Role of the cAMP-Dependent Carbon Catabolite Repression in Capsular Polysaccharide Biosynthesis in Klebsiella pneumoniae

Ching-Ting Lin1*, Yu-Ching Chen2, Tzuy-Rong Jinn1, Chien-Chen Wu3, Yi-Ming Hong1, Wen-Hao Wu1

1 School of Chinese Medicine, China Medical University, Taichung, Taiwan, Republic of China, 2 Department of Biomedical Informatics, Asia University, Taichung, Taiwan, Republic of China, 3 Department of Biological Science and Technology, National Chiao Tung University, Hsin Chu, Taiwan, Republic of China

Abstract

K. pneumoniae is the predominant pathogen isolated from liver abscesses of diabetic patients in Asian countries. Although elevated blood glucose levels cause various immune problems, its effects on K. pneumoniae virulence are unknown. This study investigated the regulation of capsular polysaccharide (CPS) biosynthesis, a major determinant for K. pneumoniae virulence, in response to exogenous glucose. We found that K. pneumoniae produce more CPS in glucose-rich medium via reduction in cyclic AMP (cAMP) levels. Individual deletion of cyaA or crp, which respectively encode adenylate cyclase and cAMP receptor protein in K. pneumoniae, markedly increased CPS production, while deletion of cpdA, which encodes cAMP phosphodiesterase, decreased CPS production. These results indicate that K. pneumoniae CPS biosynthesis is controlled by the cAMP-dependent carbon catabolite repression (CCR). To investigate the underlying mechanism, quantitative real-time PCR and promoter-reporter assays were used to verify that the transcription of CPS biosynthesis genes, which are organized into 3 transcription units (orf1-2, orf3-15, and orf16-17), were activated by the deletion of crp. Sequence analysis revealed putative CRP binding sites located on P_orf3-15 and P_orf16-17, suggesting direct CRP-CCR regulation on the promoters. These results were then confirmed by electrophoretic mobility shift assay. In addition, we found putative CRP binding sites located in the promoter region of rcsA, which encodes a cAMP transcriptional activator, demonstrating a direct repression of CRP-CCR and P_rcsA. The deletion of rcsA in mutation of crp partially reduced CPS biosynthesis and the transcription of orf1-2 but not of orf3-15 or orf16-17. These results suggest that RcsA participates in the CRP-CCR regulation of orf1-2 transcription and influences CPS biosynthesis. Finally, the effect of glucose and CCR proteins on CPS biosynthesis also reflects bacterial resistance to serum killing. We here provide evidence that K. pneumoniae increases CPS biosynthesis for successful infection in response to exogenous glucose via cAMP-dependent CCR.

Introduction

Klebsiella pneumoniae is a Gram-negative pathogen which causes suppurrative lesions, bacteremia, and urinary as well as respiratory tract infections mostly in patients with underlying diseases [1]. In Asian countries, especially in Taiwan and Korea, K. pneumoniae is the predominant pathogen responsible for pyogenic liver abscess in diabetic patients [2,3,4]. In recent years, reports of Klebsiella liver abscess (KLA) in western countries have also been accumulating [5]. Among the virulence factors identified in K. pneumoniae, capsular polysaccharide (CPS) is considered as the major determinant for K. pneumoniae virulence. Pyogenic liver abscesses isolated often carry heavy CPS loads that could protect the bacteria from phagocytosis and killing by serum factors [6,7]. The capsular serotypes of K. pneumoniae have been classified into more than 77 known types [8,9]. In Taiwan, a high prevalence of the K1 and K2 serotypes of K. pneumoniae was documented in liver abscesses of diabetes mellitus patients [10]. However, the exact mechanism of the tight association between K. pneumoniae, liver abscess, and diabetes mellitus remains unclear.

Diabetic patients have been reported to be more susceptible to infections [11,12]. It has also been demonstrated that K. pneumoniae strains are more virulent in diabetic than in normal mice [13]. The increased risk of bacterial infection in diabetic patients has been studied with regard to host immune system defects [14,15,16]; however, the alteration of gene expression patterns of pathogenic bacteria in response to elevated blood glucose levels awaits further investigation. First studied in Escherichia coli but highly conserved across bacteria, the carbon catabolite repression (CCR) regulates uptake of glucose and repression of genes required for utilization of less preferred carbon sources [17,18,19]. The CCR is generally controlled by the second messenger cyclic AMP (cAMP), which has a fundamental role in global gene regulation [20]. Bacteria grown in glucose show inhibited cAMP production, while bacteria grown in less-preferred carbon sources produce elevated levels of cAMP [17,19,21]. To balance intracellular cAMP levels, the adenylate cyclase CyaA and the cAMP phosphodiesterase CpdA, are required for cAMP biosynthesis and degradation, respectively [17,19,22,23]. The cellular target for cAMP signalling is the cAMP receptor protein (CRP). To regulate mRNA transcription, CRP
consists of a homodimer with cAMP and exhibits DNA-binding activity to the CRP binding site (TGTTA-N6-TCACA and TGCGA-N6-TGCGA) in promoter regions [24,25,26,27]. In E. coli, CRP-cAMP acts as a global regulator of gene expression by controlling the expression of almost 200 operons [28,29,30]. In addition to the regulation of carbon metabolism genes, cAMP signalling has been demonstrated to regulate the expression of various genes encoding virulence factors, such as flagella, fimbriae, protease, exotoxin, and secretion systems in bacteria [31–40]. Sequence analysis revealed a high similarity between CCR proteins (CyaA, CpdA, and CRP) in E. coli and K. pneumoniae, suggesting a conserved regulatory mechanism. In the previous study, CRP-cAMP has been demonstrated to regulate the expression of citrate fermentation genes in K. pneumoniae under fermentative conditions [41]. However, the role of CCR proteins in K. pneumoniae pathogenesis is largely uncharacterized. In this study, we aimed to examine the effect of glucose levels and CCR proteins on the regulation of K. pneumoniae CPS biosynthesis. Individual strains of K. pneumoniae CG43, a highly virulent liver abscess isolate of the K2 serotype, in which cyaA, cpdA, and cyaB had been deleted were constructed for the assessment of CPS production, and the regulatory mechanism of cAMP-dependent CCR in cps transcription was analysed.

Results

Glucose Stimulates CPS Biosynthesis

To analyse if exogenous glucose affects K. pneumoniae CPS biosynthesis, CG43S3 was grown in LB broth supplemented with increasing amount of glucose for the quantification of CPS. As shown in Fig. 1, the CPS level increased when bacteria were grown in LB broth supplemented with 0.25% and 0.5% glucose, while the addition of 0.1% glucose did not have an obvious effect. Since the presence of glucose in the growth medium has been demonstrated to inhibit the cAMP production in many bacteria [17,21], we examined whether the elevated CPS was regulated by cAMP. Increasing amounts of exogenous cAMP were added to LB broth supplemented with 0.5% glucose, and bacterial CPS production was determined. The result showed that the addition of exogenous cAMP repressed the effect of glucose on CPS production, suggesting that environmental glucose can activate K. pneumoniae CPS biosynthesis through a reduction of cAMP level.

CCR Proteins Affect CPS Biosynthesis

To confirm that K. pneumoniae CPS biosynthesis is regulated by cAMP, individual strains with deletion of cyaA and cpdA, which respectively encodes adenylate cyclase and cAMP phosphodiesterase from CG43S3, were constructed, and the effects of the deletions on CPS production were analysed. As shown in Fig. 2A, compared to wild type (WT) strain, we found that CPS levels increased in ΔcyaA, and introduction of cyaA, but not the empty vector control (pACYC184), into ΔcyaA could reverse the effect of cyaA mutation. In contrast, the deletion of cpdA caused a decreased in CPS levels, which could be complemented by introducing a plasmid-carried cpdA (pETQ-cpdA) into the ΔcpdA strain. These results confirmed that cAMP can act as a signalling molecule for regulation of CPS biosynthesis. In addition, since cAMP affects gene transcription through its effector protein CRP, we assessed the effect of deletion of cya on CPS levels. As shown in Fig. 2B, compared to WT, Δcya produced higher levels of CPS. Introduction of the complement plasmid pcrp, but not the empty vector control (pACYC184), into Δcya reversed the effect of the deletion. This result indicates that the CRP-cAMP signalling pathway is involved in the regulation of CPS biosynthesis, and that CRP acts as a negative regulator of CPS biosynthesis. In addition, the functions of CyaA, CpdA, and CRP in controlling the cAMP level in K. pneumoniae have not yet been demonstrated, enzyme-linked immunosorbent assays were performed to determine the intracellular cAMP level upon the deletion of cyaA, cpdA, or cyaB. Compared to WT (9.75 ± 0.33 nM), the intracellular cAMP level was almost undetectable in ΔcyaA (<1 nM), whereas a higher cAMP level was found in ΔcpdA (38 ± 2.8 nM) (Fig. 2C). In addition, a slight increase in the cAMP was found in Δcra (14.5 ± 0.7 nM). These result confirmed that CyaA and CpdA are responsible for CPS biosynthesis and degradation, respectively, in K. pneumoniae.

Effect of cAMP-dependent CCR on cps Transcription

The K2 cps gene cluster of K. pneumoniae Chedid contains 19 open reading frames (ORFs) organised into 3 transcription units, namely, orf1–2, orf3–15, and orf16–17 [42]. To investigate the effect of glucose and cAMP-related proteins on the expression of the 3 cps gene clusters, the mRNA level of orf1 (named galF), orf3 (named wzi), and orf16 (named manC) were measured by qRT-PCR in WT grown in LB medium containing 0.5% glucose with or without 1 mM cAMP. In addition, the effect of cyaA, cpdA, and cyaB mutation strains on the mRNA levels of galF, wzi, and manC were also determined. As shown in Fig. 3A, we found that the mRNA levels of galF, wzi, and manC was increased in glucose-rich medium (LB+0.5% glucose), whereas addition of 1 mM cAMP to the glucose-rich medium could restore the galF, wzi and manC expression, similar to the trends observed in the WT strain. Furthermore, the mRNA levels of galF, wzi, and manC was increased in glucose-rich medium (LB+0.5% glucose), whereas addition of 1 mM cAMP to the glucose-rich medium could restore the galF, wzi, and manC expression, similar to the trends observed in the WT strain. In contrast, a slight reduction in the mRNA level of cps genes was found in the ΔcpdA strain. This result indicates that cps mRNA expression is regulated by cAMP-dependent CCR, and CRP may acts a repressor of cps expression.
To further confirm whether CRP acts as a transcriptional repressor of the promoter activity of galF, wzi, and manC, the reporter plasmids pOrf12 (P_{orf1-2}:lacZ), pOrf315 (P_{orf3-15}:lacZ), and pOrf1617 (P_{orf16-17}:lacZ), each carrying a lacZ reporter gene transcriptionally fused to the putative promoter region of the K2 cps gene cluster [43], were used to transform the K. pneumoniae strains CG43S3ΔlacZ and ΔlacZΔcrp. The promoter activity measurements shown in Fig. 3B revealed that the deletion of crp in the ΔlacZ strain apparently increased the promoter activities of...
galf, wzi, and manC. These results verify that CRP represses cps expression at the transcriptional level.

**Determination of Transcriptional Start Sites on 3 Transcriptional Units in the K2 cps Gene Cluster**

Until now, the transcriptional start sites of the cps gene cluster had not been characterized. 5’ rapid amplification of cDNA ends (5’ RACE) was first performed to determine the transcriptional start sites of the 3 transcriptional units in the K2 cps gene cluster. A single DNA band was obtained for galf, wzi, and manC from the 5’ RACE analysis using either primer pair (data not shown). As shown in Fig 4A, B, and C, sequence analysis of a total of 10 clones each from galf, wzi, and manC revealed a transcriptional start site at the A nucleotide at position −61 relative to the translational start site of galf, at the G nucleotide at position −470 relative to the translational start site of wzi, and at the G nucleotide at position −56 relative to the translational start site of manC. The conserved −10 and −35 promoter sequence of −70 could be readily identified and is shown in Fig 4.

To further investigate the mechanism of CRP-cAMP regulation of cps gene transcription, the sequences of the E. coli CRP binding sites (TGTTGA-N6-TCACA and TGGGA-N6-TGGCA) were used in searching the promoter sequence of the K2 cps gene cluster of K. pneumoniae Chedlid. The maximum number of possible mismatched nucleotides was set at 3, and only the intergenic regions of the 3 transcriptional units were analysed in this search. Using these criteria, no typical CRP binding sites were located upstream of galf (Fig. 4A). However, 3 CRP binding sites were found in the sequence upstream of wzi (Fig. 4B), and one CRP binding site was found to be located at position −140 to −125 relative to the transcriptional start site of manC (Fig. 4C and D). Among the 3 CRP binding sites were located in the intergenic regions of orf2 and orf3. wzi, one was located in the 5’ mRNA region that is 137 bp upstream of the transcription start site of Wzi (at position +319 to +344 relative to the transcriptional start site of wzi), the other 2 CRP binding sites were found to be located at position −29 to −14 and −87 to −72 relative to the transcriptional start site of wzi (Fig. 4D). The result implies that CRP could bind directly to the CRP binding sites that are located in Pwzi and PmanC for controlling cps expression.

**CRP Directly Binds to the CRP Binding Sites in Pwzi and PmanC**

To demonstrate whether CRP binds directly to the upstream sequence of wzi and manC via the CRP binding sites, electric mobility shift assay (EMSA) was performed using the recombinant His6-CRP protein and different DNA fragments containing truncated sequences of Pwzi (Pwzi-1, Pwzi-2, Pwzi-3, and Pwzi-4) and PmanC (PmanC-1 and PmanC-2), as described in the Materials and Methods. Using Pwzi fragments of different lengths, binding of His6-CRP could be observed for Pwzi-1, Pwzi-2, and Pwzi-3 but not for Pwzi-4 (Fig. 5A). As shown in Fig. 5A, DNA-protein-binding complexes were observed after the incubation of 100 nM purified His6-CRP with 10 ng Pwzi-1 or Pwzi-2. However, formation of the Pwzi-3/CRP complex required the incubation with 200 nM purified His6-CRP. This suggests that the lower binding ability of His6-CRP in Pwzi-3 is due to the location of only one CRP binding site in Pwzi-3 as compared to 2 and 3 CRP binding sites in Pwzi-1 and Pwzi-2, respectively. In addition, we found that His6-CRP was able to bind to the PmanC-1 DNA fragment, but not to the PmanC-2 fragment in which the CRP binding site had been removed (Fig. 5B). This result indicates that the recombinant His6-CRP protein can bind directly to the predicted CRP binding sites located in Pwzi and PmanC.

**Expression of rcsA is Controlled by cAMP-dependent CCR and Directly Repressed by CRP**

Because no CRP binding site was found in the sequence upstream of galf, the expression of galf also appeared to be controlled by cAMP-dependent CCR, implying that CRP repression of galf transcription is indirect and that other transcription factors are involved in the CRP regulon controlling cps transcription. According to previous studies, multiple regulatory proteins, which include Fur, RcsA, RcsB, RmpA, RmpA2, KvgA, and KvhR have been shown to mediate K2 cps expression [43,44,45,46]. To further investigate whether these cps regulatory proteins are involved in CRP regulon, the CRP binding site was
searched in the upstream sequence of fur, rcsA/B, rmpA/A2, ksgA, and kshR. However, we found the 2 CRP binding sites (rcsA-1 and rcsA-2) are located at -192 to -177 and -40 to -25 relative to the translation start site of RcsA (Fig. 6A), but no typical CRP binding site was found in other upstream sequence of fur, rcsB, rmpA/A2, ksgA, and kshR. Therefore, we suggest that RcsA is a CRP-regulated transcription factor and is involved in cAMP-dependent CCR control of cps expression.

To verify this possibility, the effect of glucose and cAMP-dependent CCR on the mRNA level of rcsA was first determined by qRT-PCR. As shown in Fig. 6B, addition of 0.5% glucose to LB medium apparently increased the mRNA level of rcsA, while addition of exogenous 1 mM cAMP to glucose-rich medium could restore the level of rcsA expression level to the same as that observed in the WT strain. In addition, the mRNA level of rcsA increased in the ΔcyaA strain, while the deletion of cyaA reduced on rcdA expression. This result indicates that rcdA expression is controlled by the intracellular cAMP level in response to exogenous glucose. In addition, the deletion of cya also increased the expression of rcdA, suggesting that CRP acts a transcriptional repressor of rcdA expression. Furthermore, measurement of promoter activity confirmed the suggestion that the deletion of cya caused a higher level of expression of PrcdA (Fig. 6C).

To further investigate whether CRP binds directly to the rcdA promoter region, EMSA was performed. As shown in Fig. 6D, DNA-protein binding complexes were observed after the incubation of 100 nM purified His6-CRP with 10 ng PrcdA, but not with PrcdA in which one of the CRP binding sites was removed. Therefore, we suggested that CRP-cAMP binds directly to the CRP binding site (rcsA-1) in PrcdA to repress rcdA transcription.
regions of \textit{wzi} or \textit{manC}. Following incubation at room temperature for 30 min, the mixtures were analyzed on a 5% non-denaturing polyacrylamide gel containing 200 \textmu M cAMP. The gel was stained with SYBR Green I dye and photographed.

doi:10.1371/journal.pone.0054430.g005

\textbf{Role of RcsA in Regulation of CRP on CPS Biosynthesis}

To understand whether RcsA participates in CRP regulation of CPS biosynthesis, the level of CPS was determined in WT, \textit{ΔrcsA}, \textit{Δcrp}, and \textit{ΔcrpΔrcsA} strains. As shown in Fig. 7A, the deletion of \textit{rcsA} resulted in a slight reduction in CPS level as compared to WT strain. However, the deletion of \textit{rcsA} partially restored CPS production in the \textit{Δcrp} strain. In addition, introducing the complementary plasmid \textit{pvec} into the \textit{ΔcrpΔrcsA} strain increased CPS levels as compared to the strain carrying the empty vector control (\textit{pRK415}). These results indicate that RcsA participates in CRP regulation of CPS biosynthesis.

To further understand the role of RcsA in CRP regulation of \textit{cps} transcriptions, the promoter activity of \textit{galF} (\textit{pOrf12}), \textit{wzi} (\textit{pOrf15}), and \textit{manC} (\textit{pOrf16}) was measured in \textit{ΔlacZ\_crp}, \textit{ΔlacZ\_crp\_Δcrp}, \textit{ΔlacZ\_crp\_ΔrcsA}, and \textit{ΔlacZ\_crp\_ΔrcsA\_Δcrp} strains. As shown in Fig. 7B, the deletion of \textit{rcsA} in \textit{ΔlacZ\_crp} caused an apparent reduction on the promoter activity of \textit{galF}, but no effect on the promoter activity of \textit{wzi} and \textit{manC} was observed, indicating that RcsA plays a positive role in \textit{galF} transcription. In addition, the deletion of \textit{rcsA} in the \textit{ΔlacZ\_crp\_Δcrp} strain caused a slight reduction in the promoter activity of \textit{galF}, unlike that in the \textit{Δcrp} strain, but still showed higher promoter activity than the \textit{ΔlacZ\_crp} strain. The result implies that RcsA participates in CRP regulation of \textit{galF} transcription. However, we suggest that other unknown transcriptional regulator(s) are also involved in this regulation.

\textbf{Effect of cAMP-dependent CCR on Susceptibility to Normal Human Serum}

Since CPS has been demonstrated to protect \textit{K. pneumoniae} from killing by serum factors, we suggest that glucose, cAMP, and cAMP-related proteins may also affect the ability of \textit{K. pneumoniae}, through modulation of CPS levels, to resist the bactericidal effects of serum. To test the hypothesis, the effects of exogenous glucose and cAMP on survival rate were first determined in treatment with 75% normal human serum. We found that the survival rate of WT grown in LB supplemented with 0.5% glucose (43.2\%\%) increased about 2-fold as compared to the survival rate of WT in LB alone (21.2\%\%). Addition of 1 mM cAMP diminished the survival rate (12.7\%\%) in treatments with 75% normal human serum. Thus, the results imply that \textit{K. pneumoniae} could resist serum killing in response to higher glucose levels via the trigger of cAMP-dependent CCR.

\textbf{Discussion}

Clinically isolated \textit{K. pneumoniae} strains usually produce a large amount of CPS, which confers not only a mucoid phenotype to the bacteria but also resistance to engulfment by phagocytes or to serum bactericidal factors [7,47]. The degree of mucoidy has also

---

\textbf{Figure 5. CRP directly binds to \textit{P}_{wzi} and \textit{P}_{manC}.} Diagrammatic representation of the \textit{wzi} loci (\textit{P}_{\textit{wzi},12}) (A) and the \textit{manC} loci (\textit{P}_{\textit{manC},12}) (B). The large arrows represent the open reading frames. The relative positions of the primer sets used in PCR-amplification of the DNA probes are indicated, and the numbers denote the positions relative to the translational start site. Names of the DNA probes are shown on the left. The dashed boxes indicate the predicted CRP consensus sequences. Different concentrations of purified His6-CRP were incubated with 10 ng of various truncated DNA fragments of the upstream regions of \textit{wzi} or \textit{manC}.
Figure 6. Glucose and cAMP-related proteins affect rcsA transcription. (A) Diagrammatic representation of rcsA loci. The large arrows represent the open reading frames. The relative positions of the primer sets used in PCR amplification of the DNA probes are indicated, and the numbers denote the positions relative to the translational start site. Names of the DNA probes are shown on the left. The dashed boxes indicate the predicted CRP binding sites and the alignment is shown below. (B) qRT-PCR analysis of rcsA expression was measured in WT, ΔcyaA, ΔcpdA, and Δcrp strains in LB or indicated LB medium. The results shown are an average from triplicate measurements in one single experiment representative of three independent experiments. Error bars indicate standard deviations. *P<0.05 and **P<0.01 compared with WT. (C) The β-galactosidase activities of K. pneumoniae CG43S3ΔlacZ and the isogenic strain (ΔlacZΔcrp) carrying the reporter plasmid prcsAZ15 (PrcsA::lacZ) were determined using log-phased cultures grown in LB medium. **P<0.01 compared with ΔlacZ. (D) CRP binds directly to PrcsA. Different concentrations of purified His6-CRP were incubated with 10 ng of various truncated DNA fragments of the upstream region of rcsA. Following incubation at room temperature for 30 min, the mixtures were analyzed on a 5% non-denaturing polyacrylamide gel containing 200 μM cAMP. The gel was stained with SYBR Green I dye and photographed.

doi:10.1371/journal.pone.0054430.g006
been positively correlated with successful establishment of infection [48,49]. Although CPS has been repeatedly proven to play an important role in K. pneumoniae infections and multiple CPS regulators have been found, the environmental stimuli that modulate CPS biosynthesis has remained largely unknown. Our previous studies have reported that extracellular ferric ion could repress K. pneumoniae CPS production through Fur regulation [44,46]. Moreover, in the present study, we found that environmental glucose stimulated CPS production, which was regulated via RcsA (Fig. 7B). According to the CRP regulatory activity. In E. coli, CRP can repress cyaA transcription to down-regulate cAMP production [51]. In K. pneumoniae, a typical CRP binding box (5'-TGGTTA-AATTGA-TCAGC-3') was located at -144 to -129 relative to the translation start site of cyaA. In addition, the mRNA level of cyaA increased more than 16-fold in the Δcrp strain, unlike that in the WT strain (data not shown), indicating that the deletion of cyaA could increase cyaA expression to elevate the cAMP level in K. pneumoniae, consistent with the findings for E. coli.

To further identify the regulatory mechanisms of eps transcription, the transcriptional start sites of 3 transcriptional units in the eps gene cluster were first determined (Fig. 4). CRP binds directly to the predictive CRP binding sites and represses the transcription of wzi and manC (Fig. 3 and 5), while indirectly repressing the transcription of galF via RcsA (Fig. 3 and 7B). According to the position of the CRP binding site relative to RNA polymerase, the CRP-activated promoter is divided into the 3 classes in E. coli [27,52,53]. However, the CRP-repressed promoter displays a greater range of binding site positions [54]. In K. pneumoniae, we found CRP could directly bind to the CRP binding site of eps-2 and rcsA-1 centred at and near the -10 and -35 boxes, respectively, suggesting that CRP may interfere with RNA polymerase regulation of gene transcription (Fig. 4D and 6A). However, K. pneumoniae CRP could also repress transcription by binding directly to the predicted CRP binding site of manC, which was located at a relatively long distance upstream of the -10 and -35 boxes (-140 to -125) (Fig. 3 and 4D). Whether other additional transcriptional factors are required for CRP repression of manC transcription needs further investigation.

Multiple regulators including RcsA/B, RmpA/A2, KvhA, KvgA, and Fur have been demonstrated to control the transcription of K. pneumoniae CPS biosynthesis genes [43,44,45,46]. In this study, we also found that eps transcription is regulated by cAMP-dependent CCR. In E. coli, CRP has been reported to regulate the response to elevated blood glucose levels in diabetic patients, K. pneumoniae could produce more CPS to facilitate its persistence in the blood.

In E. coli, the expression of eps::lacZ was activated when cells were grown at a low temperature (20°C) in the presence of glucose (0.4%) as a carbon source [30]. As shown in Fig. 1, in K. pneumoniae CG43, a K2 serotype strain, we found that higher CPS production in glucose-rich medium is dependent on reducing the intracellular cAMP concentration. In addition, the activation of CPS in response to exogenous glucose was also found in K. pneumoniae NTUH-K2044, a highly virulent liver abscess isolate of K1 serotype, and addition of increasing amounts of exogenous cAMP in glucose-rich medium could completely reverse the effect of glucose on CPS production (Fig. S1). Therefore, we suggest that the regulatory role of glucose and cAMP-dependent CCR in CPS biosynthesis is conserved in K. pneumoniae stains of K1 and K2 serotype. Besides, the addition of cAMP did not completely reverse the glucose-activated CPS biosynthesis in K. pneumoniae CG43 (Fig. 1), suggesting that glucose could activate CPS biosynthesis through mechanism(s) other than repression of the cAMP-CRP regulation in K. pneumoniae CG43, which awaits further investigations.

In bacteria, CyaA and CpdA are responsible for cAMP production and degradation [22,23]. In this study, we also found that the deletion of cyaA abolished the ability of cAMP to increase CPS biosynthesis, while the deletion of cpdA elevated the cAMP level and decreased CPS biosynthesis (Fig. 2). Although the Δcrp strain has a slightly higher cAMP level than WT, CPS production is obviously high in the Δcrp strain. This result indicated that cAMP-mediated repression of CPS biosynthesis is required for CRP regulatory activity. In E. coli, CRP can repress cyaA transcription to down-regulate cAMP production [51]. In K. pneumoniae, a typical CRP binding box (5'-TGGTTA-AATTGA-TCAGC-3') was located at -144 to -129 relative to the translation start site of cyaA. In addition, the mRNA level of cyaA increased more than 16-fold in the Δcrp strain, unlike that in the WT strain (data not shown), indicating that the deletion of cyaA could increase cyaA expression to elevate the cAMP level in K. pneumoniae, consistent with the findings for E. coli.

Figure 7. RcsA is involved in CRP regulation of CPS expression. (A) CPS levels of WT, ΔrcsA, Δcrp, and ΔcrpΔrcsA strains were determined. For complementation purposes, introduction of pRK415 and prcsA into ΔcrpΔrcsA strain were also determined. Bacterial strains were grown in LB broth as indicated at 37°C with agitation. After 16 h of growth, the bacterial glucuronic acid content was determined. *P<0.05 compared to the indicated group. (B) The β-galactosidase activities of K. pneumoniae CG4353ΔlacZ and the isogenic strains (ΔlacZΔcrp, ΔlacZΔrcsA, and ΔlacZΔcrpΔrcsA) carrying the reporter plasmid pOrf12 (P_{εpsA−c}:lacZ), pOrf315 (P_{εpsA−c Rc}:lacZ), or pOrf1617 (P_{εpsA−c Rc}:lacZ) were determined using log-phased cultures grown in LB medium. The results shown are an average from triplicate measurements in one single experiment representative of three independent experiments. Error bars indicate standard deviations. *P<0.05 compared to the indicated group.

doi:10.1371/journal.pone.0054430.g007
expression of fur at the transcriptional as well as at the posttranscriptional level [55,56,57]. In addition, functional interaction of CRP and Fur has been demonstrated to coordinate the transcriptional regulation of iron and carbon metabolism [50]. In K. pneumoniae, CPS biosynthesis was modulated by iron availability and Fur has been shown to repress the transcription of rmpA/A2 and rcsA to control CPS biosynthesis [46]. As shown in this study, the expression of rcsA is also regulated by glucose and cAMP-dependent CCR (Fig. 6). In addition, RcsA is involved in the CRP regulon in regulating galF expression and CPS biosynthesis (Fig. 7). By analysing the promoter sequence of galF, a typical RecAB binding box [59] (‘TAAGATTATC’ or ‘TAC-TCA’-3') was found to be located at position -119 to -107 relative to the transcriptional start site of galF, indicating that RcsA could directly activate galF transcription. In addition, we noted that the deletion of rcsA in the ΔracZΔcyaΔF.lacZ strain retains a higher galF promoter activity compared to the ΔracZ strain, implying that CRP could directly or indirectly regulate galF expression. Although no obvious CRP binding site was found in the promoter sequence of galF, an EMSA was performed to investigate whether CRP could directly bind to PgalF. As shown in Fig. S1, DNA-protein binding complexes were observed after the incubation of 130 mM purified His₀-CRP with 10 ng PgalF, implying that CRP-cAMP could directly repress the galF transcription. However, the exact binding site of CRP in PgalF needs to be further investigated. In addition, we also found that introduction of a plasmid carrying His₆-CRP encoding gene into Δpsp strain could complement the effect of csp mutation on CPS biosynthesis (Fig. S3). The result confirmed that His₆-CRP is functional in vivo. Taken together, these findings revealed a complex regulatory circuit in these CPS regulators, which then modulate the transcription of cps genes in coordination, in response to various environmental stimuli.

In K. pneumoniae, CPS is considered to be an important virulence factor that protects the bacteria from serum killing and phagocytosis [6,7]. In this study, CAMP-dependent CCR was demonstrated to protect K. pneumoniae against serum killing, and the results suggest it plays a role in the regulation of CPS production. In addition, E. coli strains lacking cAMP-CRP are highly resistant to reactive oxygen species (ROS) containing hydrogen peroxide (H₂O₂) and hypochlorous acid (HOC) [60]. Large amounts of ROS are generated by phagocytes to inhibit bacterial colonization and survival [61]. Therefore, we suggest that cAMP-dependent CCR in K. pneumoniae not only regulates CPS production to protect the bacteria from serum killing and phagocytosis, but also alters bacterial resistance to oxidative stress to enhance the survival rate in the phagosome, and we are currently working to demonstrate this possibility. In addition, CPS and adherence factors, such as type 1 and type 3 fimbriae, have been demonstrated to play important roles in biofilm formation and pathogenesis, and their expression could be co-regulated [62]. Biofilms are surface-attached bacteria embedded in a self-produced matrix, composed mainly of polysaccharide, but also containing proteins and nucleic acids [63]. Biofilm formation promotes encrustation and protects the bacteria from the hydrodynamic forces of urine flow, host defences and antibiotics [64]. In E. coli and Serratia marcescens, glucose/CRP-cAMP has been described to regulate the expression of type 1 fimbriae and bacterial biofilm formation [36,37]; however, this regulation has not been proven in K. pneumoniae. Since glucose has also been described to repress K. pneumoniae biofilm formation [65], in addition to CPS, it is possible that glucose/CRP-cAMP is able to regulate the expression of adherence factors, which we are currently investigating.

In this study, we provide important evidence that glucose stimulates CPS biosynthesis in K. pneumoniae to protect the bacteria from serum killing, and CAMP-dependent CCR plays a profound regulatory role in CPS expression in response to glucose levels in the environment. In diabetes mellitus patients, the higher glucose level in the bloodstream is thought to have a major impact on bacterial virulence. We suggest that K. pneumoniae could evade the immune response via the regulation of cAMP-dependent CCR on CPS biosynthesis, especially during infection of diabetes mellitus patients. Future studies will include determining the role of cAMP-dependent CCR in modulating CPS biosynthesis, fimbia production, and biofilm formation in response to environmental stimuli.

Materials and Methods

Bacterial Strains, Plasmids, and Media

Bacterial strains and plasmids used in this study are listed in Table 1. Primers used in this study are list in Table 2. Bacterial strains were routinely cultured at 37°C in Luria-Bertani (LB) medium supplemented with appropriate antibiotics. The antibiotics used include ampicillin (100 μg/ml), kanamycin (25 μg/ml), streptomycin (500 μg/ml), and tetracycline (12.5 μg/ml).

Detection of cAMP

Bacteria was adjusted to 1 x 10⁹ colony forming units (cf.u./ml) and washed twice in phosphate-buffered saline (PBS). Then, the bacteria were resuspended in 300 μl of 1X lysis buffer and lysated by sonication. The lysate was centrifuged briefly at 14,000 rpm for 10 min, and the supernatant was tested for cAMP levels by using CAMP XP™ Assay Kit (Cell Signaling Technology, Inc.) and according to manufacturer’s recommendations.

Construction of the Gene-deletion Mutants and Complementation Plasmids

Specific gene deletion containing csp, cyaA, and cpdΔ was introduced into K. pneumoniae CG43S3 using an allelic exchange strategy as previously described respectively [45]. In brief, two approximately 1000 bp DNA fragments flanking both sides of the deleted region were cloned into the suicide vector pKAS6 [66], a suicide vector containing XphL, which allows positive selection with streptomycin for vector loss. The resulting plasmid was then mobilized from E. coli S17-1pir [67] to K. pneumoniae CG43S3, K. pneumoniae CG43S3ΔracZ, K. pneumoniae CG43S3ΔracA or CG43S3-derived strains, by conjugation. The transconjugants, with the plasmid integrated into the chromosome via homologous recombination, were selected with ampicillin and kanamycin on M9 agar plates. Several of the colonies were grown in LB broth supplemented with 500 μg/mL of streptomycin to log phase at 37°C and then spread onto an LB agar plate containing 500 μg/mL of streptomycin. The streptomycin-resistant and kanamycin-sensitive colonies were selected, and the deletion was verified by PCR and Southern hybridization (data not shown). The resulting K. pneumoniae mutants are listed in Table 1.

To obtain the complementation plasmids, DNA fragments containing the promoter and coding sequence of csp, cyaA, and rcsA were individually PCR-amplified with primer pairs GT131/ GT132, GT196/GT197, and HY001/GT145 (Table 2) and cloned into the shuttle vector pACYC184 or pRK415 to generate pcsp, pcyA, and pcrA, respectively. To generate the cpdΔ complement plasmid, pETQ-cpdΔ, DNA fragment containing the coding sequence of cpdΔ was individually PCR-amplified with primer pair GT210/211 (Table 2) and cloned into the expression vector pETQ. To generate the His-crp complement plasmid,
Table 1. Bacterial strains and plasmids used in this study.

| Strains or plasmids | Descriptions | Reference or source |
|---------------------|--------------|---------------------|
| K. pneumoniae       |              |                     |
| CG43S3              | CG43 Sm', K2 serotype | [71] |
| NTUH-K204444        | K1 serotype | From Dr. Jin-Town Wang |
| ΔcyaA               | CG43S3ΔcyaA | This study |
| ΔcpdA               | CG43S3ΔcpdA | This study |
| Δcrp                | CG43S3Δcrp | This study |
| ΔrcsA               | CG43S3ΔrcsA | [46] |
| ΔcrpΔrcsA           | CG43S3ΔcrpΔrcsA | This study |
| ΔlacZ               | CG43S3ΔlacZ | [43] |
| ΔlacZΔcrp           | CG43S3ΔlacZΔcrp | This study |
| ΔlacZΔrcsA          | CG43S3ΔlacZΔrcsA | This study |
| ΔlacZΔcrpΔrcsA      | CG43S3ΔlacZΔcrpΔrcsA | This study |
| ΔgalU               | CG43S3ΔgalU | [45] |
| E. coli             |              |                     |
| DH5s                | supE44ΔlacU169 (f80 lacZΔM15)hsdR1 recA1 endA1 gyrA96 thi-1 relA1 | [72] |
| BL21(DE3)           | F' ompT hsdSdR31 mcrA2 gal dcm [DE3] | New England Biolabs |
| ST7-1 Δ pir         | hsdR recA pro RP4-2 [Tc-Mu; Km-Tn7] [Δpir] | [67] |
| Plasmids            |              |                     |
| pKAS46              | Ap⁺ Km⁺, positive selection suicide vector, rpsL | [66] |
| yT&₆A              | Ap⁺, TA cloning vector | Yeastern |
| pACYC184            | Tc⁺, low copy number cloning vector | New England Biolabs |
| pRK415              | Tc⁺, broad-host-range IncP cloning vector | [73] |
| pcrp                | Km⁺, 987-bp fragment containing the upstream and coding region of crp cloned into pACYC184 | This study |
| pETQ                | Km⁺, for protein expression vector containing T5 promoter | [73] |
| pETQ-cpdA           | Km⁺, 875-bp fragment containing the coding region of cpdA cloned into pETQ | This study |
| placZ15             | Km⁺, promoter selection vector, lacZ⁺ | [43] |
| prcsA2753           | Cm⁺, 488-bp fragment containing the region upstream of rcsA cloned into placZ15 | This study |
| pOrf1617            | Cm⁺, 300-bp fragment containing the region upstream of Klebsiella K2 cps orf16-orf17 cloned into placZ15 | [43] |
| pET30b-CRP          | Km⁺, 654-bp fragment encoding full-length CRP cloned into pET30b | This study |
| pcyA04              | Ap⁺Km⁺, 2.0 kb fragment containing cyaA and its flanking regions cloned into pKAS46 | This study |
| ppcpdA04            | Ap⁺Km⁺, 2.0 kb fragment containing cpdA and its flanking regions cloned into pKAS46 | This study |
| pcrp04              | Ap⁺Km⁺, 2.0 kb fragment containing crp and its flanking regions cloned into pKAS46 | This study |
| prcsA               | Tc⁺, 1.2-kb fragment containing the upstream and coding region of rcsA cloned into pRK415 | This study |
| pcyA                | Cm⁺, 2918-bp fragment containing the upstream and coding region of cyaA cloned into pACYC184 | This study |
| pETQ-His6-crp       | Km⁺, 804-bp fragment containing the His6-crp cloned into pETQ | This study |

doi:10.1371/journal.pone.0054430.t001

pETQ-His-crp, DNA fragment containing N-terminal His-tag fused with the coding sequence of crp was individually PCR-amplified with primer pair GT132/227 (Table 2) from pET30b-CRP and cloned into the expression vector pETQ.

**Extraction and Quantification of CPS**

CPS was extracted and quantified as previously described [68]. The glucuronic acid content, represents the amount of *K. pneumoniae* K2 CPS, was determined from a standard curve of glucuronic acid (Sigma-Aldrich) and expressed as micrograms per 10⁹ c.f.u. [69].

**qRT-PCR**

Total RNAs were isolated from early-exponential-phase grown bacteria cells by use of the RNeasy midi-column (QIAGEN) according to the manufacturer’s instructions. RNA was DNase-treated with RNase-free DNase I (MoBioPlus) to eliminate DNA contamination. RNA of 100 ng was reverse-transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche) using random primers. qRT-PCR was performed in a Roche LightCycler® 1.5 Instrument using LightCycler TaqMan Master (Roche). Primers and probes were designed for selected target sequences using Universal ProbeLibrary Assay Design Center (Roche-applied science) and listed in Table 2. Data were analyzed
Table 2. Primers used in this study.

| Primer | Sequence (5′→3′) | Enzyme cleaved |
|--------|------------------|----------------|
| GT131  | GGATCCCTTACACCCATTTCCACGGG | BamHI |
| GT132  | AAGCTTCTATGGCCAGGCCTAGCAACTTT | HindIII |
| GT137  | GGATCCCTTAGTGGCCTGGCAACC | BamHI |
| GT140  | GGATCCCTTGATGTTGAGGATTTCAT | BamHI |
| GT141  | AGACTCTGGTTTCTCTCTGCTTG | BglII |
| GT145  | CAATAAGCTCTTCCCAGTACGC | BglII |
| GT154  | GGATCCGCATAACTACAGGAAAGGAA | BamHI |
| GT157  | GGATCCAGGAGGAAGGAAAGC | BamHI |
| GT159  | AGACCTGGGAGATATGGGAGGAAAGC | BglII |
| GT160  | AGACCTTTATATGGGAGGAAAGC | BglII |
| GT161  | AGACCTTTATATGGGAGGAAAGC | BglII |
| GT162  | AGACCTTTATATGGGAGGAAAGC | BglII |
| GT163  | AGACCTTTATATGGGAGGAAAGC | BglII |
| GT167  | GGCTGACAACTCCTTTCCT | |
| GT171  | TCGTGGCGACAGCTTATTAGGCT | |
| GT172  | GTCTAGATCCGAGGATATGGAATGCCA | XbaI |
| GT173  | GAATTCAACCTGGCAGTACTCAT | EcoRI |
| GT174  | GAATTCCACGACGGCAGGAGG | EcoRI |
| GT175  | CCCTGAGATGCGGAGCTCACTAAC | SacI |
| GT179  | AATCTTTCATCTGGCCGCCCAGG | |
| GT192  | TCGTGGCAATATGAGGGCCTGAGG | |
| GT196  | CGATCCCGCTCCATGGAACATCCACTTC | BamHI |
| GT197  | CAAGCTTATCCGGCCGCAATAACTAGC | HindIII |
| GT200  | TCACGGGCTCTGTCCTTTGC | |
| GT202  | CGGATCCAATATGTTCTTCAATG | BamHI |
| GT203  | GGGATCTGCAAGGCTCTGCTGCTG | BamHI |
| GT204  | GTCTAGACGCTGAGGCTTCCCTGAT | XbaI |
| GT205  | GGAGCTCGATTTATACGGCCTG | SacI |
| GT210  | ATGCAGATAAGGAGGTCGAC | |
| GT211  | GGGATCCGCGTGAATACCAATCTGAGC | BamHI |
| GT227  | GATATCGAGACCATCATCATCATCAT | EcoRI |
| CC348  | TTTCCGCCCAATTTCCCAGC | |
| CC349  | TCGAGGGTAGTCTGTTGCTGCTTGCTG | XhoI |
| CC350  | TCTGAGGGTGCTGCTGCTGCTGCTG | XhoI |
| CC351  | ATAGAAGTGATCTCAGGCCAGCAC | |
| HY001  | AAGCTTATGGCCTGCGGATTGAT | HindIII |
| YCC002 | ACTGGATCCCTGCGACCAGGATACC | BamHI |

For qRT-PCR

| Sequence (5′→3′) | TaqMan probes | Target |
|------------------|--------------|--------|
| RT03             | CGTCAATCTGCAGACCAAAGGAC | orf1 |
| RT04             | CGGTTTTTCTCAAAAATCTGAC | orf1 |
| RT05             | CGATGCCGGTCTTATAATG | orf3 |
| RT06             | CTAGGGAGATGGTTGACTGC | |
| RT07             | CGTCCACCTTTACCTTGATG | 67 orf16 |
| RT08             | AGGTAGGACCCCGACCTG | |
| RT11             | GGTAGGAGGCGCTTCTGAA | 67 23S rRNA |
| RT12             | TCACGATCATCGACTTC | |
| RT17             | TCAATAGAATTAAGCAGGAAAAAGA | 18 mmpA |
| RT18             | TTGACCTCCTCCCATCTCC | |
using the real time PCR software of Roche LightCycler® 1.5 Instrument. Relative gene expressions were quantified using the comparative threshold cycle 2ΔΔCT method with 23S rRNA as the endogenous reference.

**Measurement of Promoter Activity**

The promoter-reporter plasmids, pRcsAZ15, pOrf12, pOrf315, and pOrf1617, were individually mobilized into K. pneumoniae strains by conjugation from E. coli S17-1 λpir. The bacteria were grown to logarithmic phase in LB broth, and the β-galactosidase activity was measured as previously described [43].

**Identification of the Transcriptional Start Sites of Three Transcriptional Units in the K2 cps Gene Cluster**

For the determination of 5’ mRNA ends in the three transcriptional units in the K2 cps gene cluster, a rapid amplification of PCR was performed using 5’ RACE kit (Clontech) according to the manufacturer’s instruction as previously described [70]. A total of ten clones each of galF, wzi, and manC, respectively, were subjected to sequence analysis, and the transcriptional start sites of galF, wzi, and manC were determined. All the sequencing results indicated the same nucleotide as the transcriptional start site of galF, wzi, and manC.

**Purification of His6-CRP Protein**

The coding region of *crp* was PCR amplified with primer sets GT132/GT137 (Table 2) and cloned into the BamHI/HindIII site in pET30b (Novagen, 205 Madison, Wis). The resulting plasmid pET30b-CRP was then transformed into E. coli BL21(DE3) (New England Biolabs), and overproduction of the recombinant protein was induced by the addition of 0.1 M IPTG for 4 h at 37°C. The recombinant proteins were then purified from the soluble fraction of the total cell lysate by affinity chromatography using His-Bind resin (Novagen, Madison, Wis). Finally, the purified proteins were dialyzed against 1X TE buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 10% glycerol at 4°C overnight, and the purity was determined by SDS-PAGE.

**EMSA**

The DNA fragments of the putative promoter region of *orf1-2*, *orf3-15*, *orf16-17*, and *rscA* were respectively PCR amplified by using specific primer sets (Table 2). The purified His6-CRP was incubated with 10 ng DNA in a 15 µl solution containing 4 mM Tris-HCl (pH 7.4), 10 mM KCl, 100 mM dithiothreitol, 200 µM cAMP, and 10 µg/ml BSA at room temperature for 30 min. The samples were then loaded onto a native gel of 5% nondenaturing polyacrylamide containing 200 µM cAMP in 0.5X TB buffer (45 mM Tris-HCl, pH 8.0, 45 mM boric acid). Gels were electrophoresed with a 20-mA current at 4°C and then stained with SYBR Green I dye (Invitrogen).

**Bacterial Survival in Serum**

Normal human serum, pooled from healthy volunteers, was divided into equal volumes and stored at −70°C before use. Bacterial survival in serum was determined with minor modifications [45]. In brief, bacteria were grown in LB broth, and when growth reached mid-exponential phase, the bacteria were collected, washed twice with phosphate-buffered saline (PBS), and then adjusted to approximately 1×10^8 c.f.u./ml. The reaction mixture containing 250 µl of the cell suspension and 750 µl of pooled human serum was incubated at 37°C for 15 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 pretreatment. The assay was performed triple, each with triplicate samples. The data from one of the representative experiments are shown and expressed as the mean and standard deviation from the three samples. A CPS-deficient mutant strain, *K. pneumoniae* CG43S3ΔgalU (galU encodes for an UDP-glucose pyrophosphorylase that is responsible for supplying UDP-glucose as material for CPS biosynthesis) is served as a control.

**Statistical Method**

An unpaired t-test was used to determine the statistical significance and values of *P*<0.05 and *P*<0.01 were considered significant. The results of CPS quantification, qRT-PCR analysis, and promoter activity measurement were derived from a single experiment representative of three independent experiments. Each sample was assayed in triplicate and the mean activity and standard deviation are presented.

**Ethics Statement**

For isolation of normal human serum from healthy volunteers, the procedure and the respective consent documents were approved by the Ethics Committee of the China Medical University Hospital, Taichung, Taiwan. All healthy volunteers provided written informed consent.

**Supporting Information**

Figure S1  Glucose and cAMP affects the CPS levels of *K. pneumoniae* NTUH-K2044. CPS levels of *K. pneumoniae* NTUH-K2044 were activated by increasing environmental glucose. Bacterial strains were grown in LB broth supplemented with glucose and cAMP as indicated at 37°C with agitation. After 16 h of growth, the bacterial glucuronic acid content was

| Primer | Sequence (5’→3’) | Enzyme cleaved |
|--------|------------------|----------------|
| RT19   | AAATCATTACCCACACTAAACAAAAA | 80 | mpaA |
| RT20   | TTAGACGGCTTTTAAATTCATGG | 15B | rcsA |
| GT25   | AAAAAAGATCAAATAATGCTGCA | 20 | cyA |
| GT26   | CTTGAGATTTGCGAAGTACC |  |  |
| RT108  | AGCTGCTTCCGATCCTGA |  |  |
| RT109  | AGCAGCTACGCGTCTTCG |  |  |

Table 2. Cont.
References

1. Podschun R, Ullmann U (1998) Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev 11: 589–603.
2. Han SH (1995) Review of hepatic abscess from Klebsiella pneumoniae. An association with diabetes mellitus and septic endocarditis. West J Med 162: 280–284.
3. Lai YJ, Hu BS, Wu WL, Lin YH, Chang HY, et al. (2007) Identification of a major cluster of Klebsiella pneumoniae isolates from patients with liver abscess in Taiwan. J Clin Microbiol 45: 412–414.
4. Yang YS, Sui LY, Yang KM, Fang CP, Huang SJ, et al. (2007) Recurrent Klebsiella pneumoniae liver abscess: clinical and microbiological characteristics. J Clin Microbiol 45: 3336–3339.
5. Lederman ER, Crum NF (2005) Pyogenic liver abscess with a focus on Klebsiella pneumoniae. Infect Immun 73: 6744–6749.
6. Lin JC, Chang FY, Fang CP, Xu JL, Cheng HP, et al. (2004) High prevalence of phagocytic-resistant capsular serotypes of Klebsiella pneumonia in liver abscess. Microbes Infect 6: 1191–1196.
7. Fung CP, Hu BS, Chang FY, Lee SC, Kuo BI, et al. (2000) A 5-year study of the seroepidemiology of Klebsiella pneumoniae: high prevalence of capsular serotype K1 in Taiwan and implication for vaccine efficacy. J Infect Dis 181: 2073–2079.
8. Pan YJ, Fang HC, Yang HC, Lin TL, Heih PF, et al. (2008) Capsular polysaccharide synthesis regions in Klebsiella pneumoniae serotype K57 and a new capsular serotype. J Clin Microbiol 46: 2251–2260.
9. Fung CP, Chang FY, Lee SC, Hu BS, Kuo BI, et al. (2002) A global emerging disease of Klebsiella pneumoniae liver abscess: is serotype K1 an important factor for complicated endophthalmitis? Gut 50: 420–424.
10. Geerlings SE, Stolk RP, Camps MJ, Netten PM, Hoekstra JB, et al. (2000) Asymptomatic bacteriuria can be considered a diabetic complication in women with diabetes mellitus. Adv Exp Med Biol 485: 309–314.
11. Patterson JE, Andrade VT (1997) Bacterial urinary tract infections in diabetes. Infect Dis Clin North Am 11: 755–750.
12. Wu JH, Tsai CG (2005) Infectivity of hepatic strain K. pneumoniae. J Microbiol Immunol Infect 38: 412–414.
13. Muller LM, Gorter KJ, Hak E, Goudzwaard WL, Schellevis FG, et al. (2005) Common infections in diabetes mellitus. Clin Infect Dis 41: 281–288.
14. Muller CM, Aberg A, Straseviciene J, Emody L, Uhlin BE, et al. (2009) Type 1 fimbriae, as shown in the left panel, were determined. Bacteria were grown in LB medium with 100 μM IPTG at 37°C with agitation. **P<0.01 compared to the indicated group. (TIF)
15. Acknowledgments

We thank Professor Hwei-Ling Peng from National Chiao Tung University, Taiwan, for providing the K. pneumoniae CG43S3, ΔmucZ and ΔgalU strains and Dr. Jin-Town Wang, National Taiwan University Hospital for providing K. pneumoniae NTUH-K2044. We are also grateful to Mr. Jing-Giao Lin for his technical assistance during the study.

Author Contributions

Conceived and designed the experiments: CTL YCC TRJ CCW. Performed the experiments: CTL TRJ YMH WHW. Analyzed the data: CTL YCC. Contributed reagents/materials/analysis tools: CTL TRJ. Wrote the paper: CTL CCW.
CRP-cAMP Regulation of Capsular Polysaccharide

40. Stella NA, Kalivoda EJ, O’Dee DM, Nau GJ, Shanks RM (2008) Catabolite repression control of flagellum production by *Serratia marcescens*. Res Microbiol 159: 562–568.

41. Meyer M, Dimroth P, Bör M (2001) Catabolite repression of the citrate fermentation genes in *Escherichia coli* evidence for involvement of the cyclic AMP receptor protein. J Bacteriol 183: 5248–5256.

42. Arakawa Y, Wacharotayankun R, Nagatsu T, Ito H, Kato N, et al. (1995) Functional interactions between the carbon and iron utilization regulators, Crp and Fur, in *Escherichia coli*. J Bacteriol 177: 1786–1796.

43. Lin CT, Huang TY, Liang WC, Peng HL (2006) Homologous response regulators KvgA, KvhA and KvhR regulate the synthesis of capsular polysaccharide in *Klebsiella pneumoniae* CG43 in a coordinated manner. J Biochem (Tokyo) 140: 429–438.

44. Cheng HY, Chen YS, Wu CY, Chang HY, Lai YC, et al. (2010) RmpA regulation of capsular polysaccharide biosynthesis in *Klebsiella pneumoniae* CG43. J Bacteriol 192: 3144–3150.

45. Lai YC, Peng HL, Chang HY (2003) RmpA2, an activator of capsule biosynthesis in *Klebsiella pneumoniae* CG43, regulates K2 cps gene expression at the transcriptional level. J Bacteriol 185: 788–800.

46. Lin CT, Wu CC, Chen YS, Lai YC, Chi G, et al. (2011) Fur regulation of the capsular polysaccharide biosynthesis and iron-acquisition systems in *Klebsiella pneumoniae* CG43. Microbiology 157: 419–429.

47. Regueiro V, Campos MA, Pons J, Alberti S, Bengoechea JA (2006) The uptake of a *Klebsiella pneumoniae* capsule polysaccharide mutant triggers an inflammatory response by human airway epithelial cells. Microbiology 152: 555–566.

48. Nassif X, Honoré N, Vasselon T, Cole ST, Sansonetti PJ (1989) Positive control of colanic acid synthesis in *Escherichia coli* by cmpA and cmpR: two virulence-plasmid genes of *Klebsiella pneumoniae*. Mol Microbiol 3: 1349–1359.

49. Nassif X, Sansonetti PJ (1986) Correlation of the virulence of *Escherichia coli* with the presence of a plasmid encoding aerobactin. Infect Immun 54: 603–608.

50. Slauch JM (2011) How does the oxidative burst of macrophages kill bacteria? Still an open question. Mol Microbiol 80: 580–593.

51. Wu CC, Lin CT, Cheng WY, Huang CJ, Wang ZC, et al. (2012) Fur-dependent MrkHI regulation of type 3 fimbriae in *Klebsiella pneumoniae* CG43. Microbiology 158: 1045–1056.