Involvement of cAMP receptor protein in biofilm formation, fimbria production, capsular polysaccharide biosynthesis and lethality in mouse of Klebsiella pneumoniae serotype K1 causing pyogenic liver abscess

Qin Ou,† Jinming Fan,† Dejian Duan, Li Xu, Jie Wang, Dongsheng Zhou, Huiying Yang† and Bei Li†,*

Abstract
The global regulator cAMP receptor protein (CRP) has been shown to be required for the full virulence and/or for the expression of virulence determinants in a wide set of bacterial pathogens. In this work, the crp mutant as well as the complemented mutant was constructed from a wild-type Klebsiella pneumoniae capsular serotype K1 strain causing the primary pyogenic liver abscess. The phenotypes of wild-type strain, crp mutant and complemented mutant were characterized systematically. It was disclosed that K. pneumoniae CRP was required for the in vitro growth, fimbria production, biofilm formation and lethality in mouse, but it inhibited the capsular polysaccharide biosynthesis. These indicated the important roles of CRP in regulating the expression of virulence and biofilm genes in K. pneumoniae.

INTRODUCTION
Klebsiella pneumoniae, a rod-shaped Gram-negative bacterium that belongs to the family Enterobacteriaceae, commonly causes nosocomial infections in the urinary tract, respiratory tract and blood; on this occasion, this bacterium is considered as an opportunistic pathogen rather than a ‘true’ one, since it mostly affects debilitated patients [1]. In contrast, serious community-acquired infections including pneumonia, meningitis, necrotizing fasciitis and prostatic abscess caused by K. pneumoniae can affect previously healthy persons [2]. In particular, the K. pneumoniae-caused primary pyogenic liver abscess (PLA), which is sometimes complicated by endophthalmitis or meningitis, has emerged worldwide in recent years [3–6].

Capsular polysaccharide (CPS) is the prominent virulence factor of K. pneumoniae and protects the pathogen from opsonization and phagocytosis [7, 8]. At least 77 capsular serotypes have been recognized for K. pneumoniae [9]. Only a few serotypes including predominantly K1 and K2 are associated with the community-acquired PLA [3, 10], and these strains often give a hypermucoviscosity phenotype due to the redundant production of CPS [11]. The degree of mucoidy appears to positively correlate with the successful establishment of infection [11].

The O antigen component of LPS is responsible for serum resistance [7, 8]. Type 1 fimbriae facilitate the bacterial adherence to specific tissue surfaces in the hosts, and thus, they are required for the establishment of urinary tract infections [12]. At least three iron acquisition systems, namely Yersinia high-pathogenicity island, IucABCDIutA and IroNDCB, have been shown to be involved in the virulence of K. pneumoniae [13].

Biofilm formation is also important to the virulence of K. pneumoniae because the biofilms formed in vivo protect the pathogen from the attacks of host immune responses [14]. In addition, the ability of K. pneumoniae to form biofilms on medical devices, e.g. catheters, has a major role in the development of nosocomial K. pneumoniae infections [15]. Type 3 fimbriae, but not type 1 fimbriae, strongly promote biofilm formation in K. pneumoniae [15]. CPS also contributes to the formation of biofilm microstructures [16].

The cAMP receptor protein (CRP) is one of the best-known global regulatory proteins, and it was originally identified as a major regulator of catabolite repression, a process in which bacteria catabolize secondary sources of carbon only when the supply of the preferred substrate glucose has

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Abbreviations: CPS, capsular polysaccharide; CRP, cAMP receptor protein; PLA, pyogenic liver abscess; RT, reverse transcription.
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become depleted [17, 18]. In addition, CRP is required for the full virulence and/or for the expression of virulence determinants of a wide set of bacterial pathogens [19–25].

The CRP of *K. pneumoniae* serotype K1 strain NTUH-K2044 is conserved to that in *Escherichia coli* with an 87% sequence identity. The aim of this study is to perform the phenotypic characterization of CRP in *K. pneumoniae* NTUH-K2044. The results presented here show that the *K. pneumoniae* CRP is involved in the in *vitro* growth, biofilm formation, fimbria production, CPS biosynthesis and lethality in mice.

**METHODS**

**Bacterial strains and growth conditions**

*K. pneumoniae* NTUH-K2044 (WT), a capsular serotype K1 strain with the hypermucoviscosity phenotype, was originally isolated from a patient with community-acquired PLA and metastatic meningitis [24]. For cloning purposes, *E. coli* strain DH5α was used. Bacterial strains and plasmids used in this study are listed in Table 1. All the primers used in the present work are listed in Table 2.

For general bacterial cultivation and maintenance, bacteria were cultured in Luria–Bertani (LB) broth or on LB agar at 37°C. For the following biochemical and phenotypic assays, bacteria were pre-cultivated overnight in LB broth at 37°C with shaking at 200 r.p.m., and the resulting cell cultures were 100-fold diluted into fresh LB broth and allowed to grow under the above conditions to reach an OD_{600} of about 1.4 (at the mid-log growth phase) for cell harvest. Kanamycin was added at 50 µg ml^-1 when needed.

**Mutant construction**

Base pairs 1 to 556 of the *crp* coding region were deleted from WT to generate a mutant strain named Δ*crp* using a suicide vector pKO3-Km, as described previously [26]. Being modified from the plasmid pKO3 [27], the vector pKO3-Km [26] harboured a temperature-sensitive origin of replication and a sacB gene for positive and negative selection for chromosomal integration and excision, respectively. The flanking regions of *crp* were amplified by PCR with gene-specific primers (Table 2). Briefly, the two DNA fragments (698 and 576 bp in length, respectively) flanking the 566 bp deletion region were amplified by PCR, purified and used as the templates to create a 1274 bp deletion construct that was subsequently inserted between the *NotI* sites of pKO3-Km. The resulting plasmid was then electroporated into WT and plated on LB agar with kanamycin at the non-permissive temperature (43°C) to force the integration of plasmid into the bacterial chromosome by single crossover. During subsequent culturing, five positive colonies were picked and grown at the permissive temperature (30°C) in the presence of sucrose and absence of kanamycin to select for plasmid excision and loss. The mutant colonies were screened by PCR using the primers specific for the *crp* coding region (Table 2).

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>NTUH K-2044 (WT)</td>
<td>Wild-type strain; Ap′</td>
</tr>
<tr>
<td>Δ<em>crp</em></td>
<td>Deletion of crp from WT; Ap′</td>
</tr>
<tr>
<td>C-crp</td>
<td>Complemented crp mutant; Ap′; Km′</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>Δacr A ΔM15 Δ(lacZYA-argF)U169 endA1 recA1 hsdR17(rK−mcrA) supE44 Δ – thi −1 gyrA96 relA1 phoA</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
</tr>
<tr>
<td>pK03-Km</td>
<td>pK03-derived plasmid, with an insertion of Km resistance cassette from pUC4K into Acc1 site</td>
</tr>
<tr>
<td>pGEM-T-easy</td>
<td>pGEM-T easy with an insert of Km cassette from pUC4K into Ndel site for trans complementation</td>
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</tbody>
</table>

**Complementation of Δ*crp***

A PCR-generated DNA fragment containing the *crp* coding region together with its promoter-proximal region (889 bp upstream the coding sequence) and putative transcriptional terminator (541 bp downstream) was cloned between the *NcoI* and *SalI* sites of the vector km-pGEM-T-easy [26]. The resulting plasmid was transformed into the Δ*crp* mutant to yield the *cis–trans* complemented mutant designated as C-crp.

**RNA isolation and reverse transcription PCR**

Total RNAs were extracted using the TRIzol reagent (Invitrogen) [28]. RNA quality was monitored by agarose gel electrophoresis, and RNA quantity was determined by spectrophotometry. Contaminating DNA in the RNA samples was removed by using the Ambion DNA-free kit. cDNAs were generated by using 5 µg of RNA and 3 µg of random hexamer primers. A volume of 30 µl PCR mixture contained 50 mM KCl, 10 mM Tris/Cl (pH 8.0), 2.5 mM MgCl₂, 0.001 % gelatin, 0.1 % BSA (Sigma), 100 µM each of dATP, dCTP, dGTP and dTTP (GE Healthcare), 0.1 µM of each primer, 1 U *Taq* DNA polymerase (MBI Fermentas) and 2 µl of cDNA samples. The parameters for amplification were as follows: 95°C for 3 min, 30 cycles of 94°C for 30 s, 56°C for 45 s and a final extension step of 72°C for 5 min. The PCR products were then analysed by 1.2% agarose gel electrophoresis with ethidium bromide staining.

**Capsule assays**

For the precipitation test of mucoviscosity, 1 ml of cell cultures was centrifuged at 12 000 g for 10 min. For quantifying the extracted CPS, the *K. pneumoniae* CPS was isolated using the hot phenol–water method [29]. Briefly, about 10⁹ c.f.u. of bacterial cells were harvested and suspended in 150 µl D₂O. In parallel, serial dilutions of the bacterial culture were plated to determine the number of c.f.u. An equal volume of phenol was then added, and the mixture was incubated at 68°C for 30 min. The mixture was cooled and extracted with 150 µl chloroform and vortexed intensely.
After centrifugation, the aqueous phase was collected for measuring the amount of uronic acid as described previously [30]. The relative CPS concentration was expressed as the amount (µg) of glucuronic acid per 10^9 c.f.u. of bacterial cells.

**Biofilm assay**

When the bacterial cell cultures had achieved an OD_{600} of about 1.4, they were diluted 50-fold into 2 ml of fresh LB broth and subjected to static incubation at 37 °C for 48 h. Media and planktonic cells were removed to determine the OD_{600}. The tube with the adherent biofilm was washed three times with H_2O and was stained with 3 ml of 0.1% crystal violet for 15 min. The bound dye in each tube was dissolved with 3 ml of 95% ethanol. The OD_{570} was determined to indicate crystal violet staining. The relative capacity of biofilm formation was calculated via the following formula: 100×OD_{570}/OD_{600}. OD_{600} values were used for normalization to eliminate the effect of growth rate and cell density.

**Fimbria activity assays**

The presence and activity of fimbriae at the bacterial cell surface were assessed using the commercial baker’s yeast (Saccharomyces cerevisiae) suspended in PBS as previously described [31, 32]. Equal volumes of yeast cell suspension and bacterial suspension were mixed on a glass slide, and the aggregation was monitored visually.

**Mouse lethality assay**

The bacterial cells were washed twice with 0.95% saline and then subjective to serial 10-fold dilutions with 0.95% saline. Appropriate dilutions were plated onto the LB agar plates to calculate the numbers of c.f.u. Each of the 6- to 8-week-old female BALB/c mice was injected intraperitoneally with 0.1 ml of bacterial suspension. Five groups of mice corresponded to the doses 10^2, 10^3, 10^4, 10^5, 10^6 c.f.u., respectively: eight mice were injected at the dose of 10^6 c.f.u. and four mice for each of the other doses. The mice were monitored daily for 4 weeks, and LD_{50} was calculated as described previously [33].

**Serum resistance assay**

Bacterial survival in serum was determined as described previously [34]. Briefly, 100 µl of bacterial suspension in

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**Table 2. Primers used in this study**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequences (forward/reverse, 5¢–3¢)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Construction of mutant</td>
<td>crp GTATGCGGCCGCACTTCATAGGAGTGGGTGATG/GGTTC TGATCTTCAAGCATTGCGGTTATCTCCT CTGTAA</td>
<td>Amplification of the 698 bp DNA fragment upstream of the 566 bp target deletion region</td>
</tr>
<tr>
<td></td>
<td>crp TAACAGAGGATACCGCCGAAATCGGAGGATCACAGAACC/ GTATGCCGCCGCAACTTCATAGGAGTGGGTGATG/GTA TGCGGCCGCAACCGAGAATTAAACACG</td>
<td>Amplification of the 576 bp DNA fragment downstream of the 566 bp target deletion region</td>
</tr>
<tr>
<td></td>
<td>crp GTATGCGGCCGCACTTCATAGGAGTGGGTGATG/GTA TGCGGCCGCAACCGAGAATTAAACACG</td>
<td>Creation of the 1274 bp deletion allele</td>
</tr>
<tr>
<td>Construction of complemented mutant</td>
<td>crp GAGTCCATGGCGAGGCTCGATTATCTCG/GAGTGTCCA- CACTCCGTAGCGGTGTCG</td>
<td>Amplification of 2063 bp fragment including the crp coding region with its promoter-proximal region (889 bp upstream the coding sequence) and transcriptional terminator</td>
</tr>
<tr>
<td>Detection by PCR</td>
<td>crp TCAACCAGGGCGATTTCATC/AGCCATTTGGCGAAGAGAC</td>
<td>Amplification of a 173 bp intragenic region of crp</td>
</tr>
</tbody>
</table>

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**Fig. 1.** RT-PCR detection of crp mRNA. cDNA samples were generated from the total RNAs of WT, Δcrp and C-crp and then used as the templates for RT-PCR. The primer pairs were designed to amplify the 173 and 151 bp intragenic regions of the crp (I) and 16S rRNA (II, positive control) genes, respectively. Lane M indicates the DNA marker DL2000 (the bands from top to bottom are 2000, 1000, 750, 500, 250 and 100 bp in length). To ensure that no contamination of genomic DNA in the RT reactions would occur, RT-PCR of the negative controls was performed using the ‘cDNA’ sample generated without reverse transcriptase as a template. Reactions containing primer pairs without templates were also included as blank controls. As expected, both negative and blank controls of RT-PCR did not provide an amplicon (data not shown).

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saline was mixed with 100 µl of pooled serum from healthy volunteers, and the mixture was incubated at 37°C for 30 min. The number of viable bacteria was then determined by plate counting. The survival rate was expressed as the number of viable bacteria treated with human serum compared to the number of those incubated with PBS.

**Experimental replicates and statistical methods**

Experiments were performed with three independent bacterial cultures, and the values were expressed as mean ±standard deviation. Paired Student’s *t*-test was performed to determine statistically significant differences, and *P*<0.05 was considered to indicate statistical significance.

**RESULTS**

**Mutation and complementation**

The majority of *crp* coding region was deleted from WT by the allelic exchange to generate the Δ*crp* mutant, and then a cis–trans complemented mutant C-*crp* was constructed from Δ*crp*. The reverse transcription (RT)-PCR experiments were performed to detect the *crp* mRNA in WT, Δ*crp* and C-*crp*; as expected, the *crp* transcript was lacking in Δ*crp* but detectable in both WT and C-*crp* (Fig. 1).

**Deletion of *crp* attenuates in vitro growth**

The growth curves of WT, Δ*crp* and C-*crp* grown in the LB broth were determined, and the results showed that Δ*crp* grew much more slowly than WT (Fig. 2). After 16 h incubation on the blood agar, Δ*crp* formed colonies at a diameter of 1.0±0.1 mm, which was much smaller than that of WT (4.0±0.5 mm). For both broth and agar cultivations, the attenuated phenotype due to the *crp* deletion was restored in C-*crp*. Thus, CRP was required for the *in vitro* bacterial growth.

**Deletion of *crp* enhances CPS production**

Given that bacteria with more thick and mucoid capsules were pelleted less readily, precipitation of mid-log cell cultures was carried out to measure the effect of *crp* deletion on CPS production. After centrifugation at 12 000 *g* for 10 min, compared to Δ*crp*, WT and C-*crp* formed more dense pellets (Fig. 3a). Quantification of uronic acid content confirmed that Δ*crp* produced more CPS than WT and C-*crp* (Fig. 3b). These indicated that CRP balance negatively controlled the capsule biosynthesis.

**CRP contributes to biofilm formation**

When cultivated in LB broth in a cell-culture glass tube, a mass of cells of WT and C-*crp* attached to the liquid–solid interface, and the attached cells (i.e. *in vitro* biofilms) were steadily stained by crystal violet, and in contrast, Δ*crp* stained only a little by crystal violet (Fig. 4). These observations indicated that CRP had a stronger role in positively regulating the bacterial biofilm formation.

**CRP enhances fimbrial activity**

The fimbrial activities of WT, Δ*crp* and C-*crp* were measured by yeast agglutination. WT and C-*crp* induced a considerable agglutination of yeast cells, and in contrast, almost

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**Fig. 2.** Bacterial growth curves. Bacteria were pre-cultivated overnight in the LB broth at 37°C with shaking at 200 r.p.m., and the resulting cell cultures were 100-fold diluted into the fresh LB broth and allowed to grow under the above conditions. The OD_{600} values were monitored at 1 h interval.

**Fig. 3.** Detection of CPS. The centrifugal precipitation (a) of mid-log cell cultures was carried out to measure the effect of *crp* deletion on the CPS production, and the CPS was isolated from the pelleted bacteria cells and then determined as microgram glucuronic acid (GA) per 10^9 c.f.u. of bacterial cells (b).
no agglutination was observed for Δcrp, suggesting a huge attenuation of fimbriae activity due to the crp deletion.

**crp is essential for mouse virulence but not involved in serum resistance**

Mice were infected intraperitoneally with WT, Δcrp and C-crp and 0.95% saline (negative control), respectively. All the mice survived after the injection of 0.95% saline. The LD<sub>50</sub> values were <100, 3.2×10<sup>5</sup> and 4.5×10<sup>4</sup> c.f.u. for WT, Δcrp and C-crp, respectively; the crp deletion led to a 3 to 4 log increase in the LD<sub>50</sub> values after an intraperitoneal route of infection. In addition, the murine survival curves after the intraperitoneal infection at a dose of 10<sup>5</sup> c.f.u. were measured for WT, Δcrp and C-crp; the detected lethality in mice for Δcrp was greatly reduced compared with WT (Fig. 5). These results indicated a stronger role of CRP in positively modulating the bacterial virulence. For both LD<sub>50</sub> and murine survival curve assays, C-crp exhibited a partially restored phenotype of mouse virulence; the failure to fully restore the wild-type phenotype might be due to the lack of antibiotics for efficiently stabilizing the replication of complementation plasmid.

For the determination of serum resistance, bacterial cells were incubated with human serum samples or PBS. The relative serum resistance was determined as the number of viable bacteria treated with human serum compared to the number of those incubated with PBS. There was no obvious difference in the relative serum resistance values determined for WT, Δcrp and C-crp (Fig. 6), indicating that the crp deletion had no effect on the serum resistance.

**DISCUSSION**

Data presented here show that CRP is required for the *in vitro* growth, biofilm formation, fimbriae production and lethality in mouse, but it inhibits the CPS biosynthesis, indicating the important roles of CRP in regulating the virulence and biofilm genes in *K. pneumoniae* serotype K1.

It is amazing that Δcrp produces more CPS relative to WT, but the lethality in mouse is attenuated in Δcrp. WT and Δcrp show no difference in the serum resistance, suggesting that the virulence attenuation is not related to...
the LPS production/modification. The reasons for virulence attenuation in Δcrp were deduced to be as follows: (i) the crp deletion might reduce the in vivo bacterial growth during infection, due to the global regulatory action of CRP on metabolic pathways as characterized in other bacteria; (ii) the visualization of bacteria through Anthony’s capsule staining reveals that the K. pneumoniae morphology changes from rod to club shape after the crp deletion (data not shown), suggesting that CRP has a strong effect on the homeostasis of cell membrane/envelope; (iii) the crp deletion might attenuate the type 1 fimbriae production; and (iv) CRP positively regulates key iron uptake functions (unpublished data). The enhanced CPS production in Δcrp might not be enough to balance the above attenuating factors.

A previous signature-tagged mutagenesis screening study has shown the requirement of CRP for the biofilm formation of a K. pneumoniae serotype K2 strain [35], which was confirmed in a serotype K1 strain in the present study. Positive regulation of fimbria production by CRP has been established in E. coli [36], Salmonella enterica serovar Typhimurium [37] and Pseudomonas aeruginosa [38]. The yeast aggregation assay in this study shows that the fimbrial activity is also positively controlled by CRP in K. pneumoniae serotype K1. CRP enhances the biofilm formation most likely through regulating the production of type 3 fimbriae rather than CPS, since the CPS production is inhibited by CRP.

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Conflicts of interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

References


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