression of genes regulated by the region 3 promoter. To further confirm the impact of the global regulator CRP on the biosynthesis of heparosan, CPS was extracted from wild-type EcN and its isogenic *crp* mutant grown on glucose-defined MM plates. Both PAGE and ^1H NMR analyses were performed to analyze the extracted heparosan. As shown by PAGE, there was a substantial amount of heparosan purified from wild-type EcN, but the amount of heparosan extracted from the isogenic *crp* mutant was not detectable (Fig. 7A). Furthermore, the ^1H NMR spectrum for wild-type EcN heparosan is similar to previously reported spectra for K5 polysaccharide (Fig. 7B) (7, 26, 36). The peak at 2.04 ppm, corresponding to the methyl protons in *N*-acetyl groups of heparosan, is clearly shown for EcN heparosan. The results from both the PAGE and ^1H NMR analyses consolidate the conclusions that the biosynthesis of heparosan is stimulated by the cAMP-CRP complex.

To further address the impacts of different hexoses on the biosynthesis of heparosan, shake flask experiments were performed. Liquid MM supplemented with glucose, fructose, and mannose as the sole carbon sources was used to culture wild-type EcN and *E. coli* BL21. The cultures were shaken at 250 rpm at 37°C to an optical density at 600 nm of 1.0. Heparosan in the supernatants of bacterial cultures was purified with a DEAE-Sepharose column. The supernatant of the *E. coli* BL21 culture was purified in the same way as that for EcN and used as a negative control. ^1H NMR was utilized to analyze purified EcN heparosan (Fig. 8A). Heparosan purified from *E. coli* K5 (50 μg , 100 μg , 500 μg , 1,000 μg , 1,500 μg , and 2,000 μg) was used to develop a standard curve (Fig. 8B). Sodium terephthalate was selected as a water-soluble, stable, and nonreactive internal standard for heparosan quan-

tification, as previously described (7). The *N*-acetyl peak (2.04 ppm) area was selected and normalized to the sodium terephthalate peak area. The yields of heparosan in sugar-defined MM cultures were determined (Fig. 8C). The addition of glucose resulted in the production of 5 mg/liter of heparosan, a level which was lower than those with fructose (13 mg/liter) and mannose (10 mg/liter) (Fig. 8C).

DISCUSSION

Pharmaceutical heparin produced from porcine intestinal mucosa bears the potential risk of contamination and adulteration (2). *In vitro* chemoenzymatic synthesis of bioengineered heparin and oligosaccharides has shown promise as an alternative approach to producing the anticoagulant drug from nonanimal sources (3–5). As the starting material for the cost-effective synthesis of novel anticoagulant drugs, the availability of heparosan is a significant concern (6–8). Recent studies show that carbon sources have differential impacts on the yield of heparosan (26, 27). However, the mechanism by which carbon sources control the biosynthesis of heparosan is unclear. Furthermore, both well-known probiotic (EcN) and urinary tract pathogen (*E. coli* K5) strains share their extracellular CPS composed of heparosan as the molecular camouflage for host colonization (37, 38). An understanding of the regulation mechanism of heparosan biosynthesis will also contribute to studies of the resistance of the host to pathogens.

Our study shows that the yield of heparosan from EcN is decreased when glucose is utilized as the sole carbon source (Fig. 2A). Glucose is known to affect gene expression through the cAMP-CRP complex (32, 33). The cAMP-CRP complex binds to the consensus CRP binding motif in the promoter region and affects the affinity of RNA polymerase for the promoter DNA. The addition of glucose causes the dephosphorylation of glucose-specific phospho-enzyme IIA (P-EIIA^{Glc}) (33). The dephosphorylation process deactivates adenylate cyclase and hence decreases the intracellular concentrations of cAMP and the cAMP-CRP complex.

To address the mechanism of glucose inhibition of heparosan biosynthesis, we deleted the *crp* and *cya* genes and constructed a region 3 promoter-*lacZ* fusion plasmid. The promoter-*lacZ* fusion plasmid is derived from a low-copy-number plasmid, pFZY1, and carries a region 3 promoter. As shown by the β -galactosidase

FIG 8 Analysis of heparosan purified from shake flask cultures. EcN and *E. coli* BL21 were inoculated into MM and shaken at 250 rpm at 37°C to an optical density at 600 nm of 1.0. EcN heparosan in the supernatant was precipitated with ethanol and purified with a DEAE-Sepharose column (1.6 by 50 cm), as described in Materials and Methods. The supernatant of the *E. coli* BL21 culture was purified in the same way as that for EcN and used as a negative control. (A) ^1H NMR spectra of purified EcN heparosan from shake flask cultures compared to those for *E. coli* BL21. (B) Standard curve for the quantification of heparosan. *E. coli* K5 was grown in LB medium at 250 rpm at 37°C for 20 h. Heparosan in the supernatant was purified with a DEAE-Sepharose column and used as a standard. (C) Yield of purified EcN heparosan in MM supplemented with different carbon sources. ^1H NMR analysis was performed with sodium terephthalate as an internal standard for heparosan quantification.

activity assay, the addition of 10 mM cAMP offset the decreased β -galactosidase activity caused by glucose inhibition (Fig. 2B). Furthermore, the deletion of *crp* and *cya* blocked the expression of β -galactosidase from the region 3 promoter (Fig. 5). The in-frame deletion of *crp* and *cya* was complemented by Red-mediated homologous recombination (Fig. 3 and 4). The β -galactosidase activity of the complementary strains was restored to that of the WT strain (Fig. 5). These results clearly demonstrate that expression from the region 3 promoter is positively regulated by the cAMP-CRP complex.

In general, cAMP-CRP binds to a palindromic sequence in which two conserved motifs, TGTGA, and TCACA, are separated by a 6-bp spacer (39, 40). We performed a gel shift assay to determine whether the cAMP-CRP complex directly stimulates tran-

scription from the region 3 promoter upon binding. We compared the cAMP-CRP complex binding capability of the wild-type CRP binding motif (TGTGAtataaaTCACA) with that of the mutant CRP binding motif (cGatctataaaTtcgc). As shown in Fig. 6C, the cAMP-CRP complex directly binds to the wild-type CRP binding motif in a dose-dependent manner, and the base substitutions in the mutant CRP binding motif abolish its binding ability. Meanwhile, the mutant CRP binding motif results in failed transcription from the region 3 promoter, as shown by the dramatically decreased β -galactosidase activity (Fig. 6D).

Heparosan production was further analyzed by the PAGE and ^1H NMR analyses. The biosynthesis of heparosan was significantly inhibited by the in-frame deletion of the *crp* gene (Fig. 7A). These results further consolidate our conclusion that the binding of the

cAMP-CRP complex to the consensus CRP site is essential for transcription from the region 3 promoter. Shake flask experiments were performed to further evaluate the yield of heparosan in sugar-defined MM cultures. EcN and *E. coli* BL21 were inoculated into MM and shaken at 250 rpm at 37°C to an optical density at 600 nm of 1.0. EcN heparosan in the supernatant was purified with a DEAE-Sepharose column and analyzed by ¹H NMR. The addition of glucose resulted in a lower yield of heparosan (5 mg/liter versus 10 to 13 mg/liter) than that with fructose and mannose (Fig. 8C). Since the uptake of glucose decreases the intracellular concentration of cAMP, the decreased production of heparosan is reasonable. In the stationary phase, the depletion of glucose results in an increase in the intracellular cAMP concentration and influences heparosan production.

In summary, we have shown that the binding of the cAMP-CRP complex to the consensus CRP binding site is essential for the expression of the region 3 operon. The deletion of *crp* and *cya* and mutation of the consensus *crp* binding site inhibit expression from the region 3 promoter and prevent the biosynthesis of heparosan. Glucose has an adverse impact on intracellular cAMP concentrations and the production of heparosan. We have aligned the region 3 promoter of group 2 strains, including EcN, *E. coli* K5, *E. coli* UTI89 (O18:K1:H7), and *E. coli* K4 (O5:K4:H4) (38). EcN is a probiotic bacterium without any known toxins, while the other three bacteria are pathogenic strains. These strains have a highly conserved region 3 promoter with >95% sequence identity, while the sequence identity of the region 3 promoters of EcN and K5 is 99.3%. Both the CRP binding motif and the JUMPstart sequence are located in the region 3 promoters of these four strains. These results indicate that group 2 bacteria might use common mechanisms to escape host elimination and enhance host colonization.

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We have no conflicts of interest to declare.

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