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克雷白氏肺炎桿菌中 Fur 的功能性分析

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中文摘要:

關鍵字：螯鐵系統、莢膜多醣體、克雷白氏肺炎桿菌、Fur、*rmpA*、 *rmpA2*、*rcsA*。

螯鐵系統和莢膜多醣體 (capsular polysaccharides, CPS)都是克雷白氏肺炎桿菌 (Kp) 的重要致病因子。大部分的 Kp 臨床菌株會產生厚實的 CPS 來幫助細菌躲避免疫細胞及血清分子的攻擊，以及產生多組的螯鐵系統來獲得環境中的鐵，以幫助其生長和感染宿主。雖然在革蘭氏陰性菌中，螯鐵系統的基因表現會受到 Fur 所抑制，但在 Kp 中，Fur 的調控角色仍然還是未知的。基於過去實驗室的研究，我們發現 *fur* 突變後會增加菌體的黏度並且並提高 Kp 對於小鼠的毒性，因此推測 Fur 在毒性因子的調控上扮演重要的角色。因此在本研究中，我們完成下列的實驗來了解 Fur 在 Kp 中所扮演的角色。在顯型的分析上，我們發現 *fur* 突變株不但會減緩細菌的沉澱速度、減少莢膜多醣體並且也會抑制細菌生物膜的生合成，而經過完整 *fur* 基因的互補後即可恢復其原來的顯型表現。利用即時定量 PCR 和電泳膠遲滯實驗發現，Fur 會直接抑制參與 CPS 調控系統中 *rmpA*、*rmpA2* 以及 *rcsA* 的基因表現，然而有趣的是其中我們發現在 *fur* 的突變株下若 *rmpA* 或者 *rcsA* 再突變，則會抑制 *fur* 突變後所造成細菌中 CPS 大量表現的現象，但 *rmpA2* 突變則不會有此結果。另一方面我們也證實了鐵的存在會影響細菌中 CPS 的含量，並且 Fur 需要有鐵的狀態來調控 CPS 的生合成，然而 fur 突變後，也明顯了增加細菌中抓鐵系統的表現。進一步我們利用 Fur titration assay 和即時定量 PCR 來發現在 Kp CG43 中的八套抓鐵系統中至少有六套抓鐵系統可以直接受到 Fur 所抑制，因此我們認為在 Kp 的 Fur 在調控細菌中兩個重要的毒性因子的表現-莢膜多醣體及抓鐵系統中扮演重要的雙重角色

Abstract:

Key words: Iron-acquisition system, capsule polysaccaharides, *Klebsiella pneumoniae*, Fur, *rmpA*, *rmpA2*, *rcsA*.

Iron-acquisition system and capsule polysaccaharides (CPS) are important virulence factors in *Klebsiella pneumoniae* (Kp) infection. Most clinical isolated of *Klebsiella* strains produce large amounts of to protect the bacteria from phagocytosis and prevent from killing by serum factors, and develop several iron-acquisition systems for sequestering ferric iron from the environment to promote the growth and infection. In Gram-negative bacteria, iron-acquisition genes are negatively regulated by ferric uptake regulator (Fur). However, the regulatory roles of Fur in Kp remains large unknown. Base on the previous study, we have demonstrated that the *fur* deletion mutant rendered the bacteria more mucoid phenotype and slight reduced the LD_{50} using mouse lethality assay suggesting an involvement of Fur in virulence regulation. However, we have performed the following experiments to verify the functional role of Fur in Kp in this study. In phenotype analysis, the *fur* deletion mutant slowed the Kp precipitation rate, reduced the CPS biosynthesis, and reduced biofilm formation, while the *fur* complement stain could restore the phenotype. By using quantitative RT-PCR analyses (qPCR) and electrophoretic mobility shift assay showed that Fur repressed the expression of the CPS regulatory genes *rmpA*, *rmpA2* and *rcsA*. Interestingly, deletion of *rmpA* or *rcsA* but not *rmpA2* from the Δ*fur* strain could suppress the deletion effect of Fur. The availability of extracellular iron affected the CPS amount suggesting Fur regulates CPS biosynthesis in an Fe(II)-dependent manner. Increased production of siderophores was observed in the Δ*fur* strain suggesting the uptake of extracellular iron in Kp is regulated by Fur. Fur titration assay and qPCR analyses demonstrated that at least six of the eight putative iron-acquisition systems, identified by BLAST search in the contig database of Kp CG43, were directly repressed by Fur. Thus, we conclude that Fur has a dual role in the regulation of CPS biosynthesis and iron-acquisition in Kp.

INTRODUCTION:

Klebsiella pneumoniae is a rod-shaped Gram-negative bacterium that causes community-acquired diseases including pneumonia, bacteremia, septicemia, and urinary and respiratory tract infections, occurring particularly in immune-compromised patients (42). In Asian countries, especially in Taiwan and Korea, *Kp*is the predominant pathogen responsible for pyogenic liver abscess in diabetic patients (24, 33, 57). Among the virulence factors identified in *K. pneumoniae*, capsular polysaccharide (CPS) is considered as the major determinant for *Kp*infections. The pyogenic liver abscess isolates often carry heavy CPS that could protect the bacteria from phagocytosis and killing by serum factors (35, 44). Apart from the antiphagocytic function, *Klebsiella* CPS also helps bacterial colonization and biofilm formation at the infection sites (8, 20, 39).

Rcs system is a well-known two component system (2CS) that regulates the *cps* gene expressions in bacteria (49). Transcriptions of the *cps* genes are controlled by the response regulator, RcsB, in complex with the auxiliary regulatory protein, RcsA (22, 36). Recently, we have demonstrated that the *cps* expression in *Kp*CG43 was affected by the 2CSs KvgAS, KvhAS, and KvhR in coordination while the regulation is independent of RcsB (34). Besides RcsA, the regulators RmpA and RmpA2 were also able to interact with RcsB for the CPS biosynthesis regulation. Moreover, the *rmpA* expression was shown to be repressed by Fur, the global regulator for the expression of iron uptake systems (13). Whether Fur affects RcsA or RmpA2 is yet to be investigated.

Under iron-repletion conditions, dimeric Fur in complex with Fe(II) binds to a 19-bp consensus DNA sequence, the Fur box (GATAATGATwATCATTATC; w=A or T), in the promoters of the genes required for iron uptake, thereby preventing transcription from these genes (23). The regulation helps bacteria to avoid iron overload that may lead to hydroxyl radical formations. Multiple iron-acquisition systems are commonly present in bacteria for the uptake of iron in the environment (5). In anaerobic environment, Fe(II) is prevalent and imported into the bacterial cytoplasm via Feo system (26). However, in aerobic condition and mammalian tissues (*in vivo*), the majority of iron is found as Fe(III), and iron *in vivo* is almost entirely sequestered by iron-binding proteins (transferrin and lactoferrin) and hemoproteins (hemoglobin and myoglobin) (55).

Bacteria are generally equipped with iron/heme acquisition systems to directly

transport iron from the exogenous iron/heme sources or with siderophore and hemophore compounds released into extracellular medium to scavenge iron/heme from various sources (55). In *K. pneumoniae* NTUH-K2044, expressions of the ten putative iron acquisition genes were highly up-regulated in response to human serum and the bacterial virulence was decreased by the triple mutation of the siderophore genes (28). The siderophore genes *iucABCDiutA* and *iroNDCB* have also been reported as the determinants with statistical correlation to *K. pneumoniae*-caused liver abscess (KLA) (28, 30, 51). Nevertheless, regulation of the expressions of the iron acquisition genes in *K. pneumoniae* has not yet been studied.

In this study, regulatory roles of Fur on the expression of the *cps* regulators RmpA, RmpA2, and RcsA, and the expression of eight iron-acquisition systems in *K. pneumoniae* CG43 were investigated.

SPECIFIC AIMS

1. Investigation of regulatory roles of Fur on mediating the *cps* expression in *K. pneumoniae*;
   (1.) To characterize the functional role of Fur on the gene expressions of the CPS synthesis in *K. pneumoniae*;
   (2.) To study the interplay between Fur and other regulators involved in the *cps* expression.
2. Investigation of regulatory roles of Fur on mediating the gene expressions of iron-acquisition systems in *K. pneumoniae*;
   (1.) To determine the regulation of Fur on iron-acquisition systems by using quantitative PCR;
   (2.) To demonstrate the effect of the putative Fur box in iron-acquisition systems by promoter activity assay and EMSA.
3. Genome-wide search of the downstream genes regulated by *K. pneumoniae*;
   (1.) To screen the presence of putative Fur box in the overall genes in *K. pneumoniae* genome by bio-informatics analysis;
   (2.) To search the downstream genes regulated by Fur by using cDNA subtractive hybridization and proteomic study;

MATERIAL AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely cultured at 37℃ in Luria-Bertani (LB) medium or M9 minimal 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).

Construction of deletion mutants. Specific gene deletions were introduced into *K. pneumoniae* CG43 using an allelic exchange strategy as previously described (32). The pKAS46 system was used in the selection of the mutants (47), and the mutations were confirmed by PCR and Southern hybridization (data not shown).

Biofilm formation. Biofilm formation will be assessed by the ability of the cells to adhere to the walls of 96-well microtitre dishes made of PVC (TPP 96 flat) with some modification of the reported protocol (41). The well of plates will be coated with optimal concentration of 0.109 mg/ml human extracellular matrix (HECM) that diluted in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4℃ (8). The indicator medium (100 μl/well) contained an aliquot of 1:10 diluted overnight bacteria culture and the plate will be incubated at 37℃ with gently shaking for 48 h for biofilm formation. The unadherent bacteria will be washed triply with 200 μl PBS and then adherent bacteria will be stained with 200 μl of 0.1% (w/v) crystal violet at room temperature for 15 min. The plates will be rinsed twice with deionised water to remove excess stain. Finally, the crystal violet stained biofilm will be solubilized in 200 μl of 95% enthanol and the absorbance determined at a wavelength of 550 nm.

Real-time quantitative PCR (qPCR). Total RNAs were isolated from bacteria cells grown to early exponential phase using the RNeasy midi-column (QIAGEN) according to the manufacturer's instructions. RNA was treated with RNase-free DNase I (MoBioPlus) to eliminate DNA contamination. Hundred nanogram of RNA was reverse-transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche) using random primers. qPCR 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 are listed in Table 2. Data were analyzed using the real time PCR software of Roche LightCycler® 1.5 Instrument. Relative gene expressions were quantified using the comparative threshold cycle $2^{-\Delta\Delta CT}$ method with 23S rRNA as the endogenous reference.

Electrophoretic mobility shift assay (EMSA)

Recombinant *K. pneumoniae* Fur protein was expressed in *E. coli* and purified as previously described (13). DNA fragments of the putative promoter regions of *rmpA*, *rmpA2*, and *rcsA* were PCR amplified using specific primer sets. The purified His_6-Fur was incubated with 10-ng DNA in a 15-μl solution containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 100 mM dithiothreitol, 200 μM $MnCl_2$, and 1 μg/μl BSA at room temperature for 20 min. The samples were then loaded onto 5% native (nondenaturing) polyacrylamide gel containing 5% glycerol in 0.5× TB buffer (45 mM Tris-HCl, pH 8.0, 45 mM boric acid) and electrophoresed at 20-mA constant current at 4℃ for 2 hr. The gel was stained with SYBR Green EMSA stain (Invitrogen), and then visualized using the Safe Imager™ blue-light transilluminator.

Extraction and quantification of CPS. CPS was extracted and quantified as previously described (17). The glucuronic acid content, representing the amount of *K. pneumoniae* K2 CPS, was determined from a standard curve of glucuronic acid (Sigma-Aldrich) and expressed as micrograms per 10^9 CFU (7).

Identification of the iron acquisition genes in *K. pneumoniae* CG43. The ten genes encoding different iron acquisition systems in *K. pneumoniae* NTUH-K2044 (28) were used as query sequences to search for homologs in *Kp*CG43 contig database (unpublished results from Dr. S.-F. Tsai, National Health Research Institutes, Taiwan) as assessed by the BLAST search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (4).

Fur titration assay (FURTA). FURTA was performed according to the method described by Stojiljkovic *et al* (48). DNA sequences containing a putative Fur box were PCR amplified with specific primer sets and then cloned into pT7-7. The resulting plasmids were introduced into the *E. coli* strain H1717, and the transformants were plated onto MacConkey-lactose plates containing 100 μg/ml ampicillin and 30 μM $Fe(NH_4)_2(SO_4)_2$. The indicator strain H1717 contained a chromosomal *fhuF::lacZ* fusion, and a low affinity Fur box has been demonstrated in the *fhuF* promoter. The introduction of pT7-7 derived plasmids carrying Fur-binding sequences could thus cause the removal of Fur from the *fhuF* Fur box (27). H1717 harboring pT7-7 was used as a negative control. Colony phenotype was observed after incubation at 37℃ for 10 h. Red colony (Lac+) denoted a FURTA-positive phenotype and indicated the binding of Fur to the DNA sequence cloned into the pT7-7 plasmid.

Chrome azurol S (CAS) assay. The CAS assay was performed according to the method described by Schwyn and Neilands (46). Each of the bacterial strain was grown overnight in LB medium, and then 5 μl of culture was added onto a CAS agar plate. After 16 hr incubation at 37℃, effects of the bacterial siderophore production could be observed. Siderophore production was apparent as an orange halo around the colonies; absence of a halo indicated the inability to produce siderophores.

Statistical method. Unpaired *t* test was used to determine the statistical significance and values of $P < 0.001$ were considered significant. The results of CPS quantification and qPCR analysis were derived from a single experiment which is representative of three independent experiments. Each sample was assayed in triplicate and the average activity and standard deviation are presented.

RESULTS

The phenotype of *fur* deletion mutant in *K. pneumoniae* CG43

In this study, the *fur* complement strain was performed the construction and compared with wild type (CG43S3), *fur* deletion mutant (Δ*fur*), and *fur* complement strain (the entire *fur* gene in pRK415, named pfur) in the viscous phenotype, CPS content, and biofilm formation. During low-speed centrifugation, the *fur* mutant strain exhibited much slower precipitation than the parental strain. While the *fur* mutant and wild type stain supplied with the plasmid pfur respectively, revealed that faster precipitation via low-speed centrifugation (Fig. 1). Subsequently, the CPS production in CG43S3, Δ*fur*, and Δ*fur* [pfur] was quantified by measuring the glucuronic acid content, the core component of the K2 CPS. The production of extracellular polysaccharides in bacteria is important for the maturation of biofilms. In *K. pneumoniae*, the interaction of CPS and fimbriae was able to modulate the bacteria biofilm formation (45). Therefore, the biofilm formations in CG43S3, Δ*fur*, and Δ*fur* [pfur] were detected in polyvinylchloride (PVC) plates by staining 1% crystal violet, which could be quantitatively measured at 595 nm for the absorbance. As shown in Fig. 2, the biofilm formation was significantly lower in Δ*fur* than in wild type, and supplied with the plasmid pfur restored the ability of biofilm synthesis. These results implied that Fur is involved in the regulation of CPS biosynthesis and affects the biofilm biosynthesis in *K. pneumoniae* CG43.

Expressions of RmpA, RmpA2, and RcsA are regulated by Fur

To investigate whether Fur affects the expression of the *cps* regulatory proteins RcsA, RcsB, RmpA2, KvgA and KvhR (13, 32, 34), other than RmpA (Cheng *et al.*, 2010), qPCR analyses were carried out to compare their expression levels in *K. pneumoniae* CG43S3 and its isogenic Δ*fur* strain. As shown in Fig. 3A, when the bacteria were grown in LB, the deletion of *fur* increased the expression of not only *rmpA* but also *rmpA2* and *rcsA*. By contrast, the *fur* deletion appeared to have no effect on the expression of *rcsB*, *kvgA*, or *kvhR*. Inclusion of the iron chelator, 2, 2-dipyridyl (Dip), in the growth medium eliminated the effects caused by Fur deletion suggesting that a Fur-Fe(II) complex is involved in the regulation of the expression of *rmpA*, *rmpA2*, and *rcsA*. While expression of *rmpA* or *rcsA* was increased upon the addition of 200 micromolar Dip, no apparent change was found for the *rmpA2* expression suggesting a novel mechanism to be studied.

As in the P_{rmpA}, the promoter of *rmpA*, putative Fur box sequences could be found in the upstream regions of *rmpA2* and *rcsA* (Fig. 3B). To ascertain Fur directly affects

the expressions of *rmpA2* and *rcsA*, EMSA was performed. As shown in Fig. 3C, the purified recombinant His_6-Fur protein was able to bind to the upstream regions of *rmpA* as well as *rmpA2* and *rcsA*, but not to the P6 DNA which did not contain a Fur box (13). Addition of 200 micromolar EDTA in the reaction mixture appeared to abolish the interactions (data not shown), indicating the formation of Fur-Fe(II) complex was required for the specific binding.

Fur repressed the CPS biosynthesis via RmpA and RcsA

To investigate how Fur differentially regulates the expressions of the three CPS regulators, the double mutants with either deletion of *rmpA*, *rmpA2*, or *rcsA* from the Δ*fur* strain background were constructed and the mutation effects on the bacterial CPS biosynthesis were assessed. Consistent with the previous reports (13, 18, 32), deletion of *rmpA*, *rmpA2*, or *rcsA* caused reduction in the amount of bacterial CPS (Fig. 4). By contrast, a significant increase in the CPS amount was found in Δ*fur* strain. Interestingly, the deletion of *rmpA* or *rcsA*, but not *rmpA2* was found to suppress the *fur* deletion phenotype (Fig. 4). The results suggested that activation of the CPS biosynthesis in Δ*fur* strain is mediated by RmpA or RcsA, but not RmpA2, under the assay condition.

It has been reported that the K2 *cps* gene cluster of *K. pneumoniae* Chedid contained 19 ORFs organized into three transcription units, *orf1-2*, *orf3-15*, and *orf16-17* (6). Analysis of the *cps* promoters revealed no conserved Fur box suggesting an indirect control of Fur on the transcription of *cps*. To investigate the possibility, transcripts of *orf1*, *orf3*, and *orf16* in wild-type (CG43S3), Δ*fur*, Δ*rmpA*, Δ*rmpA2*, Δ*rcsA*, Δ*fur*Δ*rmpA*, Δ*fur*Δ*rmpA2*, Δ*fur*Δ*rcsA*, Δ*fur*Δ*rmpA*Δ*rcsA*, and Δ*fur*Δ*rmpA*Δ*rmpA2*Δ*rcsA* strains were measured by using qPCR. As shown in Fig. 5A, 5B, and 5C, all three transcripts were differentially decreased in Δ*rmpA*, Δ*rmpA2*, and Δ*rcsA* strains. Compared to either *rmpA* or *rcsA* deletion, deletion of *rmpA2* had less effect on the transcriptions of *orf1*, *orf3*, and *orf16*. Interestingly, the deletion of *rmpA* had more profound reducing effects on the transcriptions of *orf1* and *orf16* than that of the *rcsA* deletion. Moreover, the *cps* expression levels in Δ*rmpA*, Δ*rmpA*Δ*rcsA* and Δ*rmpA*Δ*rmpA2*Δ*rcsA* were similar suggesting a major regulatory role of RmpA for controlling the *cps* expression. However, RcsA and RmpA2 may also play a major role on the *cps* expression at a not yet identified condition. Moreover, whether a regulatory interaction exists between RmpA, RmpA2, and RcsA remains to be investigated.

Consistent with the results shown in Fig. 4, the deletion effect of *fur* was eliminated in Δ*fur*Δ*rmpA* or Δ*fur*Δ*rcsA* strain on the expression of *orf1* and *orf16* transcripts (Fig.

5A and 5C). Deletion of *rmpA* from the Δ*fur* strain significantly decreased the level of all three *cps* transcripts. The amounts of the *cps* transcripts in Δ*fur*Δ*rmpA*Δ*rcsA* or Δ*fur*Δ*rmpA*Δ*rmpA2*Δ*rcsA* were similar to that of the Δ*fur*Δ*rmpA* strain. These further support that RmpA plays a major role in the Fur-mediated repression of the *cps* transcription. By contrast, no apparent difference of the *cps* expression between Δ*fur* and Δ*fur*Δ*rmpA2* was observed indicating a minor role, if any, of RmpA2 on the Fur-mediated regulation of the *cps* expression. Nevertheless, the much higher expression levels of the *cps* observed in Δ*fur*Δ*rmpA*Δ*rmpA2*Δ*rcsA* than the strain Δ*rmpA*Δ*rmpA2*Δ*rcsA* suggests an unknown regulator may be involved in the Fur-mediated control of the *cps* expression.

Availability of iron affects CPS biosynthesis in *K. pneumoniae*

To determine whether Fur regulates the gene expressions in an Fe(II)-dependent manners (5, 19), we analyzed the effects of iron-depletion and iron-repletion on CPS biosynthesis. As shown in Fig. 6, when bacteria were grown in LB medium containing ~18 μM iron (1), the CPS amount was increased in the Δ*fur* strain. The *fur* deletion effect was no longer observed in the *fur*-complement strain or when Dip was added to the growth medium. Besides, the addition of 60 μM $FeSO_4$ in M9 medium caused an apparent decrease in the amount of CPS in the wild-type strain compared to that grown in M9 medium. While the Δ*fur* strain grown in M9 medium with or without $FeSO_4$ produced a higher CPS amount than the wild type strain indicating that the iron level of approximately 2 μM in M9 medium (1) appeared to be sufficient for Fur activity on the repression of CPS biosynthesis. These results suggested that the iron-repletion increased Fur activity thereby repressing the biosynthesis of CPS.

The regulatory role of Fur in iron-acquisition systems of *K. pneumoniae* CG43

To assess whether Fur affects iron-acquisition in *K. pneumoniae* in other bacteria, a CAS assay was performed to analyze the activity of siderophore secreted. As shown in Fig. 7A, an orange halo around the colony of Δ*fur* strain grown on a blue CAS plate was observed. Introduction of the complement plasmid pfur into the Δ*fur* strain appeared to diminish the orange halo phenotype. A BLAST search using the DNA sequences of the iron-acquisition systems in *K. pneumoniae* NTUH-K2044 as templates (28) for the homologs in the contig database of *K. pneumoniae* CG43 (unpublished results from Dr. S.-F. Tsai, National Health Research Institutes, Taiwan) was subsequently performed. As shown in Table 3, eight putative iron-acquisition systems were identified. Expressions of the genes (*iucA*, *fepA*, *fepB*, *entC*, *iroB*, *hmuR*, and *feoB*), corresponding to five iron-acquisition systems assessed using qPCR, were increased at least two-fold in the Δ*fur* strain. Expressions of *fhuA*, *fecA*, *fecE*, and *sitA*

were also activated in the Δ*fur* strain, although with less than two-fold increase (Table 3).

As shown in Fig. 7B, homologous sequences of the Fur box (16) could be identified in the putative promoters P_{iroB}, P_{entC}, P_{hmuR}, P_{feo}, P_{fec}, P_{fhu} and P_{sit}. A Fur box homolog was also found in the coding region of *iucA*, at the position -4 to +15 relative to the start codon. These Fur box-containing DNA fragments were then cloned into pT7-7, and the resulting plasmids were introduced individually into the *E. coli* indicator strain H1717. As shown in Fig. 7C, the *E. coli* H1717 harboring the plasmid with P_{iucA}, P_{iroB}, P_{entC}, P_{hmuR}, P_{feo}, or P_{fec}, showed FURTA-positive phenotypes. While the H1717 strains harboring pT7-7 derivatives with the upstream regions of *fhuA* or *sitA* exhibited a FURTA-negative phenotype. The results suggested that Fur is able to bind to each of the predicted Fur box sequences on *iroB*, *entC*, *iucA*, *hmuR*, *feoB*, and *fecA* to exert its regulatory function *in vivo*.

Extracellular Fe(II) has been demonstrated to be transported into bacteria via the iron acquisition systems FeoABC and SitABCD (10, 43). As shown in Fig. 7, expression of the *feo* but not the *sit* genes was affected by Fur. The *feoB* deletion mutant, which was predicted to decrease the bacterial Fe(II)-transport ability, was therefore generated to investigate if the Fe(II)-dependent regulation of the CPS biosynthesis is affected by the Feo system. However, no difference in the CPS amount between the wild-type and the Δ*feoB* strains, grown in LB or M9 supplemented with various concentrations of Dip or $FeSO_4$, was found (data not shown). It is possible that the SitABCD or other iron acquisition systems are involved in the Fur-Fe(II)-dependent regulation on the CPS biosynthesis, which may then compensate the mutation effect of *feoB*.

DISCUSSION

In this study, we have shown that the expressions of CPS regulators RmpA, RmpA2, and RcsA were all under the direct control of Fur (Fig. 3). It has been reported previously that *fur* mutation did not cause obvious change in the *rmpA2* promoter activity assessed using *lacZ* reporter system (13). By contrast, qPCR analysis revealed that the deletion of *fur* caused approximately two-fold increase in *rmpA2* mRNA (Fig. 3A). The discrepancy may be due to the dosage effect of the plasmid-based *lacZ* reporter system for an over-estimation of the β-galactosidase activity. The EMSA in Fig. 3C also supported a direct binding of Fur to the *rmpA2* promoter. Since the *rmpA2* promoter does not fit so well to the Fur of *E. coli*, whether *K. pneumoniae* Fur exerts less rigid recognition sequences is needed to be investigated.

The two homologous genes, *rmpA* and *rmpA2*, are located on pLVPK and both encode CPS regulators for the activation of the CPS biosynthesis (12, 32). Compared to RmpA, RmpA2 has an extended N-terminal region and different promoter sequence which implied differential functionality possessed by the two transcriptional factors. As shown in Fig. 4, the deleting effect of *fur* was eliminated by the further deletion of *rmpA* or *rcsA*, but not *rmpA2* suggesting their differential roles on the regulation of CPS biosynthesis. Further investigation is needed to clarify the roles of the two homologous regulators in *K. pneumoniae*.

Fur has been demonstrated as a global regulator in many bacteria (15, 37, 38). Recently, the deletion of *fur* in *Helicobacter pylori* was shown to reduce the expression of Lon protease (14). Lon is able to affect the protein stability of RcsA and RmpA2 in *E. coli* and *K. pneumoniae* (32, 53). However, no obvious effect on the expression of *lon* was found by *fur* deletion in *K. pneumoniae* CG43 (data not shown). Low identity of Fur protein sequences was found between *H. pylori* and *K. pneumoniae* (25.6% identity), suggesting a different regulatory circuit of Fur in the two bacteria.

The K2 *cps* gene cluster is predicted to encode proteins which are involved in the synthesis, transport, assembly, and modification of CPS (56). As shown in Fig. 5, the differential regulations exerted by RmpA, RmpA2, and RcsA on the *cps* expression affect both the CPS amounts and compositions. The possibility that RmpA, RmpA2, and RcsA could also affect CPS modifications to influence interactions between bacteria and host cells awaits further investigations. The mutant

Δ*fur*Δ*rmpA*Δ*rmpA2*Δ*rcsA* had a higher level of the *cps* expression than that of the mutant Δ*rmpA*Δ*rmpA2*Δ*rcsA* indicating an unknown regulator(s), besides RmpA, RmpA2, and RcsA, is likely involved in the Fur-controlling of the *cps* transcription. This shows the regulation on the *cps* expression in *K. pneumoniae* is much complexed and awaits to be further explored.

In *K. pneumoniae*, Fur has been demonstrated to regulate the expression of flavodoxin FldA and CPS biosynthesis besides regulating its own expression (2, 3, 13). Here, we showed a repressor role of Fur in the regulation of at least eight iron-acquisition systems, although at different levels, in *K. pneumoniae* CG43 (Table 3). Analysis of the putative Fur boxes on *iroB*, *entC*, *hmuR*, *iucA*, *feo*, and *fec* revealed high identities to the consensus sequence (15 to 16 out of 19 positions), while those of *fhuA* and *sitA* exhibit relatively lower identities (13 out of 19 positions). This suggested a highly conserved sequence of the nineteen base pairs sequences is required for a positive phenotype of FURTA. During infection, differential expression of the iron-acquisition systems is anticipated to provide an adaptive advantage with flexibility in response to various environmental stimuli (11, 54). Therefore, a different coordination of the eight iron-acquisition systems in CG43 is predicted. Besides, whether CG43 harbors other iron-acquisition genes remains to be investigated.

In this study, we characterized the role of Fur in the CPS regulatory circuit of *K. pneumoniae* CG43. Besides RmpA, RcsA and RmpA2 were found to be directly regulated by Fur. We also demonstrated that Fur regulates the CPS biosynthesis via RcsA or RmpA, but not RmpA2, in an Fe(II)-dependent manner. Moreover, we report the deletion effect of *fur* on the expression of the eight iron-acquisition systems identified in *K. pneumoniae* CG43.

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Table 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Descriptions	Reference or source
K. pneumoniae		
CG43S3	CG43 Sm^r	(31)
Δ*rmpA*	CG43S3Δ*rmpA*	(13)
Δ*rmpA2*	CG43S3Δ*rmpA2*	(31)
Δ*fur*	CG43S3Δ*fur*	(13)
Δ*rcsA*	CG43S3Δ*rcsA*	This study
Δ*rmpA*Δ*rcsA*	CG43S3Δ*rmpA*Δ*rcsA*	This study
Δ*rmpA*Δ*rmpA2*Δ*rcsA*	CG43S3Δ*rmpA*Δ*rmpA2*Δ*rcsA*	This study
Δ*fur*Δ*rmpA*	CG43S3Δ*fur*Δ*rmpA*	This study
Δ*fur*Δ*rmpA2*	CG43S3Δ*fur*Δ*rmpA2*	This study
Δ*fur*Δ*rcsA*	CG43S3Δ*fur*Δ*rcsA*	This study
Δ*fur*Δ*rmpA*Δ*rcsA*	CG43S3Δ*fur*Δ*rmpA*Δ*rcsA*	This study
Δ*fur*Δ*rmpA*Δ*rmpA2*Δ*rcsA*	CG43S3Δ*fur*Δ*rmpA*Δ*rmpA2*Δ*rcsA*	This study
E. coli		
DH5α	*supE44* Δ*lacU169* (*f80 lacZ*ΔM15) hsdR17 *recA1 endA1 gyrA96 thi-1 relA1*	(25)
BL21-RIL	F^- *ompT* $hsdS_B[r_B^-m_B^-]$*gal dcm* [DE3]	Laboratory stock
S17-1 λ *pir*	*hsdR recA pro* RP4-2 [Tc::Mu; Km::Tn*7*] [λ*pir*	(47)
H1717	*araD139* Δ*lacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR aroB fhuF::*λ *placMu*	(27)
Plasmids		
pKAS46	Positive selection suicide vector, *rpsL* Ap^r Km^r	(47)
pET30a-c	His-tagging protein expression vector, Km^r	Novagen
yT&A	TA cloning vector	Yeastern
pRK415	Broad-host-range IncP cloning vector, Tc^r	(29)
pT7-7	Cloning vector, Ap^r	(50)
pfur03	1.7 kb fragment containing an internal 454 bp deletion in *fur* cloned into pKAS46	(13)
prcsA03	2.0 kb fragment containing an internal 620 bp deletion in *rcsA* cloned into pKAS46	This study
piroB_2	928 bp fragment containing the putative *iroBCD* promoter, cloned into pT7-7	This study
pentC_2	284 bp fragment containing the putative *entC* promoter, cloned into pT7-7	This study
piucA_2	700 bp fragment containing the putative *iucABCD* promoter, cloned into pT7-7	This study
phmuR_2	500 bp fragment containing the putative *hmuRSTUV* promoter, cloned into pT7-7	This study
pfeo_2	564 bp fragment containing the putative *feoABC* promoter, cloned into pT7-7	This study
pfec_2	296 bp fragment containing the putative *fecIRA* promoter, cloned into pT7-7	This study
pfhuA_2	313 bp fragment containing the putative *fhuA* promoter, cloned into pT7-7	This study
psitA_2	283 bp fragment containing the putative *sitABCD* promoter, cloned into pT7-7	This study
pFT01	0.5 kb fragment containing the putative *orf1-2* promoter, cloned into pT7-7	This study
pFT02	0.9 kb fragment containing the putative *orf3-15* promoter, cloned into pT7-7	This study
pFT03	0.3 kb fragment containing the putative *orf16-17* promoter, cloned into pT7-7	This study
pFT04	0.5 kb fragment containing the putative *rmpA* promoter, cloned into pT7-7	This study
pFT05	0.5 kb fragment containing the putative *rmpA2* promoter,	This study

	cloned into pT7-7	
pFT06	0.5 kb fragment containing the putative *rcsA* promoter, cloned into pT7-7	This study

Table 2. Primers used in this study

Primer	Sequence (5'→3')	Enzyme cleaved	Target
For FURTA			
FA01	GAAGCTTGGAGCGCAGTTAGCGGAC	*Hin*dIII	P_{iroB}
FA02	CGGATCCGCCCATAGAGAGGAGGACC	*Bam*HI	
FA03	GAAGCTTCCTGGGCTGAGGTAATTCC	*Hin*dIII	P_{entC}
FA04	CGGATCCCTCAGCCAGTGACGTTTCC	*Bam*HI	
FA05	GGATCCAGAGGGTGATTTGCCAGCAT	*Bam*HI	P_{iucA}
FA06	AGATCTGGAAGCACTGAGCAGCCACA	*Bgl*II	
FA07	ACACCAAGCTTCTGACGGAG	*Hin*dIII	P_{hmuR}
FA08	CTCCGGGATCCAGACATCGC	*Bam*HI	
FA09	GGATCCCAACAGCGCGATGATGGAT	*Bam*HI	P_{feo}
FA10	AGATCTGCCAGCATGCCGAGGGAGA	*Bgl*II	
FA11	GAAGCTTGTCGCGGGCTGGATCAAG	*Hin*dIII	P_{fhuA}
FA12	CGGATCCCGCAGCGAGTGATTTGGC	*Bam*HI	
FA13	GAATTCGCAGCCTGATTGAC	*Eco*RI	P_{sitA}
FA14	GGTGTAGCATAGGATCCCTC	*Bam*HI	
For qPCR	Sequence (5'→3')	TaqMan probes	Target
GT56	ACCCCGCCAGCTTTAACTT	3	*entC*
GT57	TGTCCTTCTTTACGCAGCAG		
GT58	CAACCTGAACAGCGATTTCC	20	*fecA*
GT59	TCGGCGCTCTCTTTAACAGT		
GT62	CAGATGTCAGCGCAGATCC	20	*feoB*
GT63	CATAGGCCCGGCTGTAGA		
GT64	AAAGAGATTGGCCTCGAGTTT	20	*fepA*
GT65	TGTTGCGGTAGTCGTTGC		
GT66	AATAAACAGCTCGTTTCGTTAAAAG	160	*fepB*
GT67	GTATAGACCAGGGCGGTCAC		
GT68	GTTTGGTCGTATCGCCTGAC	3	*fhuA*
GT69	GGAAGGTGAAGTCAGTTTTATCG		
GT72	TGATGACCTACCTGCAGTACCA	20	*hmuR*
GT73	GAGCCGAGGTTCCAGGAG		
GT74	CGGAGGAACATTCGTCAAA	84	*iroB*
GT75	TTCGGAATCTAAGCCTGGTG		
GT78	TCTCCCGGCTTATTGTTGATA	67	*iucA*
GT79	GGAAGGTTTCGCAACTGGT		
GT82	GAAGATCCGTCAGACGATGG	20	*sitA*
GT83	TAGTCGCGGGCCAGATAG		
RT03	CGTCATCCAGACCAAAGAGC	83	*orf1*
RT04	CCGGTTTTTCAATAAACTCGAC		
RT05	CGATGACCGGCTTTTTAATG	83	*orf3*
RT06	CTAGCGGAGATTTGGTACTGC		
RT07	CAGTCCACCTTTATTCCGATTG	67	*orf16*
RT08	AGGTACGACCCCGACTGG		
RT11	GGTAGGGGAGCGTTCTGTAA	67	23S rRNA
RT12	TCAGCATTCGCACTTCTGAT		
RT17	TCAATAGCAATTAAGCACAAAAGAA	18	*rmpA*
RT18	TTGTACCCTCCCCATTTCC		
RT19	AAATCATTACCCACAACTAACAAAAA	80	*rmpA2*
RT20	TTAGACGGCTTTTTAATTCATGG		
GT25	AAAACAGAATCAAATATGCTGCAA	158	*rcsA*
GT26	CGTTGAGATTTGCGAAGTACC		
RT31	AAATTCACCCCGGAAAGC	120	*rcsB*
RT32	GCAGTACTTCGCTCTCTTTCG		
GT27	AAACCGTCCTGGAAAACCA	84	*kvgA*
GT28	CAACCAGCTGGATAGCATGA		
GT13	GTATTTTTATTCGCGATGTACTGC	67	*kvhR*
GT14	GCCTGAACAGCGGAGAGA		

Table 3. qPCR analyses of expressions of iron acquisition genes in *K. pneumoniae* wild-type and Δ*fur* strains

Systems	Gene	RNA expression ratio[a] Δ*fur*/wild type	Reference
Fe^{3+}			
Ferrichrome	*fhuA*	1.73±0.19	(21)
Aerobactin	*iucA*	2.42±0.18	(12)
Enterobactin	*fepA*	2.11±0.18	(40)
	fepB	2.25±0.20	(40)
	entC	3.09±0.15	(40)
Ferric citrate	*fecA*	1.61±0.16	(9)
	fecE	1.69±0.26	(9)
Salmochelin	*iroB*	6.28±0.98	(12)
Heme	*hmuR*	3.08±0.65	(52)
Fe^{2+}			
Ferrous iron	*feoB*	4.08±0.35	(10)
	sitA	1.97±0.23	(43)

a. Mean expression ratio of *fur* mutant relative to wild-type parental strain CG43S3

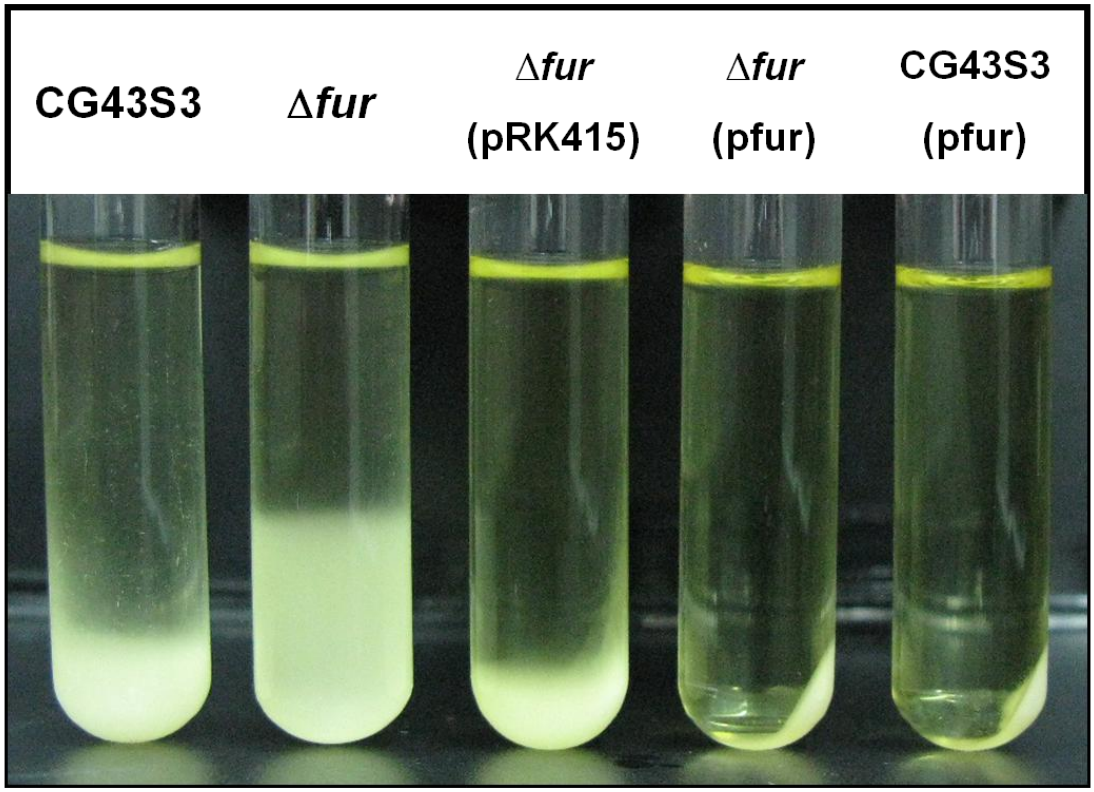


Figure 1. Kp CG43S3 *fur* gene deletion rendered a more viscous phenotype by using sedimentation assay. The bacteria containing Kp CG43S3 (wild type) and Δ*fur*, Δ*fur*[pRK415], Δ*fur*[pfur], CG43S3 [pfur] were cultured overnight in LB broth at 37°C and subjected to centrifugation at 4,000 rpm (1,500 × *g*) for 3 min.

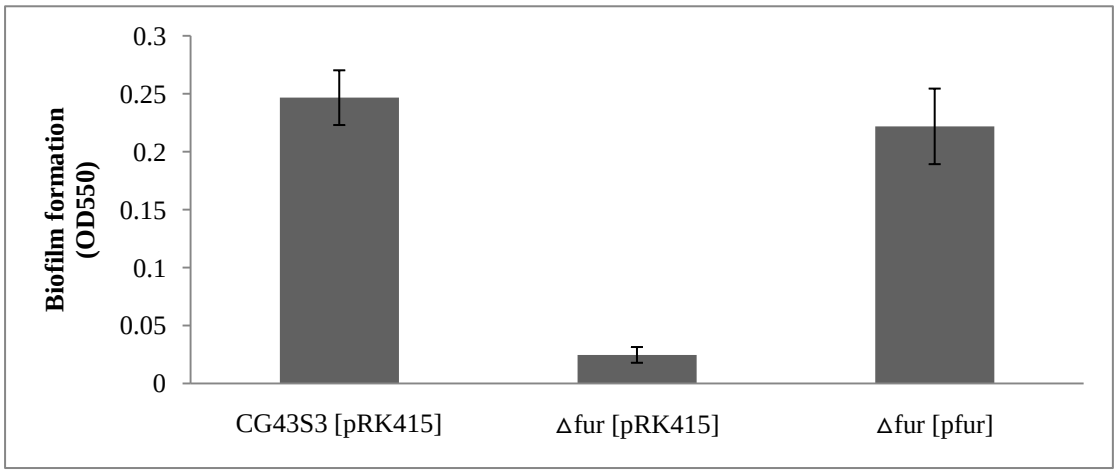
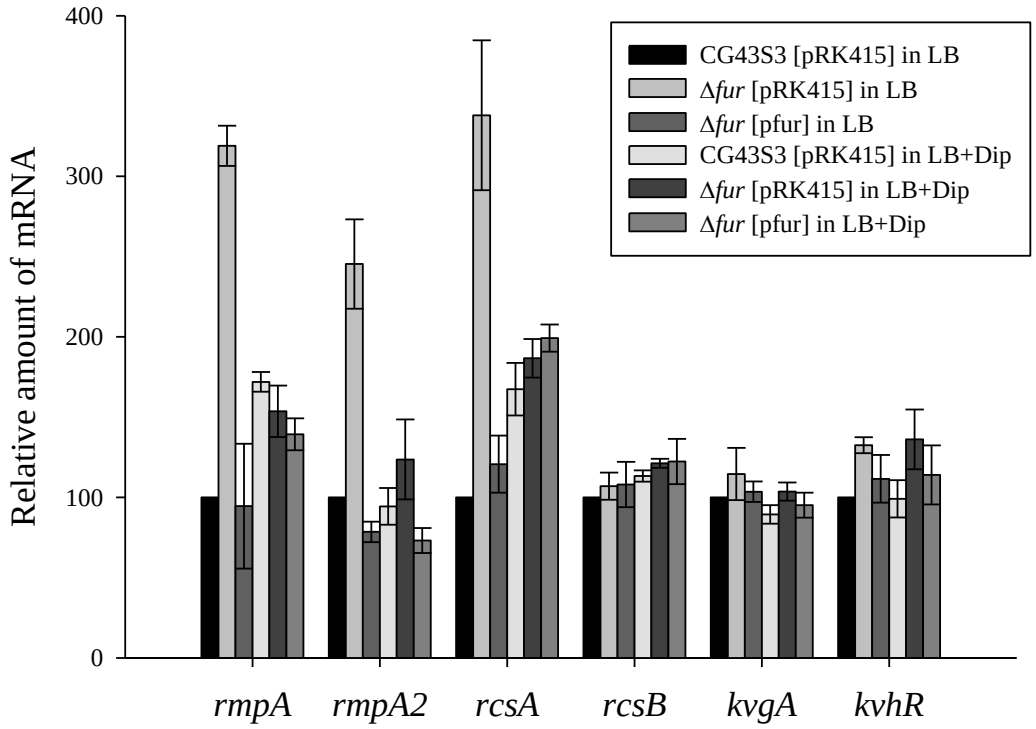
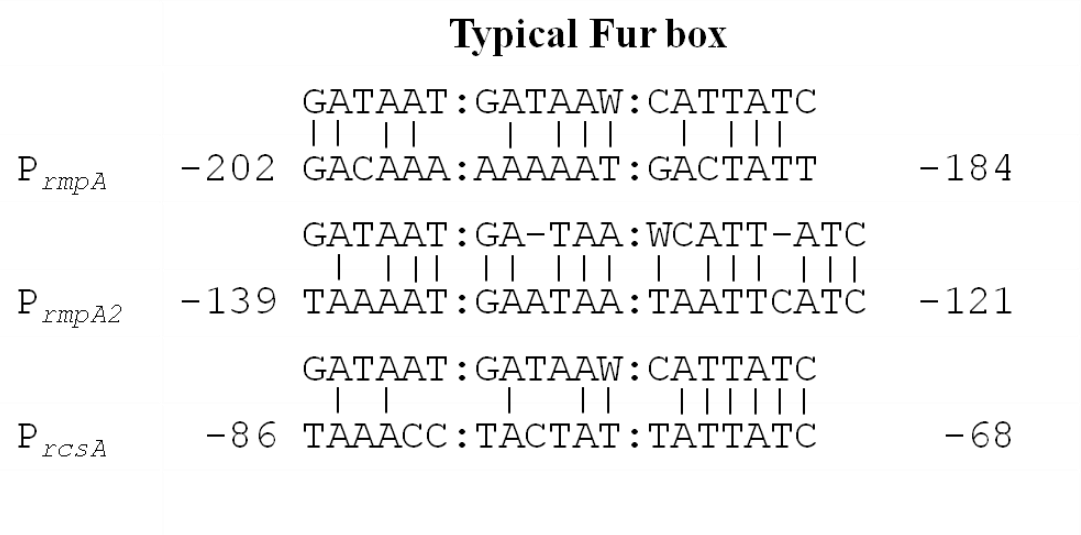


Figure 2. Knockout of *fur* decreases *K. pneumoniae* biofilm formation. Overnight cultured bacteria were 100-fold diluted and inoculated into a 96-well microtiter dish. After 48 hr static incubation at 37 ^{o}C, the bacterial biofilm formation was quantified by crystal violet staining. The values correspond to the average from triplicate samples from a single trial. Error bars, standard deviations.

(A)


400
300
200
100
0
Relative amount of mRNA
CG43S3 [pRK415] in LB
Δfur [pRK415] in LB
Δfur [pfur] in LB
CG43S3 [pRK415] in LB+Dip
Δfur [pRK415] in LB+Dip
Δfur [pfur] in LB+Dip
rmpA
rmpA2
rcsA
rcsB
kvgA
kvhR

(B)



(C)

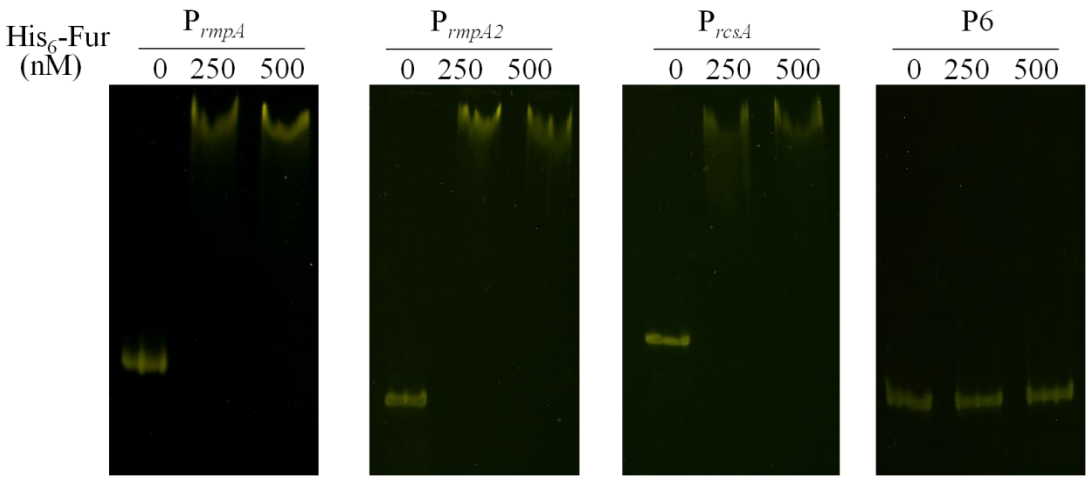


Figure 3. Fur directly repressed the expressions of *rmpA*, *rmpA2*, and *rcsA*. (A) qPCR analysis. The *K. pneumoniae* CG43S3 [pRK415], Δ*fur* [pRK415], and Δ*fur* [pfur] strains were grown overnight in LB with or without 200 micromolar 2, 2-dipyridyl (Dip), then the relative expressions of *rmpA*, *rmpA2*, *rcsA*, *rcsB*, *kvgA*, and *kvhR* in bacteria were measured by qPCR analysis. (B) DNA sequence alignment between the *E. coli* typical Fur box and the putative Fur boxes in the upstream regions of *rmpA*, *rmpA2*, and *rcsA*. The relative positions to the translational start sites are indicated. (C) EMSA of the recombinant His_6-Fur and its target promoters. DNAs of the upstream regions of *rmpA*, *rmpA2*, and *rcsA* were incubated with an increasing amount of the His_6-Fur for 30 min and then loaded onto a 5% non-denaturing polyacrylamide gel. The DNA fragment P6 was used as a negative control. The gel was stained with SYBR Green EMSA stain and imaged.

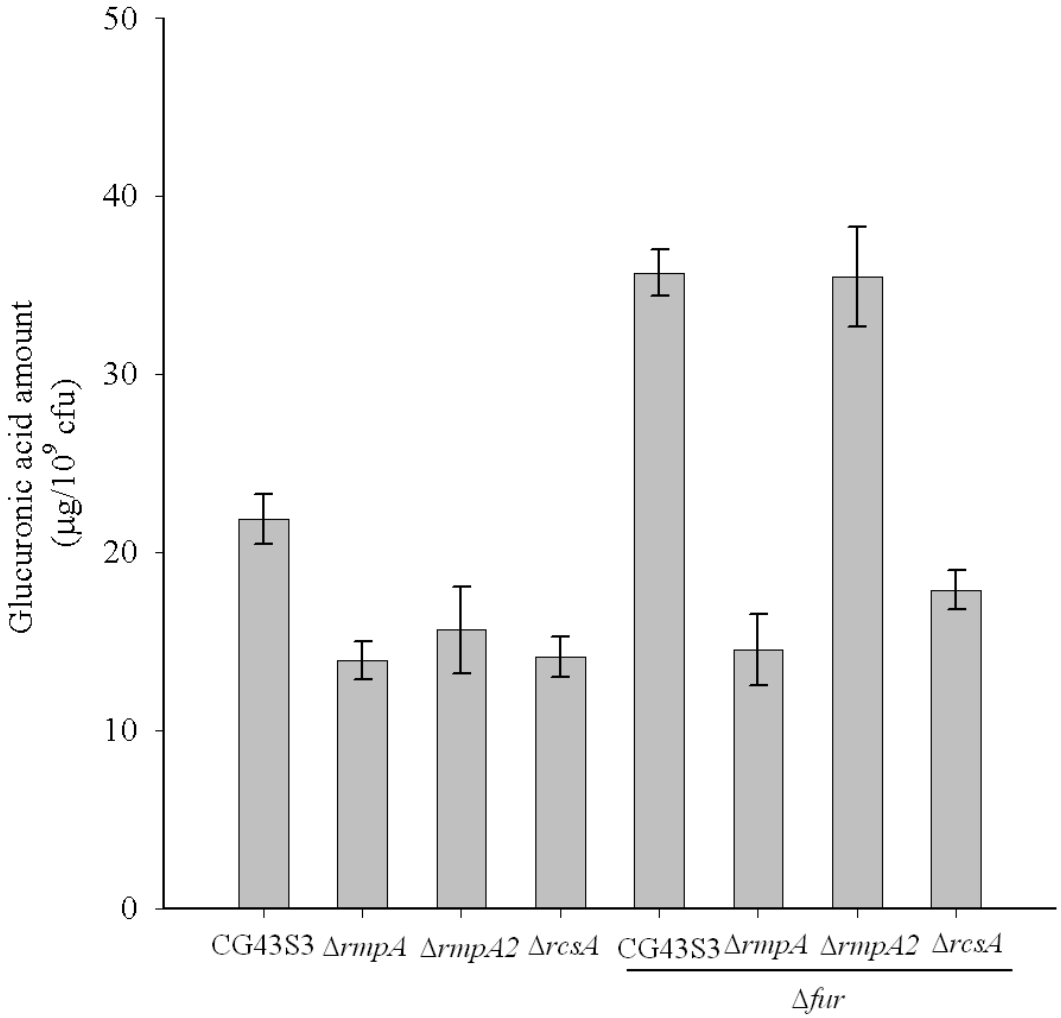
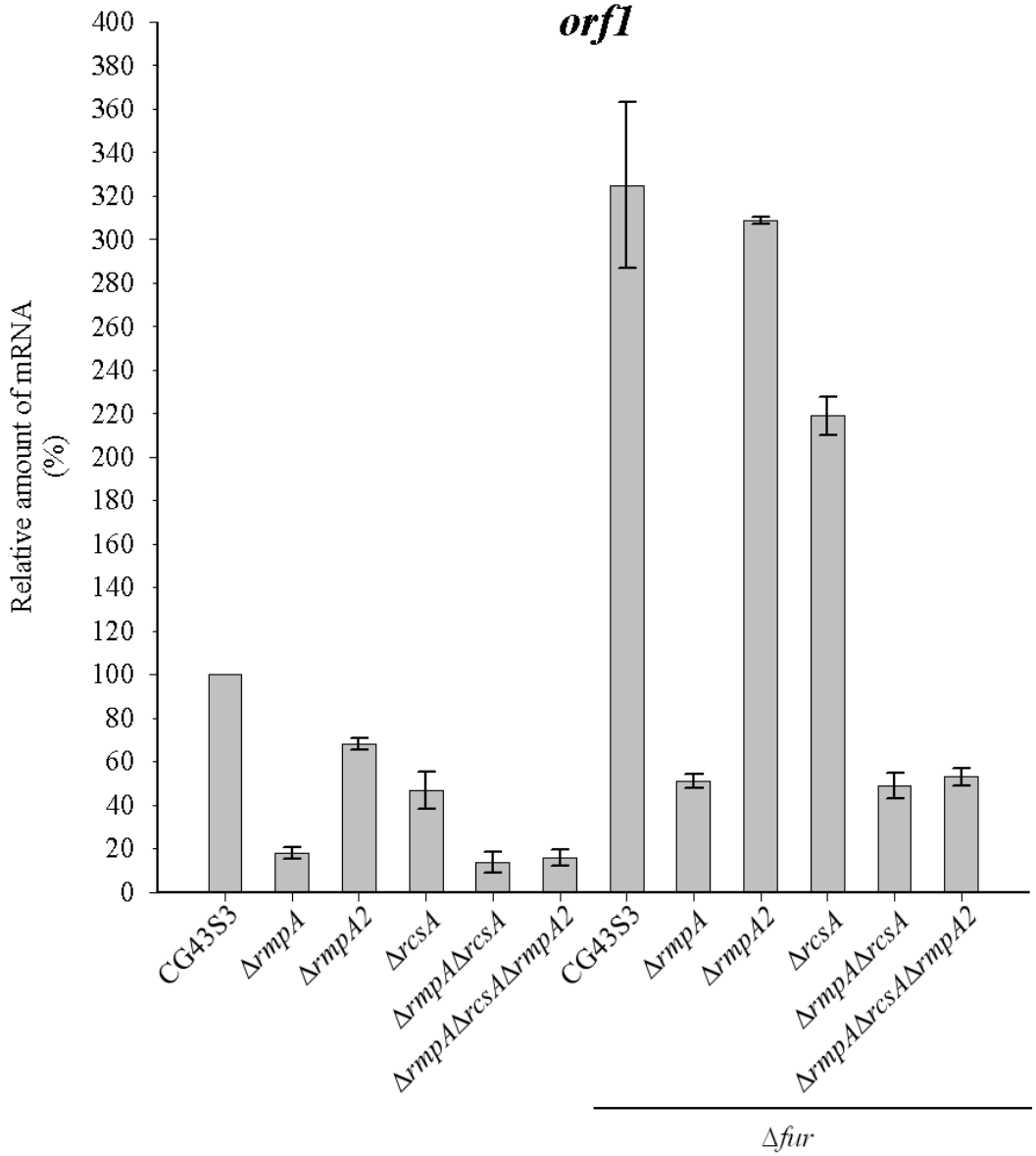
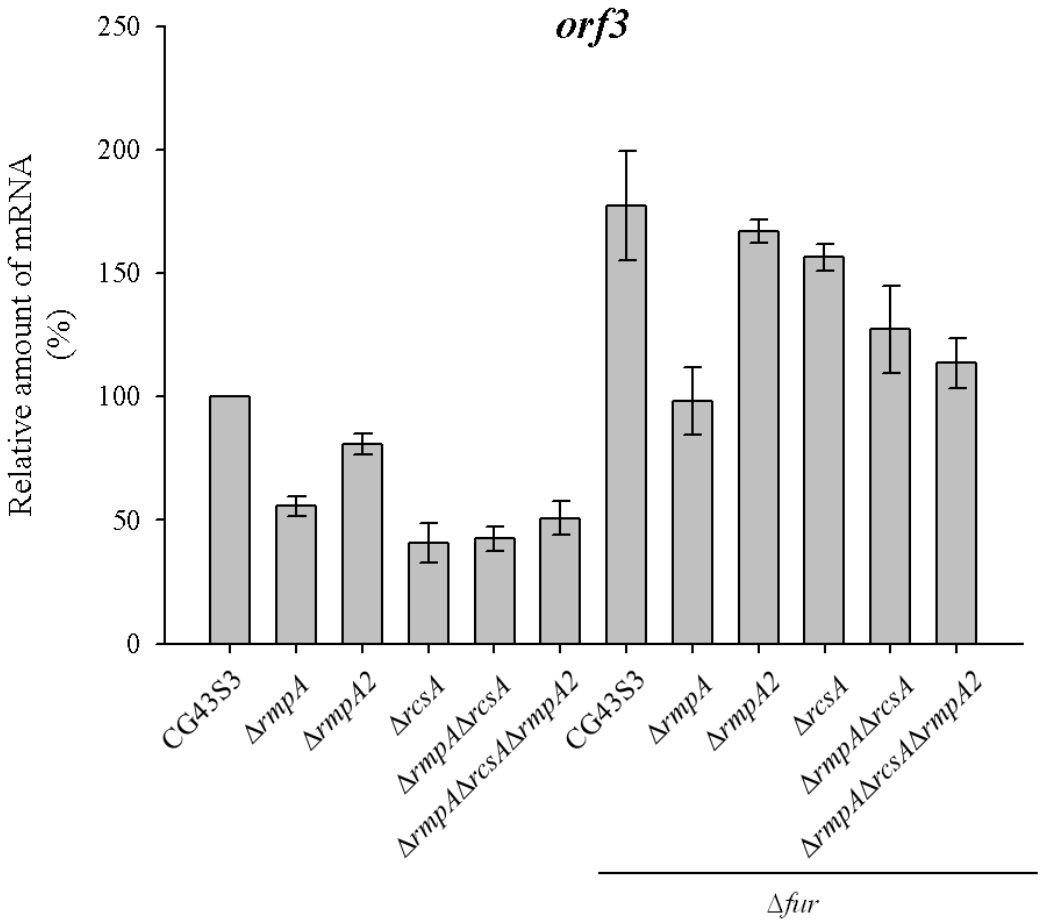


Figure 4. Fur represses the CPS biosynthesis via RmpA and RcsA. Bacteria strains, as indicated in the margin, were grown in LB medium at 37℃ with agitation. After 16 hr growth, the bacterial glucuronic acid contents were determined. Values are mean ± standard error of three independent experiments.

(A)


orf1
Relative amount of mRNA (%)
400
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
CG43S3
ΔrmpA
ΔrmpA2
ΔrcsA
ΔrmpAΔrcsA
ΔrmpAΔrcsAΔrmpA2
CG43S3
ΔrmpA
ΔrmpA2
ΔrcsA
ΔrmpAΔrcsA
ΔrmpAΔrcsAΔrmpA2
Δfur

(B)


orf3
Relative amount of mRNA (%)
250
200
150
100
50
0
CG43S3
ΔrmpA
ΔrmpA2
ΔrcsA
ΔrmpAΔrcsA
ΔrmpAΔrcsAΔrmpA2
CG43S3
ΔrmpA
ΔrmpA2
ΔrcsA
ΔrmpAΔrcsA
ΔrmpAΔrcsAΔrmpA2
Δfur

(C)

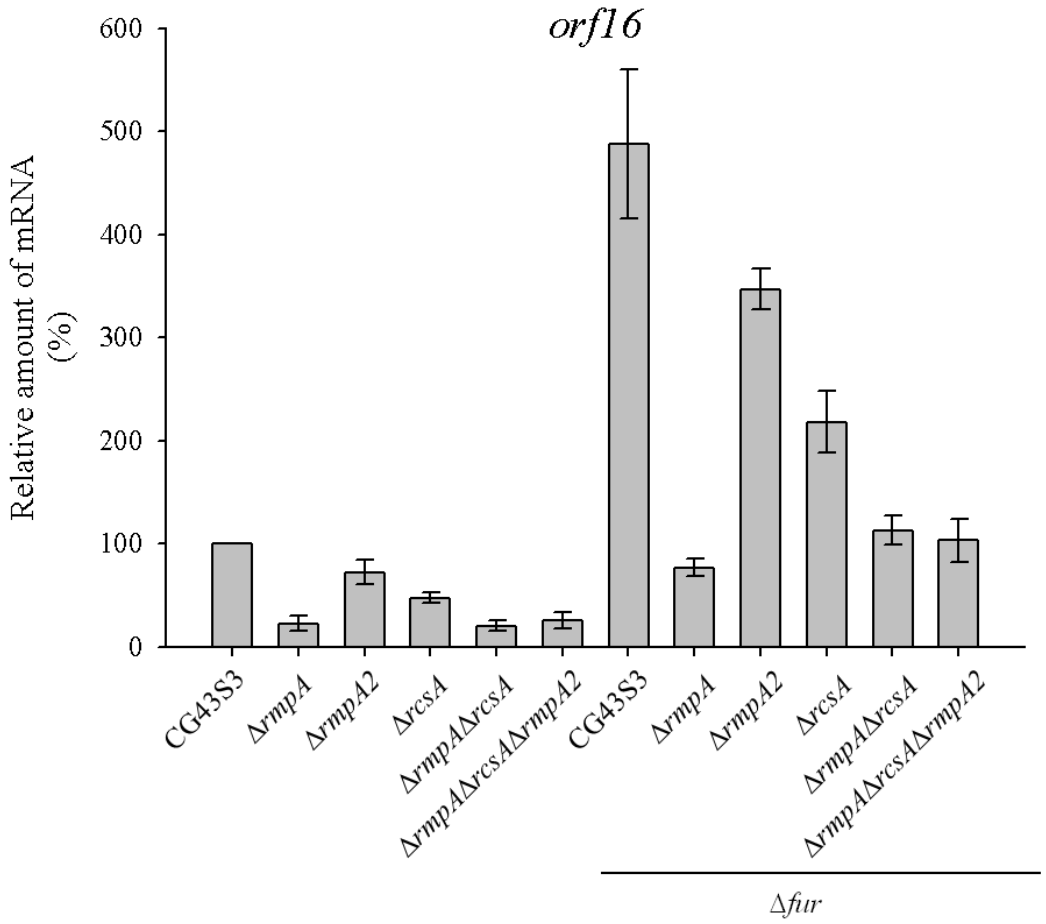


Figure 5. qPCR analyses of the expressions of the K2 *cps* genes. Bacteria strains, as indicated in the margin, were grown in LB medium at 37℃ with agitation and then subjected to qPCR analyses for the detection of *orf1* (A), *orf3* (B), and *orf16* (C) expression.

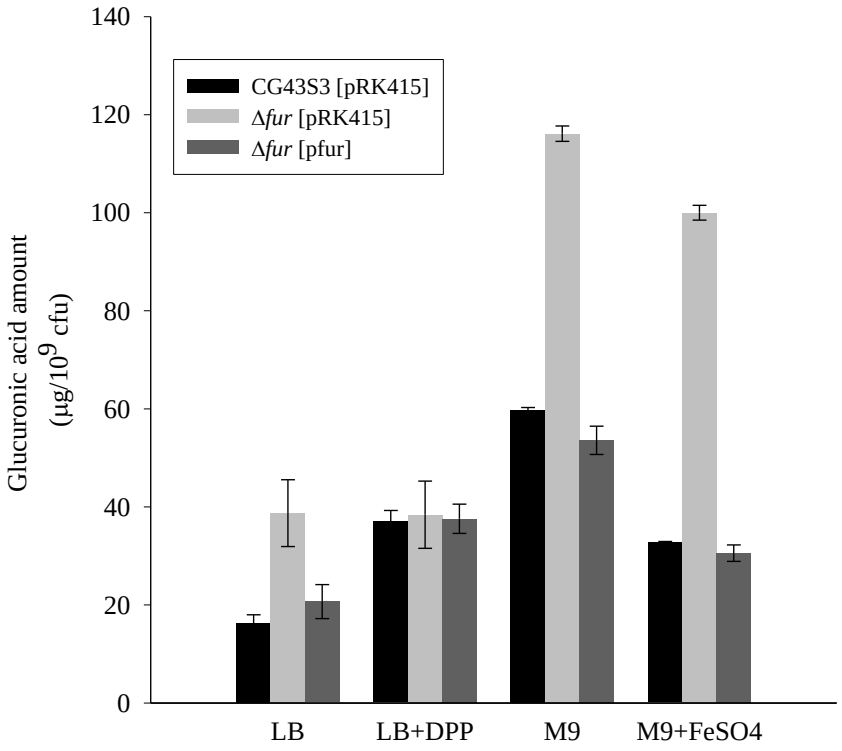
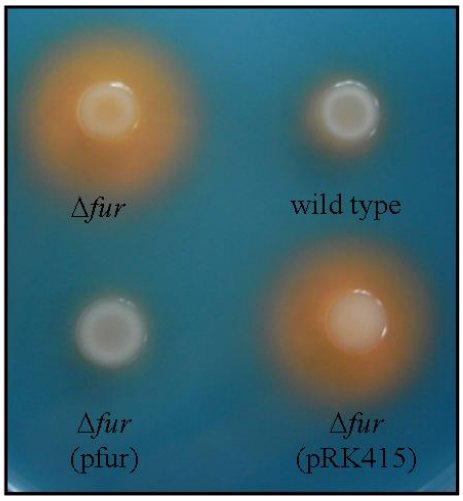


Figure 6. Fur affects the *K. pneumoniae* CPS biosynthesis in a Fe(II)-dependent manner. Bacteria were grown in media supplemented with or without 200 micromolar Dip or 60 micromolar $FeSO_4$ as indicated. After 16 hr growth, the bacterial glucuronic acid contents were determined. Values are mean ± standard error of three independent experiments.

(A)



(B)

		Typical Fur box		Consensus
		GATAAT:GATAAT:CATTATC		
P_{iroB}	-79	ACAAAT:GATAAT:CATTATC	-61	16/19
P_{entC}	-41	GATAAT:TATTCT:TAATATC	-23	15/19
P_{iucA}	-4	GATAAT:GGGAAT:CTTTATC	+15	16/19
P_{hmuR}	-124	GATAAT:ACCTAT:CATTACC	-106	15/19
P_{feoA}	-129	GATGAT:AAAAAC:CATTCTC	-111	15/19
P_{fecA}	-63	TGTAAT:GATAAC:CATTCTC	-45	15/19
P_{fhuA}	-198	CGTCAT:AATAAT:AATTCTC	-180	13/19
P_{sitA}	-99	GCAAAT:AAGAAT:TATTTTC	-81	13/19

(C)

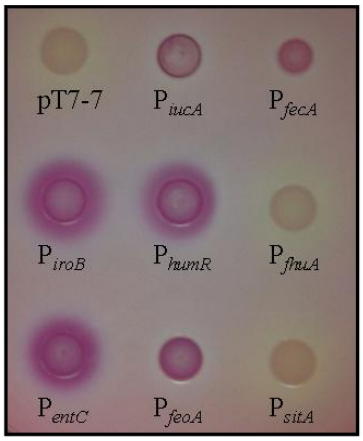


Figure 7. Fur regulation on the iron acquisition in *K. pneumoniae* CG43. (A) Deletion of *fur* increases the *K. pneumoniae* siderophore production assessed using CAS assay. Each bacterial strain assayed is indicated, and the orange halos formed around the colonies correspond to iron-chelating activity of siderophore in bacteria. (B) DNA sequence alignment between the *E. coli* typical Fur box and the putative Fur boxes in the upstream regions of the eight iron acquisition systems. Positions identical to the consensus sequences are underlined. (C) Assessment of the binding of Fur to the DNA sequences using FURTA. *E. coli* H1717 strains carrying the pT7-7 derivatives are indicated. Red colonies (Lac+) denoted FURTA-positive phenotypes. pT7-7, the FURTA-negative control.

計畫成果自評

本計畫在這一年八個月的執行過程中，順利的完成各項目標，我們利用 EMSA 和 qPCR 了解 Fur 會藉由直接抑制 RmpA 和 RcsA 的表現而影響莢膜多醣體的生合成，另外對於其他曾經被證實與莢膜多醣體生合成的相關轉錄蛋白質，包含 RcsB、KvgA、KvhR 和 Lon 皆不會受到 Fur 所調控，而我們也證實了外在鐵的存在可以幫助 Fur 對於下游基因的調控，因此在缺鐵的環境下，Fur 即缺少了對於莢膜多醣體的抑制能力，使得菌體會大量產生莢膜多醣體，這也令我們推測在克雷白氏費炎桿菌入侵到人體後，所面臨的是缺鐵環境的狀態，也因此莢膜多醣體可以大量合成，並可躲避掉吞噬細胞的攻擊。再者我們也藉由目前已解碼的克雷白氏肺炎桿菌 NTUH-K2044 (K1 serotype) 來進行分析在 CG43 (K2 serotype)中抓鐵系統的套數，其中總共有八套抓鐵系統的存在，進一步利用 Qpcr 以及跟德國的學者 Dr. Hantke 所拿到 H1717 來偵測大腸桿菌中 Fur 對於所構築出來的八套抓鐵系統的啟動子在序列的結合與調控能力，其中發現六套可以直接受到 Fur 所抑制，因此我們在這一年多以來的研究可以發現在克雷白氏肺炎桿菌的入侵過程中，Fur 扮演極為重要的角色，可以當作藥物開發的重要標的，因此未來我們希望能進一步探討環境因子對於 Fur 表現上的影響，並且找尋可以調控 Fur 表現的上游調控蛋白質，進一步可藉由促進 Fur 的表現來達到抑制菌體中毒性因子的表現。目前我們將這一年多以來的研究已經在 2009 年和 2010 年美國 Gordon research conference 所舉辦的會議發表過兩次的論文，也得到相當好的回應，而也將成果撰寫成文章投稿於 Microbiology 中，也得到了文章修正的機會，目前正在針對編輯者對實驗內容的建議進行修正，相信可以得到一個好的結果。