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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、FURTA

莢膜多醣體 (capsular polysaccharides, CPS) 和螯鐵系統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。在革蘭氏陰性菌中，螯鐵系統的基因表現會受到 Fur 所抑制。然而在克雷白氏肺炎桿菌中除了證實 Fur 可以自我調控外，對於其他的基因調控方面的研究非常的少，因此為了研究 Fur 在 Kp 上的功能，我們已經在具有高毒性 K2 血清型的 Kp CG43 中構築了 *fur* 的缺損株；其中發現 *fur* 的缺損會造成 CPS 的大量生成並提高 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。為了研究 Fur 在 *cps* 和螯鐵系統的基因調控上的角色，過去六個月我們已經完成下列的幾項實驗：

1. 我們已經構築好了 *fur* 的互補質體，並且以轉殖入 *fur* 的缺損株中觀察其顯型的變化，其中發現 *fur* 的缺損株會減緩 Kp 的沈澱速度並且減低生物膜的生成，然而 *fur* 的互補株則可恢復其顯型表現。
2. 我們構築了 Kp 中 K1 和 K2 的 *cps* 基因調控組的啟動子在 pT7-7 中，並且利用 FURTA 來偵測大腸桿菌的 Fur 對於 K1 和 K2 的 *cps* 基因調控組的啟動子之結合能力。初步的結果發現這些啟動子在 FURTA 的實驗中皆呈現陰性反應，所以大腸桿菌的 Fur 不會直接結合到這些啟動子上進行調控反應。
3. 在 *cps* 的調控迴路中，我們也構築 *rcsA* 和 *rcsB* 的啟動子在 pT7-7 中，並且利用 FURTA 來偵測大腸桿菌中 Fur 對於這兩各啟動子的結合能力，其結果也是呈現陰性反應，因此我們將進一步構築並偵測在 *cps* 的調控迴路中其他調控子的啟動子對於大腸桿菌 Fur 的結合親和力。
4. 利用生物資訊所分析出來在螯鐵系統中含有 Fur 結合區域的啟動子，我們進一步構築並利用 FURTA 來偵測 Fur 對於這些預測有 Fur 結合區域的啟動子的結合能力，其中發現只有 *iroN*、*iroB*、*entC*、*ybtP*、*fecB* 和 *kfuA* 顯示為典型的 FURTA 陽性反應，其他基因雖然有 Fur 的結合區域但是卻呈現陰性反應。

在本年度，我們已經藉由完成 *fur* 的互補質體的構築完成來確認 Fur 對於 Kp 中沈澱速率及生物膜生合成的專一性影響，並且發現大腸桿菌的 Fur 並不會直接結合至 K1 或 K2 的 *cps* 基因啟動子區域進行調控動作，而在 *cps* 的調控迴路中，*rcsAB* 的啟動子也不會受到 Fur 所結合調控。而在螯鐵系統中，我們發現並非所有帶有 Fur 結合區域的啟動子都能順利的受到 Fur 所調控，因此之後我們將利用生物資訊方式比較 Fur 固定結合區域的序列並且利用 FURTA 和電泳交遲滯實驗證實 Fur 的結合能力。下年度，我們會進一步利用二維電泳膠和即時聚合酶連鎖反應來找尋 Fur 在莢膜多醣體生合成中所調控的下游基因，綜合以上幾點，我們希望能夠瞭解 Fur 在克雷白氏肺炎桿菌的致病過程中所扮演的角色。

Abstract:

Key words: Iron-acquisition system, capsule polysaccharides, *Klebsiella pneumoniae*, Fur, FURTA

Iron-acquisition system and capsule polysaccharides (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). Due to the regulatory roles of Fur in Kp remains large unknown. We have demonstrated that the *fur* deletion mutant rendered the bacteria more mucoid phenotype and slight reduced the LD₅₀ using mouse lethality assay suggesting an involvement of Fur in virulence regulation. To study the functional role of Fur in mediating *cps* and iron-acquisition system gene clusters, we had been finish the experiments in the past six month are:

1. The construction of the *fur* complement strain was performed. The *fur* deletion mutant slowed the Kp precipitation rate and reduced biofilm formation, while the *fur* complement stain could restore the phenotype.
2. The putative promoter regions of K1 and K2 *cps* gene cluster were constructed and detected the *E. coli* Fur binding affinity by Fur titration assay (FURTA). The preliminary results showed that all the cloned promoter of either K1 or K2 *cps* gene cluster showed FURTA-negative phenotype.
3. The promoter regions of *rcaA* and *rcaB* in *cps* regulon were constructed and detected by FURTA, the results exhibited FURTA-negative phenotype implying RcsAB is not involved in Fur regulating.
4. The iron-acquisition systems containing the putative Fur boxes were constructed and detected by FURTA. The cloned promoters containing *iroN*, *iroB*, *entC*, *ybtP*, *fecB*, and *kfuA* showed typical FURTA-positive phenotypes. However, the clones containing the promoter *fepA*, *fepB*, *fyuA*, and *fhuA* exhibited negative phenotypes.

In next year, we will employ the 2D and real-time PCR to demonstrate the regulatory roles of Fur in CPS biosynthesis. However, the typical Fur box in Kp will be further analyzed and demonstrated by bioinformatics, FURTA, and EMSA. Take together, we hope to understand the functional role of Fur in Kp pathogenesis.

Introduction:

Klebsiella pneumoniae (Kp) is a common nosocomial pathogen. As an opportunistic pathogen, it often causes pneumonia, septicemia, bacteriemia, suppurative lesion, wound infection, burn infection, and urinary or respiratory tract infections in immunocompromised patients [1-4]. However, the wide spread of extended spectrum β -lactamase producing *K. pneumoniae* (ESBLKp) has prompted the search for new drugs to intervene the bacterial infections [5, 6].

Klebsiella usually have well developed polysaccharide capsules, which make their colonies characteristic mucoid. CPS is an important virulence factor in Kp infection to protect the bacteria from phagocytosis and prevent from killing by serum factors [7, 8]. Apart from their antiphagocytic function, *Klebsiella* CPS have been reported to inhibit differentiation and functional capacity of macrophage *in vitro* and mediate the resistance to antimicrobial peptides and proteins by limiting the interaction of the agents with membrane targets [9, 10]. The capsular serotypes of Kp have been classified as more than 77 recognized capsular antigens [11, 12]. In Taiwan, a high prevalence of K1 and K2 serotypes of Kp was documented in liver abscess of diabetes mellitus patients [13].

Many bacteria have evolved iron-acquisition systems for sequestering ferric iron from the environment. The iron/heme acquisition systems in bacteria can be separated into two general mechanisms. One is a direct contact between the bacteria and the exogenous iron/heme sources. Another one is compounds (siderophores and hemophores) synthesized and released by bacteria into extracellular medium to scavenge iron or heme from various sources [14]. The transport of these compounds across the outer membrane involves outer membrane receptors. The energy for siderophore transport is delivered from the inner membrane by a TonB-ExbB-ExbD complex [15]. Analysis of the genomic sequence of Kp NTUH-K2044 have found ten putative iron-acquisition systems, seven of ten putative iron-acquisition systems contain putative TonB-dependent domains [16].

To avoid iron overloading that would lead to the formation of hydroxyl radicals, microorganisms have tight regulation of iron uptake. In Gram-negative bacteria, iron-acquisition genes are negatively regulated by ferric uptake regulator (Fur) [17-19]. Under iron-replete conditions, the Fur protein functions as a dimmer with Fe^{2+} as cofactor to bind to a 19-bp consensus sequence, called the Fur box (GATAATGATwATCATTATC; w=A or T), in the promoters of genes requires for iron uptake, thereby preventing their transcription [20]. In Kp, the functions of Fur on mediating the gene expression remains large unknown. However, we further have constructed the fur deletion mutant in Kp CG43S3 to observe Fur function in Kp. Interestingly, the deletion rendered the bacteria more mucoid phenotype and reduced the LD₅₀ implying Fur has diverse regulatory functions on mediating CPS and iron-acquisition system.

In order to demonstrate the regulatory functions of Fur, the experiments we have finished in the past six month are as following:

1. Investigation of regulatory roles of Fur on mediating the *cps* expression in Kp;

- (1.)The *fur* complement in pRK415 was constructed, the comparison of wild type, fur

deletion mutant and the complement strain in the viscous phenotype, biofilm formation, and CPS content were performed.

- (2.) We have constructed the *cps* promoter region containing K1 and K2 serotype of Kp in pT7-7 vector respectively, and transferred into H1717 (*fhuF::lacZ*) to detect the direct binding affinity of Fur by Fur titration assay (FURTA).
 - (3.) The promoter region of RcsA and RcsB were also performed the construction in pT7-7 vector and transferred into H1717 to detect the direct binding affinity of Fur by FURTA.
2. Investigation of regulatory roles of Fur on mediating the gene expressions of iron-acquisition systems in Kp;
- (1.) The promoter regions of five putative iron-acquisition systems containing the putative Fur boxes in Kp NTUH-K2044 by bio-informatics analysis were constructed in pT7-7 vector and transferred into H1717. FURTA was also performed to analysis the Fur direct binding affinity.

Results and discussions:

The phenotype of fur deletion mutant in Kp CG43. In this study, the *fur* complement strain was performed the construction and compared with wild type (CG43S3), *fur* deletion mutant (F0401), and *fur* complement strain (the entire *fur* gene in pRK415, named pCY011) 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 strain supplied with the plasmid pCY011 respectively, revealed that faster precipitation via low-speed centrifugation (Fig. 1). Subsequently, the CPS production in CG43S3, F0401, and F0401 containing pCY011 will be 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 *Kp*, the interaction of CPS and fimbriae was able to modulate the bacteria biofilm formation [21]. Therefore, the biofilm formations in CG43S3, F0401, and F0401 (pCY011) 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 F0401 than in wild type, and supplied with the plasmid pCY011 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 *Kp* CG43.

The regulatory role of Fur in mediating cps gene expression in FURTA. To investigate the regulatory functions of Fur on mediating *cps* and iron-acquisition system gene expression, FURTA is a good reporter assay for examine the *in vivo* Fur binding function in the promoter regions of target genes. We asked for FURTA indicator strain, *Escherichia coli* H1717, from Dr Hantke to perform this study. H1717 contains a chromosome *fhuF::lacZ* fusion, and the low affinity Fur box in *fhuF* has demonstrated. The introduction of high-copy-number plasmids that contain Fur-binding sequences in the multiple cloning site causes removal of Fur from the *fhuF* Fur box [22]. The resultant expression of LacZ is visualized as lactose-positive colonies on MacConkey agar plates. In this study, the promoter regions of *Kp* K1 and K2 *cps* gene cluster were constructed in pT7-7 vector respectively and transferred into H1717 to assess the interaction of *cps* promoter regions with Fur. As a result, all the cloned promoter of either K1 or K2 *cps* gene cluster showed FURTA-negative phenotype, which suggests that non-direct binding of Fur on mediating the *cps* gene expression (Table 1). Furthermore, the clones carrying the promoters of *rcaA* and *rcaB* also exhibited FURTA-negative phenotype implying RcsAB is not involved in Fur regulating (Table 1). The concentration of ferric ammonium sulfate and the incubation time are critical for Fur regulating the target genes. However, the different concentration of ferric ammonium sulfate and different incubation time will be detected in this assay. The β -galactosidase activity will be detected to quantify the binding activity.

The regulatory role of Fur in mediating iron-acquisition system gene expression in FURTA. To investigate the regulatory functions of Fur on mediating iron-acquisition system gene expression, five of ten putative iron-acquisition systems containing the putative Fur boxes were first detected the direct binding affinity of Fur by FURTA. As showed in Table 2, the cloned promoters

containing *iroN*, *iroB*, *entC*, *ybtP*, *fecB*, and *kfuA* showed typical FURTA-positive phenotypes. However, the clones containing the promoter *fepA*, *fepB*, *fyuA*, and *fhuA* exhibited negative phenotypes. The results implied that Fur regulate the target gene is not dependent on the Fur box. We will attempt to align the promoter regions of *iroN*, *iroB*, *entC*, *ybtP*, *fecB*, and *kfuA* to find the conserve domain or residue for affecting the regulation of Fur. The β -galactosidase activity will be detected to quantify the Fur binding activity.

Fig. 1 Comparison of precipitation speed of Kp wild type (CG43S3), fur mutant (F0401), and the strains carried the complement plasmid (pCY011).

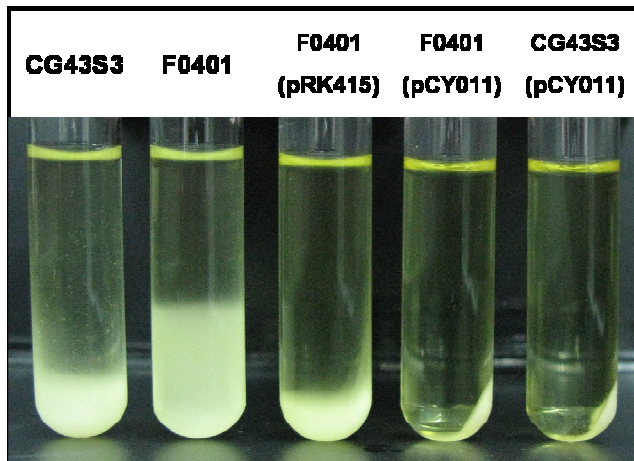


Fig. 2 Biofilm formation of Kp wild type (CG43S3), fur mutant (F0401), and the strains carried the complement plasmid (pCY011). CG43S3-pRK415, CG43-pFur, Δfur -pRK415, and Δfur -pFur (*fur* complement) strains were grown in LB broth at 37 °C for 48 h, and then measured OD550 with crystal violet stained.

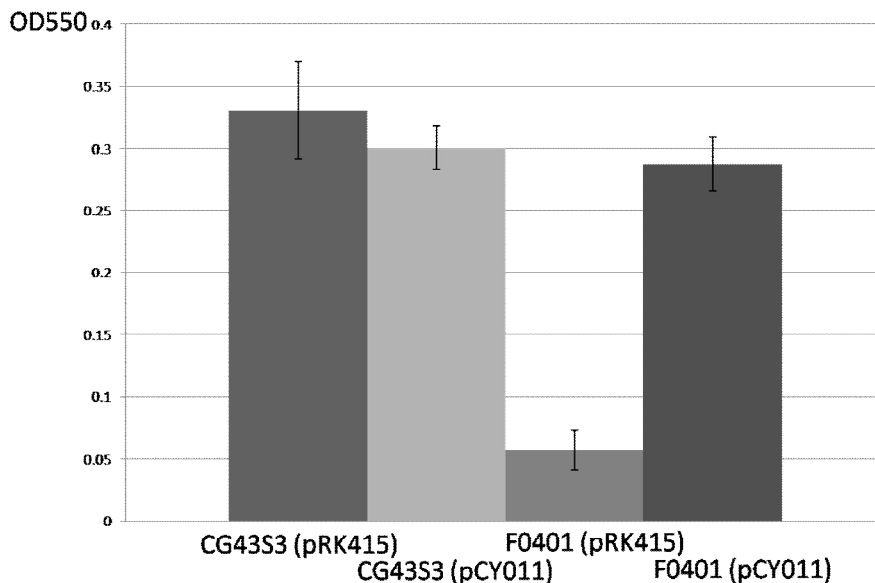


Table 1 The binding ability of Fur on the promoter regions of K1 and K2 *cps* gene cluster and *rscAB* in FURTA. The promoters were constructed to pT7-7 in *E. coli* H1717, and 5 μ l of overnight grown bacteria were spotted on MacConkey agar plate containing 30 μ M [Fe⁺³] for 16 h. + means positive result. – means negative results, and ND means not detectible.




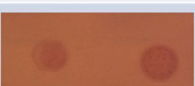
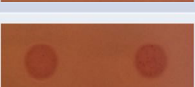
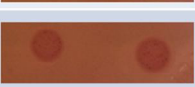


Serotype	Strain	Plasmid	Promoter region	FURTA		
					pT7-7	plasmid
K1	CG43	pYS003	ORF 1-2	-		
		pYS004	ORF 3-15	-		
		pYS005	ORF 16-17	-		
K2	NTUH-K2044	pYS006	CPS-1	-		
		pYS007	CPS-3	-		
		pYS008	CPS-5	-		
		pYS001	RcsA	-		
		pYS002	RcsB	-		

Table 2 The classification of siderophores in *K. pneumoniae* and the Fur binding ability via FURTA. The promoters were constructed to pT7-7 in *E. coli* H1717, and 5 µl of overnight grown bacteria were spotted on MacConkey agar plate containing 30 µM [Fe⁺³] for 16 h. + means positive result. – means negative results, and ND means not detectible.

System	Gene cluster	Promoter	Functions	Localization	Putative Fur box	FURTA		
							pT7-7	plasmid
Fe ³⁺								
Salmochelin	<i>iroN</i>	<i>P_{iroN}</i>	Outer membrane transporter	Plasmid	+	+		
	<i>iroBCD</i>	<i>P_{iroB}</i>	Biosynthesis	Plasmid	+	+		
Aerobactin	<i>iutA</i>	<i>P_{iutA}</i>	Outer membrane transporter	Plasmid	-	ND		
	<i>iucABCD</i>	<i>P_{iucA}</i>	Biosynthesis	Plasmid	-	ND		
Enterobactin	<i>fepABCDG</i>	<i>P_{fepA}</i>	Outer membrane transporter	Chromosome	+	-		
		<i>P_{fepB}</i>	Biosynthesis	Chromosome	+	-		
	<i>entABCDEF</i>	<i>P_{entC}</i>	Biosynthesis	Chromosome	+	+		
Yersiniabactin	<i>fyuA</i>	<i>P_{fyuA}</i>	Outer membrane transporter	chromosome	+	-		
	Yersinia HPI	<i>P_{ybtP}</i>	Biosynthesis	Chromosome	+	+		

System	Gene cluster	Promoter	Functions	Localization	Putative Fur box	FURTA		
							pT7-7	plasmid
Fe ³⁺								
Ferric citrate	<i>fecABCDE</i>	<i>P_{fecA}</i>	Outer membrane transporter	Plasmid	-	ND		
		<i>P_{fecB}</i>	Inner membrane transporter	Chromosome	+	-		
Ferrichrome	<i>fhuABCD</i>	<i>P_{fhuA}</i>	Outer and inner membrane transporter	Chromosome	-	-		
Ferric iron	<i>kfuABC</i>	<i>P_{kfuA}</i>	Ferric iron uptake	Chromosome	+	+		
Fe ²⁺								
Ferrous iron	<i>feoAB</i>	<i>P_{feoA}</i>	Ferrous iron uptake	Chromosome	-	ND		
Ferrous iron	<i>sitABCD</i>	<i>P_{sitA}</i>	Ferrous iron uptake	Chromosome	-	ND		

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計畫成果自評:

本計畫在六各月的執行過程中，順利的完成各項的基因構築的工作，其中 *fur* 的互補株的構築完成也能進一步確認 *fur* 突變後對於 Kp 顯型方面的專一性影響，而在探討 Fur 對於 *cps* 基因啟動子調控上的影響，我們也順利跟 Dr. Hantke 拿到 H1717 來偵測大腸桿菌中 Fur 對於所構築出來的啟動子序列的結合能力，因此我們分別將 K1 和 K2 的莢膜多醣體基因組的啟動子和 *rcsAB* 的啟動子進行 FURTA 的分析，雖然目前發現皆為陰性反應，即沒有受到 Fur 直接結合所調控，但是我們會進一步針對不同鐵離子濃度以及構築其他與 *cps* 調控有關的轉錄調控子的啟動子序列來進行更深入的探討。而對於螯鐵系統中，我們也發現在我們先前分析十個具有 Fur 結合區域的啟動子中，只有六個在 FURTA 的實驗中呈現陽性反應，因此我們將會進一步比較這六個陽性反應和其他四個陰性反應的啟動子在 Fur 結合區域的相異性，整體而言，本計畫的預期目標我們也已經積極的完成，因此我們在未來的一年藉由蛋白質體學和生物資訊能夠完成所規劃之目標，瞭解 Fur 在克雷白氏肺炎桿菌中所扮演的調控角色。

而在本人也將參與於今年七月二十六到三十一日於美國 Gordon research conference 所舉辦的會議 "microbial adhesion & signal transduction"。將目前所得到的實驗結果與之分享與討論，期許能更深入的加以探討並且由各學者的經驗分享能夠得到更多的經驗和學習機會。