National Chiao Tung University Thesis for the Degree of Master

國立交通大學碩士學位論文

Roles of the Two-component System Response Regulator RcsB in *Klebsiella pneumoniae* CG43

雙分子系統反應調節因子 RcsB 在克雷白氏肺炎桿菌 CG43 中所扮演的角色

分子醫學與生物工程研究所

碩士班

Student: Yenxi Tay (0157103)

學生：鄭燕曦

Advisor: Hwei-Ling Peng, PhD.

指導教授：彭慧玲博士

June, 2013

中華民國一百零二年六月

Acknowledgements（謝誌）

感謝我的指導教授彭慧玲老師，每週老師都會安排每個成員單獨跟老師討論，這個部分讓我收穫許多，除了可以練習自己去推導一些分子調控途徑之外，還練習怎麼設計實驗來解決我們想要探討的問題。我覺得這樣的練習讓我漸漸的被訓練成一個有能力在研究一個課題的過程中，找出需要改進的地方，並且在遇到問題或是瓶頸的時候，我也會以積極的態度去找出問題，進而有邏輯的去思考再去解決問題。彭老師除了在我們做研究的當兒，是我們的老師，教導我們、幫助我們在學習的路上讓我們在生科這個領域有所成長之外，很多時候，她更是扮演家長的角色，老師時常就像我們在學校的母親一樣，時時刻刻關心著我們的近況，希望我們在做研究的路上也要為自己的未來好好做規劃，並且從學校畢業之後，也會是個有能力照顧好自己、成熟的年輕人。能夠遇上這樣一位不可多得的好老師，心中充滿了無限感激。

感謝清華大學張晃猷老師以及張老師實驗室的夥伴，在實驗上給予諸多的建議及幫助。

更加感謝張老師和交通大學梁美智老師，在百忙之中撥冗擔任我的口試委員，用心的修正我論文中的錯誤及指點我實驗中未曾想過的盲點。

感謝實驗室的大家，因為有你們，這本論文才可以順利完成，也因為你們，這兩年的生活才會如此多彩多姿，在每一個實驗卡關的

時候，我才可以在你們的鼓勵之下順利突破。感謝溫柔細心的靜柔學姊，總是像我的姐姐一樣，不管在實驗上或是生活上一直給我鼓勵和支持，更是教會我很多事情；感謝哲充學長，讓我們學會很多待人處事的技巧；感謝我的好拍檔冠男，沒有我們如此精準的默契，我想我們鐵定不能這麼順利的畢業且讓人稱羨；感謝亦師亦友的子祥，我們倆總是會有不一樣的話題，我想我們三個臭皮匠的緣份一言難盡，短短的幾天就讓我們造就了今天的生死之交；感謝細心又總是考慮周全的偉豐，雖然你總是表現得默默的，但是你總是為大家付出很多，希望以後可以看到你的植物王國；感謝活潑可愛的珍儀，雖然你的課很多，我們碰面的時間不多，但是總覺得我們有時候會有莫名的默契，很懂對方是怎麼樣的人，真的很開心很幸運可以認識你這個活潑大方又很有愛心的好女生；感謝單純可愛的蕙瑜，雖然我時常以虧你為和你溝通的方式，但我真的覺得你是個很有想法、很有原則的一個人，要繼續保持這樣單純又善良的心，畢業後我們要繼續連絡喔，朋友；感謝嬌小又能幹的俐君，每次都讓我覺得你又知性又很有智慧，緣份讓我們在台灣相遇，只可惜我們相處的時間總是嫌少，我們一拍即合，一直也覺得很幸運可以跟你成為朋友！keep in touch! 感謝文靜又聰明的豐碩，雖然不知道你會不會看到我的感謝，但是真的很開心時常有你這個學弟和我們一起討論實驗、互相切磋，一起出遊真的很開心；感謝搞笑卻認真的家睿，雖然每次喜歡虧你在狀況外，但卻被你對於實驗的認真心態很是感動，博班生涯也許時常會遇到瓶頸，但是一定要勇敢的把它完成，加油！

還有感謝吳東昆老師、廖光文老師、楊昀良老師、曾慶平老師、黃兆祺老師以及各位老師實驗室的成員，無私的提供我支援，給予我在碩士生涯的指點與實驗上的幫助。

最重要的是，要感謝我親愛的家人，因為你們的支持與鼓勵，我才能有今天這個碩士學位。感謝媽媽在我成長的道路上，讓我可以不斷的往自己的嚮往的方向，不管在甚麼領域都可以自由發揮。感謝弟弟，在我隻身在外求學的時候，擔起照顧媽媽的責任，讓我這 5 年來可以專注於課業。特別感謝鄭嵩一家人，在論文定稿這段期間的照顧，如果沒有你們就不會有這本我人生重要的著作。還要感謝鄭嵩，一直在身邊支持和陪伴我，讓我疲憊、無助的時候，仍然可以堅持著，並全力衝刺論文和碩士學位的最後關鍵，直到終點。

最後，我也要感謝我自己，成功並且順利的以一年的時間完成碩士學位，並且在過程中不斷的堅持、永不放棄，最後才有今天的自己。畢業後的日子，一定還有更多的挑戰和值得期待的未來等著我去面對、等著我去創造，我會繼續努力、一直提醒自己莫忘初中，直到夢想實現！

燕曦

論文摘要

當細菌感受到外界環境壓力後，雙分子調控系統（Two-component system）的偵測激酶 RcsC 和 RcsD 將訊號傳遞給下游之反應調控蛋白 RcsB，磷酸化該蛋白的第 56 個氨基酸天冬氨酸鹽（Aspartate）並使 RcsB 活化。 過去研究顯示，RcsB 與 RcsA 形成雙合體來調控細菌莢膜多醣體的基因表現。在肝膿瘍克雷白氏肺炎桿菌分離株 CG43 中，*rcsB* 基因的缺失可導致莢膜多醣體的生合成、第三型線毛的表現和抗酸能力明顯下降。本論文中，我們擬探討 RcsB 的磷酸化是否會影響莢膜多醣體、第三型線毛和抗酸能力相關基因的表現。本研究首先構築了兩個定點突變的 RcsB，分別為 RcsB-D56A 和 RcsB-D56E。RcsB-D56A 模擬無法接受磷酸根的 RcsB；而 RcsB-D56E 則是模擬持續磷酸化狀態的 RcsB。CG43S3Δ*rcsB* 回補 pRK415-RcsB-D56A 和 pRK415-RcsB 結果相近，均提高莢膜多醣體的生合成和細菌的抗酸能力。相對地，回補 pRK415-RcsB-D56E 則恢復了第三型線毛的主要單元蛋白 MrkA 的表現量。此結果暗示著 RcsB 的磷酸化影響此蛋白和 DNA 或是其他蛋白質的結合，進而決定它的調控目標。我們以西方點墨分析發現 RcsB-D56A 正向調控酸逆境伴隨蛋白，而啟動子活性分析顯示其對抗酸相關的蛋白 YfdX 的為轉錄層面的影響。同時，我們藉大腸桿菌來大量表現 RcsB 的 N 端並純化之後免疫兔子後取得多株抗體，有趣的是，我們發現此多株抗體只能偵測 RcsB 和 RcsB-D56A 卻無法辨識 RcsB-D56E，此暗示 RcsB-D56E 殘基改變可能影響其結構而致失去抗體辨識的抗原決定部位 (epitope)。

不過，西方墨點法顯示 RcsB 可受酸誘導表現，而此抗體將可以用於免疫共沉澱找出 RcsB 協同作用的蛋白質。這些結果暗示著 RcsB 的磷酸化狀態對於克雷白氏肺炎桿菌 CG43 的各種致病方式的調控扮演著非常重要的角色，並且可能會是其致病的關鍵點，往後 RcsB 的協同作用蛋白的發現將可以幫助我們進一步去更瞭解 RcsB 的調控路徑。

Abstract

The two-component system (TCS) response regulator RcsB is activated by phosphorylation at Asp56 upon receiving stress signals transferred from the sensor kinase RcsC and RcsD. RcsB has been demonstrated to be required for CPS (capsular polysaccharide) gene expression through heterodimer forming with RcsA. In *Klebsiella pneumoniae* CG43, a liver abscess isolate, deletion of *rcsB* reduced the CPS expression, type 3 fimbriae pilin MrkA production, and acid stress response. This study, investigates if RcsB phosphorylation plays a role in regulating CPS production, MrkA expression, and acid stress response. Two site-directed mutants RcsB-D56A which is unable to accept the signal transferred, and RcsB-D56E, a phosphorylation mimetic form of RcsB, have been generated. Comparing with CG43S3Δ*rcsB*[pRK415], CG43S3Δ*rcsB*[pRK415-RcsB-D56A] and CG43S3Δ*rcsB*[pRK415-RcsB] exhibit an increased the CPS production and acid survival rate. By contrast, CG43S3Δ*rcsB*[pRK415-RcsB-D56E] restored the MrkA production. These findings suggest that the phosphorylation status of RcsB affects its DNA binding or protein binding activity thereafter determines its regulation targets. Western blot analysis showed that RcsB-D56A positively regulates

the acid stress chaperone YfdX expression and promoter activity assay suggested that the RcsB-mediated regulation is at the transcriptional level. In addition, the N-terminal region of RcsB was overexpressed in *E. coli* and the protein was purified to immunize rabbit for polyclonal antibody generation. The raised antibody could recognize RcsB and RcsB-D56A but not RcsB-D56E. This result implies that the amino acid D56E substitution of RcsB alters the protein conformation, and hence the loss of the specific epitope for the antibody. Western blot analysis also revealed that RcsB was acid inducible and co-immunoprecipitation analysis would be feasible for the identification of the RcsB interacting proteins. The findings indicated that the phosphorylation status of RcsB might be the key of the regulation of different virulence factors in *K. pneumoniae* CG43, and further identification of the RcsB-interacting protein will help toward understanding the regulatory pathway of RcsB.

Table of Contents

List of Tables

List of Figures

Abbreviations

Asp	Aspartate
bp(s)	base pair(s)
CPS	capsular polysaccharide
CFU	colony forming unit
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
ESBL	extended-spectrum β-lactamase
ETC	electron transport chain
HK	histidine kinase
HTH	helix-turn-helix
HV	hypermucoviscosity
IP	immunoprecipitation
IPTG	isopropyl 1-thio-β-$_D$-galactopyranoside
kb	kilobase(s)
kDa	kilodalton(s)
LB	Luria-Bertani
LPS	lipopolysaccharide

μg	microgram
μL	microliter
mL	milliliter
mM	millimolar
μM	micromolar
NDM-1	New Delhi metallo-β-lactamase 1
ng	nanogram
OD	optical density
ONPG	o-nitrophenyl-β-$_D$-galactopynoside
PAGE	polyacrylamide gel electrophoresis
PAI	pathogenecity island
PCR	polymerase chain reaction
PLA	pyogenic liver abscess
Rcs	regulator of capsule synthesis
RR	response regulator
rpm	revolutions per minutes
SDS	sodium dodecyl sulfate
TCS	two-component system
X-gal	5-bromo-4-chloro-3-inodolyl-β-$_D$-galactopyranoside

1. Introduction

1.1 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative bacterium. It is facultative anaerobic, non-motile, rod-shaped, heavily encapsulated, which belongs to the *Enterobacteriaceae* that is under the gamma subdivision of the phylum of *Proteobacteria* which include genus such as *Escherichia*, *Salmonella*, *Shigella* and *Yersinia* [1]. The *Klebsiella* spp. are ubiquitous in nature, with two plausible habitats: the surface water, soil, sewage, plants [2-6] and the mucosal surfaces of the animal host, such as human respiratory tract and intestinal tract [7].

1.2 Infections of *K. pneumoniae*

K. pneumoniae is an opportunistic pathogen, which usually caused infections found in immuno-compromised individuals who are hospitalized and suffered from severe underlying diseases, such as diabetes mellitus or chronic pulmonary obstruction. In respect of bacteremia caused by

nosocomial Gram-negative pathogens, *Klebsiella* is second only to *Escherichia coli* [8]. *K. pneumoniae* usually causes variety of diseases including suppurative lesions, bacteriemia, urinary tract infections, pneumonia, and sometimes life-threatening septic shock [7, 9-12]. Reported carrier rates in hospitalized patients are 77% in the stool, 19% in the pharynx and 42% on the hands of patients [13, 14]. However, such high rates of nosocomial colonization were likely due to the use of antibiotics rather than the factors with delivery of care in the hospital [15, 16], especially the extended-spectrum β-lactamase (ESBL)-producing strains [17-21]. Carbapenems are considered to be the preferred agents for treatment of serious infections caused by ESBL-producing *K. pneumoniae* because of their high stability to β-lactamase hydrolysis and observed retained susceptibility of ESBL producers [22], however, carbapenems-resistant *K. pneumoniae* have been reported worldwide since 2000s [23-26]. In 2008, an emergence of multidrug resistant superbug, so-called NDM-1 was first detected in *K. pneumoniae* isolate from a Swedish patient of Indian origin traveled to New Delhi. A carbapenem-resistant *K. pneumoniae* strain bearing the novel gene was identified from the Sweden patient [24]. All of the NDM-1 isolates were resistant to multiple different

classes of antibiotics, including β-lactams, fluoroquinolones, and aminoglycosides, but most were still susceptible to colistin [27].

During the last decade, *K. pneumoniae* infections causing community-acquired primary pyogenic liver abscess (PLA) have also become an emerging disease receiving increasing attention. Distinct from the classical PLA, which is a complication of intra-abdominal or biliary tract infections resulting from multiple aerobic and aerobic bacterial strains [28], PLA caused by primary infection of *K. pneumoniae* as a single pathogen is often cryptogenic and complicated with metastatic lesions [29-31]. Most cases have been reported from Taiwan [32], which were associated with a distinct invasive syndrome in liver abscess, meningitis and endophthalmitis. Attempts have been made to identify the risk factors for *K. pneumoniae* infections associated with abscess-formation, especially diabetes mellitus is the most tightly associated with *K. pneumoniae* PLA among the host factors.

1.3 Virulence Factors of *Klebsiella pneumoniae*

The factors that contributing to *K. pneumoniae* pathogenicity that had been identified, includes the capsular polysaccharide (CPS), lipopolysaccharide (LPS), adhesion factors, iron acquisition systems and some other stress responses.

1.3.1 Capsular Polysaccharide (CPS) and lipopolysaccharide (LPS)

Clinically isolated *K. pneumoniae* usually produces large amounts of CPS and therefore forms large glistering colonies with viscid consistency, e.g. the isolation that used in study *K. pneumoniae* CG43, an isolation from Chang Gung Memorial Hospital, which causes liver abscess in diabetes mellitus patients. As a major virulence factor, CPS acts to protect the bacteria from phagocytosis, killing by polymorphonuclear granulocytes and bactericidal serum factors [33-35]. Besides physical hindrance to fimbrial binding, the role of *Klebsiella* CPS in mediating the bacterial resistance to antimicrobial peptides has also been reported [36, 37]. There are 77 serotypes of CPS had been identified, reported that K1 and K2 CPS are the most virulent to mice [38]. Moreover, the hypermucoviscosity (HV)

phenotype of *K. pneumoniae* isolates resulting from a profound expression of CPS has also been correlated with the development of invasive syndrome [39]. The LPS O-antigen and the lipid-A are major component of Gram-negative bacterial cell walls and establishment of septic shock [33, 40]. The O-antigen is thought to play a role in resistance to complement killing [40] and to contribute to bacteremia as well as lethality during murine pneumonia infections [41]. The lipid-A (endotoxin) region is reported as the primary inflammatory component of LPS because of specific and sensitive recognition by the innate immune system [42].

1.3.2 Adhesion Factors

A number of adhesins have been suggested as potential virulence factors, including type 1 [43-45] and type 3 fimbriae [46-48], non-fimbrial adhesion CF29K [49] and KPF28 [50, 51], were associated with the initial attachment and subsequent colonization of *K. pneumoniae* in the respiratory and urinary tract [52]. Type 1 fimbriae have been most extensively studied in *E. coli*, and the corresponding structures of *K. pneumoniae* are highly similar with regard to genetic composition and

regulation [44, 53-55]. The fimbrial rod consists of the major subunits FimA and the minor subunits FimI, FimF and FimG. However, several reports indicated that *K. pneumoniae* poorly expresses type 1 fimbriae in vitro [44, 55, 56], the thick capsule of *K. pneumoniae* has been shown to impede the activity of type 1 fimbriae and also to retard the assembly of type 1 fimbrial subunits from periplasm to cell surface [55, 57, 58], suggesting a cross-regulation of the expression of fimbriae and capsule for an efficient infection.

Several studies have also demonstrated an important role for type 3 fimbriae in biofilm formation on biotic and abiotic surfaces [45, 47, 59-64]. Biofilm are recognized as surface-attached bacteria embedded in a self-produced matrix, composed mainly of polysaccharide, but also containing proteins and nucleic acids [65]. Biofilm formation promotes encrustation and protects the bacteria form hydrodynamic forces of urine flow, host defenses and antibiotics [66]. The ability of bacteria to form biofilm on medical devices is believed to play major role in development of nosocomial infections, including the catheter-associated urinary tract infections, which is frequently caused by *K. pneumoniae* [7, 66-68]. In addition, type 3 fimbriae mediate adhesion to epithelial cells, from the

respiratory and urinary tracts and extracellular matrix proteins, such as collagen V, in vitro [62, 69-73]. Type 3 fimbriae are encoded by *mrkABCDF* operon [46, 74, 75]. MrkA and MrkF are the major and minor subunits, respectively, which constitute the fimbrial rod and facilitate biofilm formation [46, 47].

1.3.3 Stress Responses

Besides those bacteria structural components that suggested being pathogenic, there are some mechanisms of *K. pneumoniae* that responsible for stress resistances, in order to survive under variety of environmental conditions, such as different pH condition, different concentration of oxygen, changes of osmotic pressure etc. Surviving under acidic condition in human stomach is the prior ability for *Enterobacteriaceae* to cause further infection. Therefore, before entering into the intestinal tract, which is in high pH value, there must be some specific mechanisms for *K. pneumoniae* to cope with the low pH stomach condition, which composed of gastric acid. One of the members of *Enterobacteriaceae*, *E. coli* had been identified to have 4 clusters of acid resistant system, whereas acid

resistant mechanism of *K. pneumoniae* not yet been identified completely. Even so, reported in 2007, RcsB is responsible for the glutamate-dependent acid resistant system in *E. coli* [76], which also found in *K. pneumoniae* CG43 in our study. Suggesting that RcsB might plays role for the acid stress response in *K. pneumoniae* CG43.

Oxidative stresses threaten bacteria by damaging DNA, protein, cell membrane, and affect bacterial growth and replication. During infection, bacteria usually living at a condition with full of oxidative stress, including intracellular oxidative stress i.e. oxygen gas produced by the electron transport chain (ETC), environmental existing oxidative stress i.e. environmental redox products or come from other competing pathogen during infection, and oxidative stress producing during phagocytosis occurred in host cell to attack bacteria [77]. Under oxidative stress, bacteria have several ways to cope with, such as oxidant-scavenger regulators and some antioxidant enzymes. Previously our lab members had suggested that *sodA*, *sodB*, *sodC*, *katE*, *katG* and *rpoS*, which are reported as oxidative resistant related genes clearly in *E. coli*, are also involved in the oxidative resistant event in *K. pneumoniae* CG43.

1.4 Two-Component System (TCS)

All organisms must respond to changing environmental conditions quickly and efficiently. While higher order organisms like plants and animals have adaptive responses that include complex behaviors such as migration or adaptation over generations, lower order and unicellular organisms including bacteria and yeast must sense and respond to changes their environmental using alternative strategies. Bacterial adaptation occurs on the level of individual genes and proteins; the level of global regulons; the whole-cell level (via motility or sporulation); or at multicellular level (via quorum sensing and biofilm, formation) [78]. TCSs are found ubiquitously in prokaryotes, archaea, fungi, yeast, and some plants. In bacteria, TCS responsible to sense, respond, and adapt to a wide range of environments, stresses, and growth conditions [79]. In the prototypical two-component system, comprised of sensor histidine kinase (HK) and their cognate response regulator (RR) substrates, this signaling system have been implicated in mediating the response of bacteria to a wide range of signals and stimuli, including nutrients, cellular redox state, changes in osmolarity, quorum signals, antibiotics, and more [80].

Most of the TCS signal transduction mechanisms prevailing to control gene expression in bacteria. Typically, a signal transduction pathway is initiated when the sensor histidine kinase stimulated by stress signals. The sensor kinase catalyzes its autophosphorylation and then subsequently transfers the phosphoryl group to a response regulator, which can then effect changes in cellular physiology, often by regulating gene expression [81].

1.5 Regulator of Capsule Synthesis (Rcs) System

The Rcs system, first identified by its role in the transcriptional regulation of the genes for capsular polysaccharide in *E. coli*, is proving to be an unexpected complex example of such a system. Besides, several bacteria such as *Salmonella typhi* [82], *K. pneumoniae* [83], the plant pathogenic bacteria *Erwinia amylovora* [84, 85] and *Pantoea stewartii* [86], the biosynthesis of CPS is controlled by the Rcs regulatory system [81], implicating that the regulating mechanism is highly conserved among these pathogenic bacteria . The Rcs system is composed of RcsA, RcsB, RcsD, and RcsF. RcsF is an outer membrane protein that is responsible for

receiving extracellular stimulation. RcsC acts as an inner membrane sensor kinase in this system, with a periplasmic domain [87]. RcsD (previously called YojN) is a phosphotransfer protein, which presented adjacent to RcsC at inner membrane, it transfers phosphoryl group from sensor kinase RcsC to response regulator RcsB in this system. In phosphorelays of this system, transfer of phosphate from His to Asp is conserved, phosphate travels from His to Asp to His to Asp [81]. Generally, phosphorylation and dephosphorylation of the response regulator, in turn, change the activity of this protein, frequently by modulating its ability to bind to DNA and act as a regulator of transcription. There are plenty of studies showing that Rcs system is presented in several members of *Enterobacteriaceae* bacterial regulatory mechanisms. In *E. coli*, Rcs system regulates CPS production [87], cell division [88], motility [89], type 1 pili [90] and glutamate-dependent acid resistant [76]. In *Salmonella*, Rcs system involved in the regulation on the gene that encoding capsule synthesis [91], virulence factors related genes [92, 93]. In *K. pneumoniae*, regulation of Rcs system on CPS production was reported clearly [94-96], however, suggesting other regulations still pending on further investigation.

1.6 The Response Regulator -- RcsB and RcsB-dependent Regulation

RcsB is a typical cytoplasmic activator for CPS biosynthesis [87]. According to the sequence alignment, it is highly conserved (~90% sequence identity) among different species, such as *E. coli*, *S. typhi*, *E. amylovora* and *K. pneumoniae* [85]. It has been proved that RcsB is activated by membrane-bound sensor RcsC and RcsD through phosphotransferring to a highly conserved Asp residue in the N-terminal domain of RcsB [87, 97]. RcsB is composed of two conserved motifs: an N-terminal domain for receiving phosphate group at the 10^{th}, 11^{th}, 56^{th} Asp residues as well as interacting with other regulator proteins; the C-terminal as an effector domain, which is a DNA binding motif that places this RcsB in the LuxR family of helix-turn-helix proteins [87].

1.6.1 Role of RcsB phosphorylation

The RcsB protein contains a LuxR-like helix-turn-helix DNA binding domain. Several members of the LuxR family are response regulators (RR), which act as transcriptional activators or repressors. As receiving phosphate group from the upstream kinase is the way for signal reception

of RcsB, phosphorylation status of RcsB determines downstream gene expression. This can be exemplified by the regulation of CPS production in *E. coli*, phosphorylated form of RcsB stimulates expression of the *cps* operons [98]. Previous study showed that, *Salmonella* biofilm development depends on the phosphorylation status of RcsB [99]; the phosphorylated RcsB inhibits biofilm development, while unphosphorylated RcsB induces. Rcs system responsible for wide range of regulation among various species of bacteria besides those had been mentioned above, it is able to receive lot of different stimulation, and then transfer the signals to downstream in order to regulate on corresponding genes.

1.6.2 RcsB interacting protein

RcsB is a global regulator allows multiple different regulatory inputs into its activation of specific targets, providing a simple mechanism for regulation of subsets of the RcsB-regulated genes. Interaction of RcsB with coregulator, will affect its binding to target genes. There are several types of RcsB-dependent regulation among the family of *Enterobacteriaceae*. The first type is exemplified by the regulation of *cps* in *E. coli* and related capsule genes in the plant pathogens *E. amylovora* and *P. stewartii* [81]. In these cases, RcsA is required to form heterodimer with RcsB. A necessary

site for RcsB and RcsA action was mapped approximately 100 nt upstream of the transcription start site had been demonstrated in *Erwinia amylovora* [100]. A consensus site for this binding has been determined from mutagenesis studies. Additional sites are present upstream of the *rcsA* promoter of *E. coli*, *Salmonella*, *Klebsiella*, which is autoregulated by RcsA and RcsB [101, 102]; together, these have been used to define an "RcsAB box" with a consensus sequence of TaAGaatatTCctA. In vitro studies demonstrate a tenfold higher binding for RcsA/RcsB than for RcsB alone at the regulatory sites for capsule synthesis [103]. Although the auxiliary regulator has been studied most thoroughly is RcsA, a number of other proteins have been found to cooperate with RcsB to stimulate transcription at specific promoters. The colanic acid, which is the main component of capsule, has not been identified as a virulent factor in mammals, related *E. coli* and *Klebsiella* species use RcsB to regulate synthesis of capsules that are important for virulence [104-106]. In some cases, these organisms have evolved somewhat different helper functions for RcsB. In *Salmonella typhi*, while a functional *rcsA* gene is present, the virulence-associated Vi antigen is synthesized in a process that is dependent on RcsB and independent of RcsA. The TviA protein, encoded

by the first gene of the ten-gene Vi cluster, is necessary for activation [82, 107]. Although no DNA binding has yet been demonstrated for TviA, its ability to suppress an rcsB missense mutation in the DNA binding domain may suggest that it interacts with RcsB and stimulates its ability to bind its DNA site [82]. For targets that depend on other auxiliary protein, the conditions of its induction, and the sensitivity of the target to RcsB and the helper protein will determine the expression pattern. In E. coli O157:H7, RcsB interacts with GadE, which is a central regulator involved in the glutamte-dependent acid resistance system AR2, to mediate acid resistance in stationary phase [108]. Some more in *E. coli*, another LuxR-type family transcriptional regulator, BglJ, interact with RcsB to form heterodimer that presumably bind upstream of the *bgl* promoter, and then relieves the H-NS (histone-like nucleoid-structuring protein) -mediated repression of *bgl* operon [109]. In conclusion, RcsB is a regulator that requires auxiliary proteins while acting, and can act with many different such auxiliary proteins that activated by specific signals, hence, providing the corresponding output.

1.7 Previous Studies - RcsB-dependent regulation in *K. pneumoniae*

Previous studies in our lab showed that, RcsB regulatory function is involved in the CPS biosynthesis [94, 110], type 3 major pilin MrkA production [111] and acid stress resistance [112] in *K. pneumoniae* CG43.

The understanding about the RcsB-dependent regulation on CPS biosynthesis is more thoroughly among the other RcsB-dependent regulation in *K. pneumoniae*. As known, RcsB requires auxiliary protein RcsA to regulate CPS biosynthesis in *E. coli* as well as. Moreover, synthesis of the K2 capsule in depends on a large virulence plasmid pLVPK, which has rendered an ~1,000 fold decrease in mouse virulence in curing [113]. pLVPK encodes RmpA and RmpA2, both are transcriptional activators that had been demonstrated to interact with RcsB on the regulation of the CPS biosynthesis [114, 115]. It had been showed that, the level of *K. pneumoniae* CG43 CPS production significantly reduced in the absence of RcsB (Appendix II) [110].

Type 3 fimbria is the adhesion component that involved in the biofilm formation of *K. pneumoniae.* The major subunit MrkA is usually used as

the indicator for the type 3 pilin expression. In the western blot that incubated with the MrkA polyclonal antibodies, the level of MrkA expression reduced in the RcsB deletion mutant *K. pneumoniae* CG43 (Appendix III) [111]. During infection, an enteric bacterium has to cope with the acidic condition in human stomach before it reaches the intestinal tract. Therefore, high number of the survived *K. pneumoniae* is required after passing through the stomach. An experiment demonstrated that, the acid survival rate of RcsB deletion mutant *K. pneumoniae* CG43 became lower comparing to the wild type *K. pneumoniae* CG43 (Appendix IV) [112]. Hence, indicating that RcsB is an important global regulator in *K. pneumoniae* CG43, which involved in the regulation of several virulence factors.

1.8 Specific Aims

Phosphorylation of RcsB has been demonstrated to be required for its regulatory function on bacterial *cps* gene expression [98, 116]. We intend to investigate if phosphorylation of RcsB affects its regulation on the suspected RcsB-regulated gene expressions. RcsB consists of N terminal

receiver domain and C-terminal DNA-binding domain. The N-terminal phosphoreceiver domain belongs to the CheY family and REC superfamily [117], and the highly conserved aspartate residue at position 56 (residue position is based on the amino acid sequence of *K. pneumoniae* RcsB) has been proposed as a phosphorylation site in those CheY family regulators [118]. An aspartate-glutamate substitution at the position 56 of RcsB also leads to constitutive gene expression, implying this position is a potential phosphorylation site [116, 119]. Two site-directed mutations RcsB-D56E and RcsB-D56A will be generated to mimic the phosphorylated form of RcsB and the RcsB that is unable to accept phosphate group, and then examine whether these two mutations affect the phenotypes of *K. pneumoniae* CG43.

As mentioned, RcsB outputs the received signal by stimulating the *cps* operon expression as a transcription factor [87]. Hence, a LacZ reporter system will be set up to confirm that, RcsB affects *yfdX* (which suggested to be involved in the acid stress response) expression at the transcriptional level. Furthermore, electrophoretic mobility shift assay (EMSA) will be demonstrated to investigate whether RcsB regulates the transcription of *yfdX* directly binding to their promoters. Since RcsB activates gene

expression by binding to promoter as a hetero-/homodimer, while acting with different auxiliary proteins allows RcsB activates different specific target genes. Co-immunoprecipitation (Co-IP) will be done to identify the auxiliary protein, which cooperates with RcsB to regulate the target gene that is stimulated under specific condition.

2. Materials and Methods

2.1 Plasmids, primers, bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1 and Table 2, and the primers used are listed in Table 3. *K. pneumoniae* CG43, a clinical isolate of serotype K2, is high virulent to mice [120]. *E. coli* and *K. pneumoniae* strains were generally propagated at 37°C in Luria-Bertani (LB) broth. M9 minimal medium was also used in some specific assay. Bacterial growth was assessed by measuring the absorbance of optical density at 600 nm (OD_{600}). The antibiotics used include ampicillin (100 μg/ml), chloramphenicol (35 μg/ml), kanamycin (25 μg/ml), tetracycline (5 μg/ml) and streptomycin (500 μg/ml). Microaerobic culture for the MrkA expression was added with mineral oil (M5310) which was purchased from Sigma.

2.2 Construction of the site-directed mutants RcsB

pHY121 that carrying *rcsB* and its approximately 600 bp adjacent regions was used as the template. The plasmids with site-directed mutations were constructed by quick change method. pHY121 was amplified with the complementary primer sets rcsB-D56E(＋)/rcsB-D56E(－) , wc30/wc31 and wc32/wc33 encompassing the mutation site (167th bp for D56A and 168th bp for D56E of pHY121insert) by using *PfuUltra* II Fusion HS DNA polymerase (Agilent Technologies) to generate mutant alleles of *rcsB* with the D56E or D56A mutations. The PCR product was resolved on an agarose gel, recovered, treated with DpnI for 2 hr to remove the template plasmid and transformed into *E. coli* JM109. The plasmid, pHY121*, carrying the mutation allele encoding RcsB (RcsB*, D56E and D56A mutations) was then prepared from the transformant colony and confirmed by sequence analysis. The mutated fragment RcsB-D56E and RcsB-D56A were subcloned into pRK415 to yield pYX001 and pYX002, respectively. pYX001 and pYX002 were then individually mobilized from *E. coli* S17-1 λ*pir* to the *K. pneumoniae* CG43S3 Δ*rcsB* strain by conjugation, and the subsequent selection was performed as described above. Each site-directed mutation in *K. pneumoniae* was confirmed by DNA sequencing.

2.3 Measurement of bacterial growth

Cultures of the parental strain *K. pneumoniae* CG43S3, along with *rcsB* deletion mutant strains, Δ*rcsB*[pRK415-RcsB], Δ*rcsB*[pRK415-RcsB-D56E], Δ*rcsB*[pRK415-RcsB-D56A] complement strains were grown overnight in LB medium. Twenty microliters of ovenight LB cultures of *K. pneumoniae* strains was used to inoculate 4 ml of LB broth. The cultures were incubated at 37°C with shaking, and the optical density was recorded as the absorbance at 600 nm at the indicated time points. Values were the average and standard deviation from triplicate samples from one of the three independent trials.

2.4 Extractions and Quantification of CPS

Bacterial CPS was extracted using the method described [121]. Briefly, 500 μl of overnight grown bacterial was mixed with 100 μl of 1% Zwittergent 3-14 (Sigma-Aldrich, Milwaukee, WI, USA) in 100mM citric acid (pH 2.0) and incubated at 50°C for 20 min. After centrifugation, 250 μl of the supernatant was transferred to a new tube, and the CPS was precipitated with 1 ml of absolute ethanol. The pellet was dried, dissolved

in 200 μl de-ionized water, and then 1200 μl of 12.5 mM borax in H_2SO_4 was added. The mixture was vigorously mixed, boiled for 5 min, cooled down and then 20 μl of 0.15% 3-hydroxydiphenol (Sigma-Aldrich, Milwaukee, WI, USA) was added. The absorbance at 520 nm was measured, and the glucuronic acid content was determined from a standard curve of glucuronic acid and expressed as μg per 10^9 CFU or 10^{10}CFU.

2.5 Western Blot Analysis

Samples for Western blot analysis were prepared by obtaining whole bacterial lysates, cells were grown in LB medium at 37°C for overnight. Each culture was harvested for 400 μl, after centrifugation, cells were resuspended in 100 μl of de-ionized water, and then were boiled at 100°C for 10 min. Quantification was taken to equalized the concentration of all cultures, and added with 2 × protein dye that containing 20% of DTT (Dithiothreitol) to boil again at 100°C for 10 min. Proteins were separated on SDS-polyacrylamide gels (13.5% to 5%). For Western blotting, proteins were transferred onto PVDF (Polyvinylidene fluoride) membranes by electroblotting. When blotting was completed, add blocking buffer which

contains 5% milk in TBS buffer (20 mM Tris-HCl, 50 mM NaCl, pH 7.5) and blocked for 16 hr at 4°C. Probing was carried out with diluted antibodies: anti-GAPDH, anti-RcsB N-terminal, anti-MrkA, anti-FimA or anti-YfdX at a ratio of 1: 10000 for 2 hr at room temperature. Before incubating the blot in second antibody, wash the membrane twice by TBST buffer (TBS buffer with 0.1% tween-20) for 10 min per time, and then wash by TBS buffer for 10 min to remove the non-specific binding of first antibody. Secondary hybridization was incubated with diluted anti-rabbit IgG conjugated with alkaline phosphatase at a ratio of 1: 5000 for 1 hr at room temperature. Bound ligands were detected by using the alkaline phosphatase staining method, which contain alkaline phosphate buffer (150 mM Tris-HCl, 5 mM $MgCl_2 \bullet 6H_2O$, pH 9.5), BCIP (50 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in 95% dimethylformamide) and NBT (50 mg/ml of p-nitro-blue tetrazolium chloride in 70% dimethylformamide).

2.6 Biofilm Formation Assay

Overnight grown bacteria were diluted 1: 100 in LB broth supplemented with appropriate antibiotic and then inoculated into each well

of a 96-well microtiter dish (Orange Scientific) for statically incubation at 37°C for 12 hr and 48 hr After removal of the bacteria, the plate was washed by de-ionized water once, and 150 μl of 1% (w/v) crystal violet was added to each well. The plate was incubated at room temperature on an orbital shaker for 30 min, and then washed three times. The dye was solubilized in 1% (w/v) SDS, and absorbance at 595 nm was determined.

2.7 Yeast-cell Agglutination

Agglutination of yeast *Saccharomyces cerevisiae* AH109 was carried out as described [122]. Briefly, bacteria (~10^8 CFU/ml) were suspended in saline (0.85% of NaCl) with or without 5% mannose and then mixed with 1% of yeast (Sigma-Aldrich) on a glass slide. After 5 min incubation at room temperature on an orbital shaker, agglutination of yeast caused by bacteria could be assessed.

2.8 Disc Diffusion Assay

Bacteria were overnight grown with 1: 20 dilution in LB broth supplemented with appropriate antibiotic and then incubated at 37°C until OD_{600} reached 0.3- 0.4. Spread 100 μl of each strain of culture on M9 agar plate, and then place a nitrate disc on the center of the plate. Add 5 μl of 10 mM paraquat on the nitrate disc and place the agar plate by staying the agar at the bottom in 37°C incubator for overnight.

2.9 Acid Stress Response

Bacterial resistance to acid challenge was determined essentially as previously described [123]. Overnight grown bacteria were diluted 1: 20 in LB broth supplemented with an appropriate antibiotic and then incubated at 37°C until OD_{600} reached 0.4 - 0.6. All strains were further divided into two groups of tubes, while each tube containing 1 ml of bacteria culture. Both groups of culture was centrifuged and resuspended in 1 ml of pH 4.4 LB medium for a 1 hr adaption period before the acid challenge. After adaptation, each culture from one of the group was centrifuged and resuspended in 1 ml of pH 3.0 M9 medium for 45 min. After the acid

challenge, 100 μl of each culture in the second group was immediately removed for serial tenfold dilution in saline, and 100 μl of the 10^{-6} diluted sample was plated onto LB agar plates at what was considered to be the initial time point (t_0). The group with pH 3.0 acid challenge was removed 100 μl immediately and serially diluted, 100 μl of each culture to the 10^{-6} dilution was plated onto LB agar plates and incubated at 37°C overnight. The survival rates of each strain were then calculated by dividing the number of colonies with acid challenge by the number of colonies at t_0.

2.10 Measurement of Promoter Activity

- A laboratory stocked plasmid, placZ15-P_{yfdX}, which is the promoter selection plasmid placZ15 that inserted with the *yfdX* promoter region in front of a promoter-less *lacZ* gene [124]. placZ15-P_{yfdX} was then mobilized from *E. coli* S17-1 λ*pir* to *K. pneumoniae* wild type and Δ*rcsB* strains by conjugation. β-galactosidase activity was determined as previously described [124]. In brief, overnight cultures were diluted 1: 20 in LB broth supplemented with appropriate antibiotic and then incubated at 37°C until OD_{600} reached 0.8 - 1.0. Harvest 1 ml of each

culture in a tube, and wash with 1 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) for twice. All cultures were resuspended after washed, 100□μl of each culture was taken to mix with 900 μl of Z buffer, 17 μl of 0.1% SDS and 35 μl of chloroform, mixture was shaken vigorously. After incubation in 30°C water bath for 10 min, the reaction was initiated by adding 200 μl of 4 mg/ml ONPG (o-nitrophenyl-β-D-galactopyranoside) (Sigma-Aldrich). Upon the yellow-colored appearance, the reaction was terminated by adding 500 μl of 1 M Na_2CO_3. OD_{420} of each strain was recorded and the β-galactosidase activity was expressed as Miller units (1 Miller unit = $1000 * \frac{\mathrm{OD420}}{(t*v*\mathrm{OD600})}$; OD_{420} is the absorbance of the yellow o-nitrophenol; t = reaction time in minutes; v = volume of culture assayed in milliliters; OD_{600} = reflects optical density of bacteria culture) [125]. Each sample was assayed in triplicate, at least three independent experiments were carried out. The data shown were calculated from one representative experiment and shown as the means and standard deviation from triplicate samples.

2.11 RcsB N-terminal region antiserum preparation

The N-terminal domain of *rcsB* was PCR amplified using primers Yen001 and Yen002 and cloned into the PCR cloning vector yT&A (Yeastern Biotech, Taiwan). The NdeI/SalI fragments form the resulting plasmid (yT&A-RcsB N-terminal) were then cloned individually into pET30a (Novagen, Madison, Wis, USA) to yield pET30a-RcsB N-terminal allowing the in-frame fusion to the N-terminus His-tag. Plasmid pET30a-RcsB-Nterminal was then introduced into *E. coli* BL21 (DE3) (Invitrogen, USA) for overexpression of the His_6::RcsB N-terminal recombinant protein. The recombinant protein was over-produced from the mid-log phased culture (OD_{600} around 0.4 - 0.6) by induction with 1 mM IPTG (isopropyl-1-thio-β-$_D$-galactopyranoside) for 4 hr at 37°C. The proteins were then purified from total cell lysate by affinity chromatography using His-Bind resin (Novagen, Madison, Wis). After purification, the eluent was dialyzed against 1× protein storage buffer (10 mM Tris-HCl pH 7.5, 138 mM NaCl, 2.7 mM KCl, and 10% glycerol) at 4°C overnight. RcsB N-terminal region antiserum was prepared by immunizing a New Zealand white rabbit with 2.5 mg of the purified His_6::RcsB N-terminal recombinant protein and the immunized rabbit was exsanguinated on day 60.

2.12 Statistical Methods

The results of the biofilm-forming activity and β-galactosidase activity assays were derived from a single experiment that was representative of three independent experiments. Each sample was assayed in triplicate and the data were presented as the mean ± standard deviation (SD). Differences between groups were evaluated by a two-tailed Student's *t*-test. *P*-values less than 0.05 were considered statistically significant difference.

3. Results

3.1 Roles of phosphorylation of RcsB in *K. pneumoniae* CG43

3.1.1 Generation of specific point-mutation on RcsB

As a LuxR family response regulator, a common post-translational modification of phosphorylation at a conserved Asp in the N-terminal receiver domain performed by a sensor histidine kinase (HK) or by cytoplasmic phosphodonors, acetyl-phosphate. Phosphorylation of the conserved Asp in the N-terminal receiver domain of response regulator activates the protein by inducing conformational changes which facilitate interaction of the response regulator with the target DNA [117, 126]. Furthermore, some response regulators have been shown to autophosphorylate in vitro in the presence of acetyl phosphate [127-129], which has been proposed to be global signal in bacteria [130]. Three aspartate residues at position 10^{th}, 11^{th}, 56^{th} are highly conserved in an N-terminal phosphorylation motif and Asp56 has been proved as a

phosphorylated site (Appendix I) [81]. As shown in Fig. 1, to determine the role of the conserved D56 on RcsB regulation in *K. pneumoniae* CG43, the site-directed mutants with single amino acid substitution of the D56 to alanine (D → A) to prevent it from phosphorylation [131, 132], or to glutamte (D → E) to mimic the phosphorylated state [133-136], were created. The mutated sequences were identified by the Tri-I Biotech, Inc. to confirm the single nucleotide substitution. The point-mutated RcsB was then individually introduced into *K. pneumoniae* CG43 Δ*rcsB* mutant strain.

3.1.2 RcsB D56A as well as RcsB retarded the growth of *K. pneumoniae* CG43 Δ*rcsB*

To investigate the effect of RcsB phosphorylation state on bacterial growth, *K. pneumoniae* CG43 wild type, Δ*rcsB* mutant and Δ*rcsB* complement strains were grown in LB medium for 24 hours. The optical density at 600 nm of each bacteria culture was recorded every hour. As shown in Fig. 2, the growth rate of *K. pneumoniae* CG43 Δ*rcsB* [pRK415-RcsB] and *K. pneumoniae* CG43 Δ*rcsB* [pRK415-RcsB-D56A] were significantly lower comparing to the wildtype *K. pneumoniae* CG43. We

assumed that *K. pneumoniae* CG43 with high level of RcsB or unphosphorylated RcsB expression directs the carbon metabolic pathway to CPS biosynthesis and, therefore resulting of the retarded growth rate.

3.1.3 RcsB D56A as well as RcsB restored the production of capsule polysaccharide in *K. pneumoniae* CG43 Δ*rcsB*

K. pneumoniae CG43 is a highly encapsulated virulent strain [120]. In order to verify whether complementation with the phosphorylated or unphosphorylated RcsB restores the production of CPS, [pRK415-RcsB-D56E] and [pRK415-RcsB-D56A] were complemented into Δ*rcsB* mutant, and the amounts of CPS produced were compared with that of Δ*rcsB*[pRK415]. All of the strains were streaked on LB agar for comparing their colony morphology, the colony surface of Δ*rcsB*[pRK415-RcsB-D56E] appeared to be less mucoid than that of Δ*rcsB*[pRK415] (Fig. 3A), and the degree of viscosity was reduced significantly as determined by a string test [114], which refers to the ability to form a string when the bacterial colony was picked with toothpick. The CPS-deficient phenotype is evident as assessed using sedimentation assay and the amount of K2 CPS

produced. In the sedimentation test, all of the samples were centrifuged at 4,000 rpm for 5 min. As shown in Fig. 3B, the Δ*rcsB*[pRK415-RcsB-D56E] as well as Δ*rcsB*[pRK415] could be rapidly precipitated. In the CPS quantification analysis, the content of main substance, which is the glucuronic acid, was extracted for quantification. As shown in Fig. 3C, Δ*rcsB*[pRK415] and Δ*rcsB*[pRK415-RcsB-D56E] had reduced at least 50% of the CPS contents in comparing with Δ*rcsB*[pRK415-RcsB] and Δ*rcsB*[pRK415-RcsB-D56A]. The amount of CPS could be restored by RcsB-D56A but not by RcsB-D56E, suggesting that the regulation of RcsB on CPS biosynthesis may require the unphosphorylated form of RcsB.

3.1.4 Complementation with the phosphorylated RcsB restored the expression of type 3 fimbriae in *K. pneumoniae* CG43 Δ*rcsB*

Type 3 fimbriae are 4 nm wide and 0.5-2 μm long surface organelles that are originally characterized in *Klebsiella* strains by their ability to mediate mannose-resistant agglutination of tannic acid-treated human

erythrocytes (MR/K haemagglutination) [75, 137]. *K. pneumoniae* type 3 fimbriae are encoded by *mrkABCDF* gene cluster [74], several studies have demonstrated an important role for type 3 fimbriae in biofilm formation on biotic surfaces [45, 138]. The ability of bacteria to form biofilm on medical devices is believed to play a major role in development of nosocomial infections. In addition, type 3 fimbriae have been demonstrated to mediate bacterial attachments to several cell types including tracheal epithelial cells, renal tubular cells, extracellular matrix proteins, and components of basement membranes of human lung tissue [69, 72, 73]. According to the previous study, the level of type 3 fimbriae expression reduced in the RcsB deletion mutant *K. pneumoniae* CG43. As shown in Fig. 4A, the Western blot hybridization using MrkA antiserum revealed that the expression of the type 3 major pilin, MrkA was increased approximately 1.53 fold in the Δ*rcsB*[pRK415-RcsB-D56E], while Δ*rcsB*[pRK415-RcsB-D56A] expressed a small amount of MrkA (approximately 39%). As type 3 fimbriae is the adhesion component that involved in the biofilm formation of *K. pneumoniae*, the biofilm formation assay was performed in the 96-plastic well and glass tubes. Consistently, Δ*rcsB*[pRK415-RcsB-D56E] exhibited a higher level of biofilm-forming activity than Δ*rcsB*[pRK415-

RcsB-D56A], as assessed by quantitative measurement with crystal violet staining. However, the biofilm-forming activity assessed by glass tube exhibited an inverse effect. Thus, Δ*rcsB*[pRK415-RcsB-D56A] was not a biofilm former compared to the Δ*rcsB*[pRK415-RcsB-D56E]. This suggested that, the expression of type 3 fimbriae is regulated by the phosphorylated form of RcsB, and the phosphorylated state of RcsB maydetermine the adhesion of *K. pneumoniae* CG43 on different surfaces.

3.1.5 RcsB D56E increases the expression of type 1 fimbriae in *K. pneumoniae* CG43 Δ*rcsB*

Type 1 fimbriae are approximately 7 nm wide and 1-2 μm long surface organelles found in virtually all members of the family *Enterobacteriaceae* [139, 140]. They are expressed by *fimACDFGHIK* gene cluster [141] and well known for the ability to bind to mannose-containing structures on host cells and extracellular matrix. Bacteria expressing type 1 fimbriae are able to cause mannose-sensitive agglutination of yeast cells or erythrocytes (mannose-sensitive haemagglutination, MSHA) from guinea pig. Previous study showed that,

the deletion of *mrkA* increased the expression of type 1 fimbriae, while the overproduction of FimB recombinase repressed MrkA expression. Together, both indicated that the expression of type 3 fimbriae and type 1 fimbriae are regulated in a coordinate manner in *K. pneumoniae* [142]. Western blot analysis using anti-FimA serum, Fig. 5A revealed that, FimA did not express in *K. pneumoniae* CG43 wild type strain, but express only in Δ*rcsB* mutant and Δ*rcsB*[pRK415-RcsB-D56E]. The activity of type 1 fimbriae was assessed using mannose sensitive yeast agglutination analysis. Each strains of bacteria culture was added with 1% of yeast for reaction in a single well of 24-well plate, and also another reaction with 5% of mannose for the mannose competition assay. As shown in Fig. 5B, agglutination could be observed in the well with Δ*rcsB* mutant and Δ*rcsB*[pRK415-RcsB-D56E]. In the mannose-sensitive agglutination activity assay (Fig. 5C), mannose could inhibit the agglutination activity of Δ*rcsB* mutant and Δ*rcsB*[pRK415-RcsB-D56E]. The result indicates that, RcsB phophorelay status affects the expression of type 1 fimbriae, and therefore effect on the adhesion of *K. pneumoniae* CG43.

3.1.6 RcsB D56A as well as RcsB promoted oxidative stress resistance of *K. pneumoniae* CG43 Δ*rcsB*

Once entering to the host, bacterial pathogens must circumvent the attack of reactive oxygen species produced by the immune cells. Therefore, to examine whether RcsB phosphorelay status could play role in the regulation of oxidative stress response, each overnight grown strain was refreshed cultured until OD_{600} around 0.3-0.4, and 100μl of each culture spread on LB agar plate supplemented with tetracycline. A nitrocellulose disc containing 10μM of paraquat, a superoxide generator, as then placed onto the bacterial lawn. All plates were incubated at 37°C for 16 hr., the diameters of the inhibition zone were measured and recorded. As shown in Fig. 6, the inhibition zones on the LB agar plate with the Δ*rcsB*[pRK415-RcsB] and Δ*rcsB*[pRK415-RcsB-D56A] were smaller in comparing to the other strains. This indicated that the unphosphorylated RcsB might be able to restore the oxidative stress resistivity of *K. pneumoniae* CG43.

3.1.7 RcsB D56A as well as RcsB restored the acid stress resistance activity under shaking cultured condition

Previous study revealed that, *rcsB* deletion decreases the ability of glutamate-dependent acid resistance in *E. coli* dramatically [76]. However, *K. pneumoniae* is unable to survive in pH 2.5 M9 medium. Therefore, all *K. pneumoniae* CG43 cultures were undergoing adaption at pH 4.4 in LB medium according to the related study [123],. The acid resistance of *K. pneumoniae* CG43 was determined after 90 min challenge with pH 3.0 in M9 medium. The result in Fig. 7A showed that the Δ*rcsB*[pRK415-RcsB] and Δ*rcsB*[pRK415-RcsB-D56A] complementation strains restored the bacterial survival rates significantly, while Δ*rcsB* mutant strain that transformed with RcsB-D56E only recovered slightly level of acid survival rate. Previous study showed that, the level of *rcsB* expression reported by the promoter activity analysis of P_{rcsDB} is not affected by the alteration of pH value in LB shaking culture. Using bioinformatics tool, 6 RcsB-dependent genes were identified. One of these genes, namely *yfdX* had been reported to endode a chaperone required for the acid stress response. The ability of acid resistance was significantly decreased in *K. pneumoniae* CG43 Δ*yfdX* mutant [112], indicating that *yfdX* is involved in the acid

resistant system. As shown in Fig. 7B and Fig. 7C, the acid survival rate and the western blot hybridization with anti-YfdX revealed that, both Δ*rcsB*[pRK415-RcsB-D56E] and Δ*rcsB*[pRK415-RcsB-D56A] complementation strains restored YfdX expression under microaerobic incubation. However, in highly aerated culturing condition, only the Δ*rcsB*[pRK415-RcsB-D56A] complementation strain was able to restore the expression of YfdX. It is hence that, the unphosphorylated RcsB is required for the acid resistance, while in the microaerobic environment, acid resistant system could be regulated neglecting the phosphorelay status of RcsB in microaerobic environment.

3.1.8 Effect of different phosphorylation states of RcsB on *yfdX* gene expression.

Due to there are several RcsB binding boxes among the *yfdX* promoter region were predicted previously [112], we aimed to determine whether unphosphorylated or phosphorylated RcsB could regulate the *yfdX* transcription. The promoter region of *yfdX* gene was transcriptionally fused to the reporter plasmid, carrying a *lacZ* reporter gene. This recombinant

plasmid (P_{yfdX}::*lacZ*) was then transformed into *K. pneumoniae* strains CG43S3Δ*lacZ* and Δ*rcsB*Δ*lacZ* individually. The promoter activity measurements shown in Fig. 10A reveal that, the deletion of *rcsB* reduced the activity of P_{yfdX}::*lacZ*. As shown in Fig. 10B, the complementary effects of RcsB are different under different oxygen level of cultivation. Under static culture (microaerobic), all the RcsB complementary strains up-regulated the P_{yfdX} activity comparing to Δ*rcsB* mutant strain, while RcsB-D56E complemented strain was unable to recover P_{yfdX} activity during shaking culture (sufficient oxygen). This suggests that the P_{yfdX} activity is mediated by RcsB in acidic condition, whereas this regulation manner is determined by the phosphorylation state of RcsB only when *K. pneumoniae* is cultured with sufficient oxygen.

3.2 Different roles of phosphorylated and unphosphorylated RcsB.

According to the results of the phenotype assays, we proposed a model of RcsB to show the likely regulatory pathway on different phenotypes or virulence factors in *K. pneumoniae* CG43. Urinary catheters

are standard medical devices utilized in both hospital and nursing home settings, but are associated with a high frequency of catheter-associated urinary tract infections (CAUTI). In particular, biofilm formation on the catheter surface by *K. pneumoniae* causes severe problems. Previous study showed that, type 1 and type 3 fimbriae expressed by *K. pneumoniae* enhance biofilm formation on urinary catheters in a catheterized bladder model that mirrors the physico-chemical conditions present in catheterized patients [143]. In this study, our result presented RcsB-D56E increased the expression of type 1 fimbriae major pilin FimA, and type 3 fimbriae major pilin MrkA, suggesting that constitutively phosphorylated RcsB might induces the biofilm development via positively regulating the expression of type 1 and type 3 fimbriae. As known, *mrkH, mrkI,* and *mrkJ* are the regulatory genes of type 3 fimbriae, and a few RcsB binding boxes could be found on the putative promoter region of mrkH and mrkI. It is hence we propose that RcsB might regulates type 3 fimbriae via interacting with these regulatory proteins. Curli fibers are a major adhesin factor to surfaces, and also affect cell aggregation and biofilm formation in many enterobacteria, such as *Salmonella* and pathogenic *E. coli* strains [144-147]. Expression of both curli fibers and cellulose depends on the CsgD protein,

a response regulator of the LuxR family. The CsgD is also a positive regulator for the expression of type 3 fimbriae [148]. If RcsB interacts with CsgD for regulating the type 3 fimbriae expression remains to be investigated.

The complementation with RcsB-D56A recovered and/or increased CPS production, oxidative stress and acid stress responses, which suggesting that, the unphosphorylated form of RcsB plays positive role for these genes expression. Therefore, we proposed the unphosphorylated RcsB is important in the stress resistance response regulation that protects the bacteria in *K. pneumoniae* CG43 from stress damages.

3.3 Identification of the RcsB interacting protein under acidic condition.

3.3.1 RcsB interacting proteins in the regulatory pathway of CPS synthesis are different from that involved in regulating the acid stress resistance.

The interacting proteins interacting with RcsB in the regulation of the CPS synthesis are known in *K. pneumoniae*. Here, we intended to examine whether the RcsB interacting proteins for the CPS synthesis regulation are also required for the acid stress resistant regulation. As shown in Fig. 10C, neither Δ*rcsA*, Δ*rmpA* nor Δ*rmpA2* mutant strain shows the same effect on the YfdX expression as that of Δ*rcsB* in Western blot analysis using anti-YfdX serum. The result indicates that the RcsB on the regulation of the CPS synthesis and acid stress resistant require different interacting proteins.

3.3.2 Generate an antibody specific to RcsB N-terminal region

In order to capture the RcsB interacting protein in co-immunoprecipitation assay, the first 343 nucleotides of *rcsB* was fused into an expression plasmid pET30a, and the resulting recombinant plasmid pET30a-RcsB-N (Table 2) was used to transform *E. coli* BL21 (DE3). The expression condition of the recombinant RcsB N-terminal was then

analyzed. As shown in Fig. 11A, an IPTG-induced overexpression of the His_6-RcsB-N could be observed. The purified His_6-RcsB-N of approximately 17 kDa (Fig. 11B) was used to immunize rabbit for raising anti-RcsB antibody. The specificity of anti-RcsB-N antibody was tested by a 10000-fold dilution at room temperature. As shown in Fig. 12, the anti-RcsB-N antibody could specifically bind to the recombinant RcsB N-terminal polypeptide.

3.3.3 Expression of RcsB under acidic growth conditions

The RcsB-N antiserum was generated from the rabbit with 13 boosts of immunization. The RcsB has to be highly expressed for the isolation of the RcsB interacting protein by co-immunoprecipitation. According to Fig. 13, RcsB was induced in *K. pneumoniae* CG43 under acid treatment. This can be observed for Δ*rcsB*[pRK415-RcsB] and Δ*rcsB*[pRK415-RcsB-D56A], but not in Δ*rcsB*[pRK415-RcsB-D56E]. Nevertheless, Coomassie blue staining of the SDS-PAGE samples as shown in Fig. 13B revealed that the protein expression of Δ*rcsB*[pRK415-RcsB-D56E] was similar to

Δ*rcsB*[pRK415-RcsB-D56A] when the bacteria cultivated under static culture. This implies that the raised antibody could not recognize the acid induced expressed RcsB-D56E protein.

4. Discussion

The complementation analysis of *rcsB* deletion showed a similar trend in most of the phenotype assays between the activity of RcsB and RcsB-D56A. Both RcsB and RcsB-D56A decrease the growth, while increase the CPS production, acid stress survivals, oxidative stress response, biofilm formation on glass tube. By contrast, RcsB-D56E increased the production of type 1 and type 3 major pilin. The increased expression of CPS, which causing energy consumed, probably renders the bacteria slow growth rate. The acid survival analysis revealed that oxygen level of the growth condition is an important factor for the RcsB-dependent acid stress resistance and this might be closely dependent on the acid stress response-related protein, YfdX. The promoter activity assay further confirmed that RcsB regulates YfdX expression at the transcriptional level. In summary, we found that the phosphate signaling relay on RcsB confers both positive and negative regulation on the downstream genes.

RcsB had been studied for several regulations in other *Enterobacteriaceae* members. In *E. coli* and *Salmonella*, phosphorylated

RcsB as activated form regulates the CPS synthesis [81]. However, there are some evidences showing that, RcsB can be activated in unphosphorylated form. For this case, phosphorylated RcsB up-regulates the biofilm formation in *E. coli*, while in *Salmonella*, unphosphorylated RcsB is required to induce biofilm development. Here, we have shown that RcsB positively regulates CPS biosynthesis, acid stress response and oxidative stress response possibly in unphosphorylated form; while in phosphorylated form to activate the expression of type 1 fimbriae, type 3 fimbriae and biofilm formation. The phosphorylation status of RcsB on the CPS synthesis regulation in *K. pneumoniae* is different from that in *E. coli* and *Salmonella*. We hypothesize that, *K. pneumoniae* RcsB interacts with RmpA or RmpA2 at 37°C, instead of RcsA at 30°C, to form heterodimer, in which RcsB might be in unphosphorylated form, while the phosphorylated RcsB interacts with RcsA in phosphorylated form. Another possible explanation for this is that, the RcsB phosphorylation shift is determined by kinase or phosphatase activity of RcsC that has been shown to key factor for the regulation of biofilm formation in *E. coli* [149]. We can envision at least two mechanisms that could lead to different outcome among different bacteria species: first, the phosphatase activity of RcsC

prevails over the kinase activity. Alternatively, RcsB might be prone to accepting phosphoryl groups from other phosphodonors. Nevertheless, different phosphorylation levels of RcsB conferring opposite regulations had also been reported in *E. coli* and *Salmonella* [99]. Hence, further studies of the RcsB phosphorylation levels in vivo as well as in the strain with point-mutated RcsC at the residue which determines the kinase/phosphatase activity are necessary to validate this hypothesis.

The previous structural study demonstrated that phosphorylated RcsB is able to induce conformational change in the N-terminal domain and then triggers the formation of tetramer [150]. This suggests that the tetrameric structure of RcsB affects its regulation by altering the binding to target DNA or interacting with coregulators. Further investigation requires a chromosomal complementation analysis, as RcsB-D56E and RcsB-D56A are plasmid form which may cause artificial effects such as the selection by tetracycline resulted in compromising the phosphorelay status or the copy number effect. It is possible that the multi-copy of the phosphorylated RcsB leads to excessive phosphorelay activity and therefore represents an overexpression condition.

Altogether, our results agree with the model in which RcsB regulates the expression of the virulence factors in a phosphorylation-dependent manner at the transcriptional level.

5. Perspectives

5.1 To study whether the RcsB interacting with RcsA, RmpA and RmpA2 is in phosphorylated or unphosphorylated form

5.1.1 Identifying the interaction between RcsB-D56E/A and RcsA, RmpA, RmpA2 using Bacterial Two-Hybrid System.

5.1.1 Evaluating the phosphorylation effect on the binding of RcsB to DNA by EMSA (electrophoretic mobility shift assay) analysis.

5.2 To investigate the effect of RcsB phosphorylation on acid stress response

5.2.1 Determining the acid survival rate of Δ*rcsB*[pRK415-RcsB-D56E] and Δ*rcsB*[pRK415-RcsB-D56A] strains under static cultivation.

5.2.2 Examining whether RcsB phosphorylation regulates the expression of the acid stress chaperone protein.

5.3 To identify the phosphorylated and non-phosphorylated RcsB interacting proteins

5.3.1 Identifying the RcsB interacting protein(s) using immunoprecipitation (IP) and mass spectrometry from the total proteins of the bacteria grown specifically under acidic conditions or the conditions optimal for type 3 fimbriae expression.

5.3.2 Study further for the co-regulator mechanisms.

6. References

1. Topley WWC, Wilson GS, Collier LH, Balows A, Sussman M, Topley WWC: **Topley & Wilson's microbiology and microbial infections**, 9th edn. London; New York; Arnold; Oxford University Press; 1998.
2. Bagley ST, Seidler RJ, Talbot HW, Jr., Morrow JE: **Isolation of Klebsielleae from within living wood**. *Appl Environ Microbiol* 1978, **36**(1):178-185.
3. Brown C, Seidler RJ: **Potential pathogens in the environment: Klebsiella pneumoniae, a taxonomic and ecological enigma**. *Appl Microbiol* 1973, **25**(6):900-904.
4. Edberg SC, Piscitelli V, Cartter M: **Phenotypic characteristics of coliform and noncoliform bacteria from a public water supply compared with regional and national clinical species**. *Appl Environ Microbiol* 1986, **52**(3):474-478.
5. Seidler RJ, Knittel MD, Brown C: **Potential pathogens in the environment: cultural reactions and nucleic acid studies on Klebsiella pneumoniae from clinical and environmental sources**. *Appl Microbiol* 1975, **29**(6):819-825.
6. Matsen JM, Spindler JA, Blosser RO: **Characterization of Klebsiella isolates from natural receiving waters and comparison with human isolates**. *Appl Microbiol* 1974, **28**(4):672-678.
7. Podschun R, Ullmann U: **Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors**. *Clin Microbiol Rev* 1998, **11**(4):589-603.

8. Yinnon AM, Butnaru A, Raveh D, Jerassy Z, Rudensky B: **Klebsiella bacteraemia: community versus nosocomial infection**. *QJM* 1996, **89**(12):933-941.
9. Bartlett JG, O'Keefe P, Tally FP, Louie TJ, Gorbach SL: **Bacteriology of hospital-acquired pneumonia**. *Arch Intern Med* 1986, **146**(5):868-871.
10. Jarvis WR, Munn VP, Highsmith AK, Culver DH, Hughes JM: **The epidemiology of nosocomial infections caused by Klebsiella pneumoniae**. *Infect Control* 1985, **6**(2):68-74.
11. Ko WC, Paterson DL, Sagnimeni AJ, Hansen DS, Von Gottberg A, Mohapatra S, Casellas JM, Goossens H, Mulazimoglu L, Trenholme G *et al*: **Community-acquired Klebsiella pneumoniae bacteremia: global differences in clinical patterns**. *Emerg Infect Dis* 2002, **8**(2):160-166.
12. Paterson DL, Ko WC, Von Gottberg A, Mohapatra S, Casellas JM, Goossens H, Mulazimoglu L, Trenholme G, Klugman KP, Bonomo RA *et al*: **International prospective study of Klebsiella pneumoniae bacteremia: implications of extended-spectrum beta-lactamase production in nosocomial Infections**. *Ann Intern Med* 2004, **140**(1):26-32.
13. Davis TJ, Matsen JM: **Prevalence and characteristics of Klebsiella species: relation to association with a hospital environment**. *J Infect Dis* 1974, **130**(4):402-405.
14. Selden R, Lee S, Wang WL, Bennett JV, Eickhoff TC: **Nosocomial klebsiella infections: intestinal colonization as a reservoir**. *Ann Intern Med* 1971, **74**(5):657-664.

15. Pollack M, Charache P, Nieman RE, Jett MP, Reimhardt JA, Hardy PH, Jr.: **Factors influencing colonisation and antibiotic-resistance patterns of gram-negative bacteria in hospital patients**. *Lancet* 1972, **2**(7779):668-671.

16. Rose HD, Schreier J: **The effect of hospitalization and antibiotic therapy on the gram-negative fecal flora**. *Am J Med Sci* 1968, **255**:228-236.

17. Flidel-Rimon O, Leibovitz E, Juster-Reicher A, Amitay M, Miskin A, Barak Y, Mogilner B: **An outbreak of antibiotic multiresistant Klebsiella at the Neonatal Intensive Care Unit, Kaplan Hospital, Rehovot, Israel, November 1991 to April 1992**. *Am J Perinatol* 1996, **13**(2):99-102.

18. French GL, Shannon KP, Simmons N: **Hospital outbreak of Klebsiella pneumoniae resistant to broad-spectrum cephalosporins and beta-lactam-beta-lactamase inhibitor combinations by hyperproduction of SHV-5 beta-lactamase**. *J Clin Microbiol* 1996, **34**(2):358-363.

19. Keynan Y, Rubinstein E: **The changing face of Klebsiella pneumoniae infections in the community**. *Int J Antimicrob Agents* 2007, **30**(5):385-389.

20. Medeiros AA: **Nosocomial outbreaks of multiresistant bacteria: extended-spectrum beta-lactamases have arrived in North America**. *Ann Intern Med* 1993, **119**(5):428-430.

21. Meyer KS, Urban C, Eagan JA, Berger BJ, Rahal JJ: **Nosocomial outbreak of Klebsiella infection resistant to late-generation cephalosporins**. *Ann Intern Med* 1993, **119**(5):353-358.

22. Colodner R, Raz R, Chazan B, Sakran W: **Susceptibility pattern of extended-spectrum beta-lactamase producing bacteria isolated from inpatients to five antimicrobial drugs in a community hospital in Northern Israel**. *Int J Antimicrob Agents* 2004, **24**(4):409-410.
23. Hirsch EB, Tam VH: **Detection and treatment options for Klebsiella pneumoniae carbapenemases (KPCs): an emerging cause of multidrug-resistant infection**. *J Antimicrob Chemother* 2010, **65**(6):1119-1125.
24. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan S *et al*: **Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study**. *Lancet Infect Dis* 2010, **10**(9):597-602.
25. Nordmann P, Cuzon G, Naas T: **The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria**. *Lancet Infect Dis* 2009, **9**(4):228-236.
26. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR: **Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India**. *Antimicrob Agents Chemother* 2009, **53**(12):5046-5054.
27. **[New Delhi-metallo-beta-lactamase 1 (NDM-1)-producing, multiple drug resistant Enterobacteriaceae]**. *The Japanese journal of antibiotics* 2010, **63**(5):365-367.

28. McDonald MI: **Pyogenic liver abscess: diagnosis, bacteriology and treatment**. *Eur J Clin Microbiol* 1984, **3**(6):506-509.
29. Lee CH, Leu HS, Wu TS, Su LH, Liu JW: **Risk factors for spontaneous rupture of liver abscess caused by Klebsiella pneumoniae**. *Diagn Microbiol Infect Dis* 2005, **52**(2):79-84.
30. Lindstrom ST, Healey PR, Chen SC: **Metastatic septic endophthalmitis complicating pyogenic liver abscess caused by Klebsiella pneumoniae**. *Aust N Z J Med* 1997, **27**(1):77-78.
31. Wang JH, Liu YC, Lee SS, Yen MY, Chen YS, Wang JH, Wann SR, Lin HH: **Primary liver abscess due to Klebsiella pneumoniae in Taiwan**. *Clin Infect Dis* 1998, **26**(6):1434-1438.
32. Chuang YP, Fang CT, Lai SY, Chang SC, Wang JT: **Genetic determinants of capsular serotype K1 of Klebsiella pneumoniae causing primary pyogenic liver abscess**. *J Infect Dis* 2006, **193**(5):645-654.
33. Evrard B, Balestrino D, Dosgilbert A, Bouya-Gachancard JL, Charbonnel N, Forestier C, Tridon A: **Roles of capsule and lipopolysaccharide O antigen in interactions of human monocyte-derived dendritic cells and Klebsiella pneumoniae**. *Infect Immun* 2010, **78**(1):210-219.
34. Flannagan RS, Cosio G, Grinstein S: **Antimicrobial mechanisms of phagocytes and bacterial evasion strategies**. *Nat Rev Microbiol* 2009, **7**(5):355-366.
35. Lin JC, Chang FY, Fung CP, Xu JZ, Cheng HP, Wang JJ, Huang LY, Siu LK: **High prevalence of phagocytic-resistant capsular**

serotypes of Klebsiella pneumoniae in liver abscess. *Microbes Infect* 2004, **6**(13):1191-1198.

36. Campos MA, Vargas MA, Regueiro V, Llompart CM, Alberti S, Bengoechea JA: **Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides**. *Infect Immun* 2004, **72**(12):7107-7114.
37. Llobet E, Tomas JM, Bengoechea JA: **Capsule polysaccharide is a bacterial decoy for antimicrobial peptides**. *Microbiology* 2008, **154**(Pt 12):3877-3886.
38. Mizuta K, Ohta M, Mori M, Hasegawa T, Nakashima I, Kato N: **Virulence for mice of Klebsiella strains belonging to the O1 group: relationship to their capsular (K) types**. *Infect Immun* 1983, **40**(1):56-61.
39. Lee HC, Chuang YC, Yu WL, Lee NY, Chang CM, Ko NY, Wang LR, Ko WC: **Clinical implications of hypermucoviscosity phenotype in Klebsiella pneumoniae isolates: association with invasive syndrome in patients with community-acquired bacteraemia**. *J Intern Med* 2006, **259**(6):606-614.
40. Merino S, Camprubi S, Alberti S, Benedi VJ, Tomas JM: **Mechanisms of Klebsiella pneumoniae resistance to complement-mediated killing**. *Infect Immun* 1992, **60**(6):2529-2535.
41. Shankar-Sinha S, Valencia GA, Janes BK, Rosenberg JK, Whitfield C, Bender RA, Standiford TJ, Younger JG: **The Klebsiella pneumoniae O antigen contributes to bacteremia and lethality during murine pneumonia**. *Infection and immunity* 2004, **72**(3):1423-1430.

42. Medzhitov R, Janeway C, Jr.: **Innate immune recognition: mechanisms and pathways**. *Immunological reviews* 2000, **173**:89-97.
43. Stahlhut SG, Tchesnokova V, Struve C, Weissman SJ, Chattopadhyay S, Yakovenko O, Aprikian P, Sokurenko EV, Krogfelt KA: **Comparative structure-function analysis of mannose-specific FimH adhesins from Klebsiella pneumoniae and Escherichia coli**. *J Bacteriol* 2009, **191**(21):6592-6601.
44. Struve C, Bojer M, Krogfelt KA: **Characterization of Klebsiella pneumoniae type 1 fimbriae by detection of phase variation during colonization and infection and impact on virulence**. *Infect Immun* 2008, **76**(9):4055-4065.
45. Struve C, Bojer M, Krogfelt KA: **Identification of a conserved chromosomal region encoding Klebsiella pneumoniae type 1 and type 3 fimbriae and assessment of the role of fimbriae in pathogenicity**. *Infect Immun* 2009, **77**(11):5016-5024.
46. Huang YJ, Liao HW, Wu CC, Peng HL: **MrkF is a component of type 3 fimbriae in Klebsiella pneumoniae**. *Res Microbiol* 2009, **160**(1):71-79.
47. Langstraat J, Bohse M, Clegg S: **Type 3 fimbrial shaft (MrkA) of Klebsiella pneumoniae, but not the fimbrial adhesin (MrkD), facilitates biofilm formation**. *Infect Immun* 2001, **69**(9):5805-5812.
48. Stahlhut SG, Chattopadhyay S, Struve C, Weissman SJ, Aprikian P, Libby SJ, Fang FC, Krogfelt KA, Sokurenko EV: **Population variability of the FimH type 1 fimbrial adhesin in Klebsiella pneumoniae**. *J Bacteriol* 2009, **191**(6):1941-1950.

49. Di Martino P, Bertin Y, Girardeau JP, Livrelli V, Joly B, Darfeuille-Michaud A: **Molecular characterization and adhesive properties of CF29K, an adhesin of Klebsiella pneumoniae strains involved in nosocomial infections**. *Infect Immun* 1995, **63**(11):4336-4344.

50. Di Martino P, Livrelli V, Sirot D, Joly B, Darfeuille-Michaud A: **A new fimbrial antigen harbored by CAZ-5/SHV-4-producing Klebsiella pneumoniae strains involved in nosocomial infections**. *Infect Immun* 1996, **64**(6):2266-2273.

51. Di Martino P, Sirot D, Joly B, Rich C, Darfeuille-Michaud A: **Relationship between adhesion to intestinal Caco-2 cells and multidrug resistance in Klebsiella pneumoniae clinical isolates**. *J Clin Microbiol* 1997, **35**(6):1499-1503.

52. Lawlor MS, Hsu J, Rick PD, Miller VL: **Identification of Klebsiella pneumoniae virulence determinants using an intranasal infection model**. *Molecular microbiology* 2005, **58**(4):1054-1073.

53. Clegg S, Purcell BK, Pruckler J: **Characterization of genes encoding type 1 fimbriae of Klebsiella pneumoniae, Salmonella typhimurium, and Serratia marcescens**. *Infection and immunity* 1987, **55**(2):281-287.

54. Gerlach GF, Clegg S, Allen BL: **Identification and characterization of the genes encoding the type 3 and type 1 fimbrial adhesins of Klebsiella pneumoniae**. *Journal of bacteriology* 1989, **171**(3):1262-1270.

55. Schembri MA, Blom J, Krogfelt KA, Klemm P: **Capsule and fimbria interaction in Klebsiella pneumoniae**. *Infection and immunity* 2005, **73**(8):4626-4633.

56. Ryan RP, Fouhy Y, Lucey JF, Crossman LC, Spiro S, He YW, Zhang LH, Heeb S, Camara M, Williams P *et al*: **Cell-cell signaling in Xanthomonas campestris involves an HD-GYP domain protein that functions in cyclic di-GMP turnover**. *Proceedings of the National Academy of Sciences of the United States of America* 2006, **103**(17):6712-6717.

57. Matatov R, Goldhar J, Skutelsky E, Sechter I, Perry R, Podschun R, Sahly H, Thankavel K, Abraham SN, Ofek I: **Inability of encapsulated Klebsiella pneumoniae to assemble functional type 1 fimbriae on their surface**. *FEMS microbiology letters* 1999, **179**(1):123-130.

58. Sahly H, Podschun R, Oelschlaeger TA, Greiwe M, Parolis H, Hasty D, Kekow J, Ullmann U, Ofek I, Sela S: **Capsule impedes adhesion to and invasion of epithelial cells by Klebsiella pneumoniae**. *Infection and immunity* 2000, **68**(12):6744-6749.

59. Boddicker JD, Anderson RA, Jagnow J, Clegg S: **Signature-tagged mutagenesis of Klebsiella pneumoniae to identify genes that influence biofilm formation on extracellular matrix material**. *Infection and immunity* 2006, **74**(8):4590-4597.

60. Burmolle M, Bahl MI, Jensen LB, Sorensen SJ, Hansen LH: **Type 3 fimbriae, encoded by the conjugative plasmid pOLA52, enhance biofilm formation and transfer frequencies in Enterobacteriaceae strains**. *Microbiology* 2008, **154**(Pt 1):187-195.

61. Di Martino P, Cafferini N, Joly B, Darfeuille-Michaud A: **Klebsiella pneumoniae type 3 pili facilitate adherence and biofilm**

formation on abiotic surfaces. *Research in microbiology* 2003, **154**(1):9-16.

62. Jagnow J, Clegg S: **Klebsiella pneumoniae MrkD-mediated biofilm formation on extracellular matrix- and collagen-coated surfaces**. *Microbiology* 2003, **149**(Pt 9):2397-2405.
63. Ong CL, Beatson SA, McEwan AG, Schembri MA: **Conjugative plasmid transfer and adhesion dynamics in an Escherichia coli biofilm**. *Applied and environmental microbiology* 2009, **75**(21):6783-6791.
64. Schroll C, Barken KB, Krogfelt KA, Struve C: **Role of type 1 and type 3 fimbriae in Klebsiella pneumoniae biofilm formation**. *BMC microbiology* 2010, **10**:179.
65. Sutherland I: **Biofilm exopolysaccharides: a strong and sticky framework**. *Microbiology* 2001, **147**(Pt 1):3-9.
66. Warren JW: **Catheter-associated urinary tract infections**. *International journal of antimicrobial agents* 2001, **17**(4):299-303.
67. Maki DG, Tambyah PA: **Engineering out the risk for infection with urinary catheters**. *Emerging infectious diseases* 2001, **7**(2):342-347.
68. Ronald A: **The etiology of urinary tract infection: traditional and emerging pathogens**. *Disease-a-month : DM* 2003, **49**(2):71-82.
69. Hornick DB, Allen BL, Horn MA, Clegg S: **Adherence to respiratory epithelia by recombinant Escherichia coli expressing Klebsiella pneumoniae type 3 fimbrial gene products**. *Infect Immun* 1992, **60**(4):1577-1588.

70. Huang YJ, Wu CC, Chen MC, Fung CP, Peng HL: **Characterization of the type 3 fimbriae with different MrkD adhesins: possible role of the MrkD containing an RGD motif**. *Biochemical and biophysical research communications* 2006, **350**(3):537-542.

71. Sebghati TA, Clegg S: **Construction and characterization of mutations within the Klebsiella mrkD1P gene that affect binding to collagen type V**. *Infection and immunity* 1999, **67**(4):1672-1676.

72. Tarkkanen AM, Allen BL, Westerlund B, Holthofer H, Kuusela P, Risteli L, Clegg S, Korhonen TK: **Type V collagen as the target for type-3 fimbriae, enterobacterial adherence organelles**. *Mol Microbiol* 1990, **4**(8):1353-1361.

73. Tarkkanen AM, Virkola R, Clegg S, Korhonen TK: **Binding of the type 3 fimbriae of Klebsiella pneumoniae to human endothelial and urinary bladder cells**. *Infect Immun* 1997, **65**(4):1546-1549.

74. Allen BL, Gerlach GF, Clegg S: **Nucleotide sequence and functions of mrk determinants necessary for expression of type 3 fimbriae in Klebsiella pneumoniae**. *J Bacteriol* 1991, **173**(2):916-920.

75. Duguid JP: **Fimbriae and adhesive properties in Klebsiella strains**. *J Gen Microbiol* 1959, **21**:271-286.

76. Castanie-Cornet MP, Treffandier H, Francez-Charlot A, Gutierrez C, Cam K: **The glutamate-dependent acid resistance system in Escherichia coli: essential and dual role of the His-Asp phosphorelay RcsCDB/AF**. *Microbiology* 2007, **153**(Pt 1):238-246.

77. Imlay JA: **Pathways of oxidative damage**. *Annu Rev Microbiol* 2003, **57**:395-418.
78. Galperin MY, Higdon R, Kolker E: **Interplay of heritage and habitat in the distribution of bacterial signal transduction systems**. *Mol Biosyst* 2010, **6**(4):721-728.
79. Perry J, Koteva K, Wright G: **Receptor domains of two-component signal transduction systems**. *Mol Biosyst* 2011, **7**(5):1388-1398.
80. Laub MT, Goulian M: **Specificity in two-component signal transduction pathways**. *Annu Rev Genet* 2007, **41**:121-145.
81. Majdalani N, Gottesman S: **The Rcs phosphorelay: a complex signal transduction system**. *Annu Rev Microbiol* 2005, **59**:379-405.
82. Virlogeux I, Waxin H, Ecobichon C, Lee JO, Popoff MY: **Characterization of the rcsA and rcsB genes from Salmonella typhi: rcsB through tviA is involved in regulation of Vi antigen synthesis**. *J Bacteriol* 1996, **178**(6):1691-1698.
83. McCallum KL, Whitfield C: **The rcsA gene of Klebsiella pneumoniae O1:K20 is involved in expression of the serotype-specific K (capsular) antigen**. *Infect Immun* 1991, **59**(2):494-502.
84. Bernhard F, Poetter K, Geider K, Coplin DL: **The rcsA gene from Erwinia amylovora: identification, nucleotide sequence, and regulation of exopolysaccharide biosynthesis**. *Mol Plant Microbe Interact* 1990, **3**(6):429-437.
85. Bereswill S, Geider K: **Characterization of the rcsB gene from Erwinia amylovora and its influence on exoploysaccharide synthesis and virulence of the fire blight pathogen**. *J Bacteriol* 1997, **179**(4):1354-1361.

86. Poetter K, Coplin DL: **Structural and functional analysis of the rcsA gene from Erwinia stewartii**. *Mol Gen Genet* 1991, **229**(1):155-160.
87. Stout V, Gottesman S: **RcsB and RcsC: a two-component regulator of capsule synthesis in Escherichia coli**. *J Bacteriol* 1990, **172**(2):659-669.
88. Carballes F, Bertrand C, Bouche JP, Cam K: **Regulation of Escherichia coli cell division genes ftsA and ftsZ by the two-component system rcsC-rcsB**. *Mol Microbiol* 1999, **34**(3):442-450.
89. Francez-Charlot A, Laugel B, Van Gemert A, Dubarry N, Wiorowski F, Castanie-Cornet MP, Gutierrez C, Cam K: **RcsCDB His-Asp phosphorelay system negatively regulates the flhDC operon in Escherichia coli**. *Mol Microbiol* 2003, **49**(3):823-832.
90. Schwan WR, Shibata S, Aizawa S, Wolfe AJ: **The two-component response regulator RcsB regulates type 1 piliation in Escherichia coli**. *J Bacteriol* 2007, **189**(19):7159-7163.
91. Cano DA, Dominguez-Bernal G, Tierrez A, Garcia-Del Portillo F, Casadesus J: **Regulation of capsule synthesis and cell motility in Salmonella enterica by the essential gene igaA**. *Genetics* 2002, **162**(4):1513-1523.
92. Garcia-Calderon CB, Casadesus J, Ramos-Morales F: **Rcs and PhoPQ regulatory overlap in the control of Salmonella enterica virulence**. *J Bacteriol* 2007, **189**(18):6635-6644.
93. Erickson KD, Detweiler CS: **The Rcs phosphorelay system is specific to enteric pathogens/commensals and activates ydeI, a**

gene important for persistent Salmonella infection of mice. *Mol Microbiol* 2006, **62**(3):883-894.

94. Wacharotayankun R, Arakawa Y, Ohta M, Hasegawa T, Mori M, Horii T, Kato N: **Involvement of rcsB in Klebsiella K2 capsule synthesis in Escherichia coli K-12**. *J Bacteriol* 1992, **174**(3):1063-1067.
95. Nassif X, Honore N, Vasselon T, Cole ST, Sansonetti PJ: **Positive control of colanic acid synthesis in Escherichia coli by rmpA and rmpB, two virulence-plasmid genes of Klebsiella pneumoniae**. *Mol Microbiol* 1989, **3**(10):1349-1359.
96. Nassif X, Fournier JM, Arondel J, Sansonetti PJ: **Mucoid phenotype of Klebsiella pneumoniae is a plasmid-encoded virulence factor**. *Infect Immun* 1989, **57**(2):546-552.
97. Jayaratne P, Keenleyside WJ, MacLachlan PR, Dodgson C, Whitfield C: **Characterization of rcsB and rcsC from Escherichia coli O9:K30:H12 and examination of the role of the rcs regulatory system in expression of group I capsular polysaccharides**. *J Bacteriol* 1993, **175**(17):5384-5394.
98. Chen MH, Takeda S, Yamada H, Ishii Y, Yamashino T, Mizuno T: **Characterization of the RcsC-->YojN-->RcsB phosphorelay signaling pathway involved in capsular synthesis in Escherichia coli**. *Biosci Biotechnol Biochem* 2001, **65**(10):2364-2367.
99. Latasa C, Garcia B, Echeverz M, Toledo-Arana A, Valle J, Campoy S, Garcia-del Portillo F, Solano C, Lasa I: **Salmonella biofilm development depends on the phosphorylation status of RcsB**. *J Bacteriol* 2012, **194**(14):3708-3722.

100. Wehland M, Kiecker C, Coplin DL, Kelm O, Saenger W, Bernhard F: **Identification of an RcsA/RcsB recognition motif in the promoters of exopolysaccharide biosynthetic operons from Erwinia amylovora and Pantoea stewartii subspecies stewartii**. *J Biol Chem* 1999, **274**(6):3300-3307.

101. Ebel W, Trempy JE: **Escherichia coli RcsA, a positive activator of colanic acid capsular polysaccharide synthesis, functions To activate its own expression**. *J Bacteriol* 1999, **181**(2):577-584.

102. Wehland M, Bernhard F: **The RcsAB box. Characterization of a new operator essential for the regulation of exopolysaccharide biosynthesis in enteric bacteria**. *J Biol Chem* 2000, **275**(10):7013-7020.

103. Pristovsek P, Sengupta K, Lohr F, Schafer B, von Trebra MW, Ruterjans H, Bernhard F: **Structural analysis of the DNA-binding domain of the Erwinia amylovora RcsB protein and its interaction with the RcsAB box**. *J Biol Chem* 2003, **278**(20):17752-17759.

104. Rahn A, Whitfield C: **Transcriptional organization and regulation of the Escherichia coli K30 group 1 capsule biosynthesis (cps) gene cluster**. *Mol Microbiol* 2003, **47**(4):1045-1060.

105. Russo TA, Singh G: **An extraintestinal, pathogenic isolate of Escherichia coli (O4/K54/H5) can produce a group 1 capsule which is divergently regulated from its constitutively produced group 2, K54 capsular polysaccharide**. *J Bacteriol* 1993, **175**(23):7617-7623.

106. Whitfield C, Roberts IS: **Structure, assembly and regulation of expression of capsules in Escherichia coli**. *Mol Microbiol* 1999, **31**(5):1307-1319.

107. Arricau N, Hermant D, Waxin H, Ecobichon C, Duffey PS, Popoff MY: **The RcsB-RcsC regulatory system of Salmonella typhi differentially modulates the expression of invasion proteins, flagellin and Vi antigen in response to osmolarity**. *Mol Microbiol* 1998, **29**(3):835-850.

108. Johnson MD, Burton NA, Gutierrez B, Painter K, Lund PA: **RcsB is required for inducible acid resistance in Escherichia coli and acts at gadE-dependent and -independent promoters**. *J Bacteriol* 2011, **193**(14):3653-3656.

109. Venkatesh GR, Kembou Koungni FC, Paukner A, Stratmann T, Blissenbach B, Schnetz K: **BglJ-RcsB heterodimers relieve repression of the Escherichia coli bgl operon by H-NS**. *J Bacteriol* 2010, **192**(24):6456-6464.

110. Cheng H-Y: **Regulation of Two-component Systems in the Polymyxin B Resistance and Capsular Polysaccharide Biosynthesis in Klebsiella pneumoniae CG43**. *PhD thesis.* Taiwan R.O.C.: National Chiao Tung University; 2010.

111. Wu C-C: **Prevalence Analysis and Expressional Control of Klebsiella pneumoniae Fimbriae**. *PhD thesis.* Taiwan R.O.C.: National Chiao Tung University; 2010.

112. Lin C-H: **Role of RcsB in acid-resistance in Klebsiella pneumoniae CG43**. *Master thesis.* Taiwan R.O.C.: National Chiao Tung University; 2009.

113. Fang CT, Chuang YP, Shun CT, Chang SC, Wang JT: **A novel virulence gene in Klebsiella pneumoniae strains causing primary liver abscess and septic metastatic complications**. *J Exp Med* 2004, **199**(5):697-705.
114. Lai YC, Peng HL, Chang HY: **RmpA2, an activator of capsule biosynthesis in Klebsiella pneumoniae CG43, regulates K2 cps gene expression at the transcriptional level**. *J Bacteriol* 2003, **185**(3):788-800.
115. Cheng HY, Chen YS, Wu CY, Chang HY, Lai YC, Peng HL: **RmpA regulation of capsular polysaccharide biosynthesis in Klebsiella pneumoniae CG43**. *J Bacteriol* 2010, **192**(12):3144-3158.
116. Gupte G, Woodward C, Stout V: **Isolation and characterization of rcsB mutations that affect colanic acid capsule synthesis in Escherichia coli K-12**. *J Bacteriol* 1997, **179**(13):4328-4335.
117. Stock AM, Robinson VL, Goudreau PN: **Two-component signal transduction**. *Annu Rev Biochem* 2000, **69**:183-215.
118. Volz K: **Structural conservation in the CheY superfamily**. *Biochemistry* 1993, **32**(44):11741-11753.
119. Lukat GS, Stock AM, Stock JB: **Divalent metal ion binding to the CheY protein and its significance to phosphotransfer in bacterial chemotaxis**. *Biochemistry* 1990, **29**(23):5436-5442.
120. Chang HY, Lee JH, Deng WL, Fu TF, Peng HL: **Virulence and outer membrane properties of a galU mutant of Klebsiella pneumoniae CG43**. *Microb Pathog* 1996, **20**(5):255-261.

121. Domenico P, Schwartz S, Cunha BA: **Reduction of capsular polysaccharide production in Klebsiella pneumoniae by sodium salicylate**. *Infect Immun* 1989, **57**(12):3778-3782.
122. Blumer C, Kleefeld A, Lehnen D, Heintz M, Dobrindt U, Nagy G, Michaelis K, Emody L, Polen T, Rachel R *et al*: **Regulation of type 1 fimbriae synthesis and biofilm formation by the transcriptional regulator LrhA of Escherichia coli**. *Microbiology* 2005, **151**(Pt 10):3287-3298.
123. Hall HK, Foster JW: **The role of fur in the acid tolerance response of Salmonella typhimurium is physiologically and genetically separable from its role in iron acquisition**. *J Bacteriol* 1996, **178**(19):5683-5691.
124. Lin CT, Huang TY, Liang WC, Peng HL: **Homologous response regulators KvgA, KvhA and KvhR regulate the synthesis of capsular polysaccharide in Klebsiella pneumoniae CG43 in a coordinated manner**. *J Biochem* 2006, **140**(3):429-438.
125. Miller JH: **Experiments in Molecular Genetics.** . *Cold Spring Harbor Laboratory Press* 1972.
126. Maris AE, Sawaya MR, Kaczor-Grzeskowiak M, Jarvis MR, Bearson SM, Kopka ML, Schroder I, Gunsalus RP, Dickerson RE: **Dimerization allows DNA target site recognition by the NarL response regulator**. *Nat Struct Biol* 2002, **9**(10):771-778.
127. Cheng HP, Walker GC: **Succinoglycan production by Rhizobium meliloti is regulated through the ExoS-ChvI two-component regulatory system**. *J Bacteriol* 1998, **180**(1):20-26.

128. Lukat GS, McCleary WR, Stock AM, Stock JB: **Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors**. *Proc Natl Acad Sci U S A* 1992, **89**(2):718-722.
129. Schroder I, Wolin CD, Cavicchioli R, Gunsalus RP: **Phosphorylation and dephosphorylation of the NarQ, NarX, and NarL proteins of the nitrate-dependent two-component regulatory system of Escherichia coli**. *J Bacteriol* 1994, **176**(16):4985-4992.
130. McCleary WR, Stock JB, Ninfa AJ: **Is acetyl phosphate a global signal in Escherichia coli?** *J Bacteriol* 1993, **175**(10):2793-2798.
131. Dahl MK, Msadek T, Kunst F, Rapoport G: **Mutational analysis of the Bacillus subtilis DegU regulator and its phosphorylation by the DegS protein kinase**. *J Bacteriol* 1991, **173**(8):2539-2547.
132. Ma S, Selvaraj U, Ohman DE, Quarless R, Hassett DJ, Wozniak DJ: **Phosphorylation-independent activity of the response regulators AlgB and AlgR in promoting alginate biosynthesis in mucoid Pseudomonas aeruginosa**. *J Bacteriol* 1998, **180**(4):956-968.
133. Gueriri I, Bay S, Dubrac S, Cyncynatus C, Msadek T: **The Pta-AckA pathway controlling acetyl phosphate levels and the phosphorylation state of the DegU orphan response regulator both play a role in regulating Listeria monocytogenes motility and chemotaxis**. *Mol Microbiol* 2008, **70**(6):1342-1357.
134. Klose KE, Weiss DS, Kustu S: **Glutamate at the site of phosphorylation of nitrogen-regulatory protein NTRC mimics aspartyl-phosphate and activates the protein**. *J Mol Biol* 1993, **232**(1):67-78.

135. Lan CY, Igo MM: **Differential expression of the OmpF and OmpC porin proteins in Escherichia coli K-12 depends upon the level of active OmpR**. *J Bacteriol* 1998, **180**(1):171-174.
136. Lauriano CM, Ghosh C, Correa NE, Klose KE: **The sodium-driven flagellar motor controls exopolysaccharide expression in Vibrio cholerae**. *J Bacteriol* 2004, **186**(15):4864-4874.
137. Gerlach GF, Allen BL, Clegg S: **Type 3 fimbriae among enterobacteria and the ability of spermidine to inhibit MR/K hemagglutination**. *Infect Immun* 1989, **57**(1):219-224.
138. Ong CL, Ulett GC, Mabbett AN, Beatson SA, Webb RI, Monaghan W, Nimmo GR, Looke DF, McEwan AG, Schembri MA: **Identification of type 3 fimbriae in uropathogenic Escherichia coli reveals a role in biofilm formation**. *J Bacteriol* 2008, **190**(3):1054-1063.
139. Hanson MS, J. Hempel, and C. C. Brinton, Jr.: **Purification of the Escherichia coli type 1 pilin and minor pilus protein and partial characteriaztion of the adhesin**. *J Bacteriol* 1988, **170**(3350-8).
140. Klemm P, and M. A. Schembri.: **Bacterial adhedins: function and structure.** *Int J Med Microbiol* 2000, **290**:27-35.
141. Klemm P, and K. A. Krogfelt: **Type 1 fimbriae of Escherichia coli**. *Boca Raton, FL: CRC Press* 1994:9-26.
142. Huang Y-J: **Characterization of type 3 fimbriae in Klebsiella pneumoniae**. *PhD thesis.* Taiwan R.O.C.: National Chiao Tung University; 2007.
143. Stahlhut SG, Struve C, Krogfelt KA, Reisner A: **Biofilm formation of Klebsiella pneumoniae on urethral catheters requires either**

type 1 or type 3 fimbriae. *FEMS Immunol Med Microbiol* 2012, **65**(2):350-359.

144. Cookson AL, Cooley WA, Woodward MJ: **The role of type 1 and curli fimbriae of Shiga toxin-producing Escherichia coli in adherence to abiotic surfaces**. *Int J Med Microbiol* 2002, **292**(3-4):195-205.

145. Olsen A, Arnqvist A, Hammar M, Normark S: **Environmental regulation of curli production in Escherichia coli**. *Infectious agents and disease* 1993, **2**(4):272-274.

146. Prigent-Combaret C, Brombacher E, Vidal O, Ambert A, Lejeune P, Landini P, Dorel C: **Complex regulatory network controls initial adhesion and biofilm formation in Escherichia coli via regulation of the csgD gene**. *Journal of bacteriology* 2001, **183**(24):7213-7223.

147. Romling U, Bian Z, Hammar M, Sierralta WD, Normark S: **Curli fibers are highly conserved between Salmonella typhimurium and Escherichia coli with respect to operon structure and regulation**. *Journal of bacteriology* 1998, **180**(3):722-731.

148. Cheng W-Y: **Regulatory roles of MrkH, MrkI, MrkJ and CsgD on the expression of type 1 and type 3 fimbriae of Klebsiella penumoniae CG43**. Hsinchu, Taiwan: National Chiao Tung Univerisity; 2011.

149. Ferrieres L, Thompson A, Clarke DJ: **Elevated levels of sigma S inhibit biofilm formation in Escherichia coli: a role for the Rcs phosphorelay**. *Microbiology* 2009, **155**(Pt 11):3544-3553.

150. Lee C-Y: **Structural and functional characterization of the regulator RcsB for capsule synthesis in Klebsiella pneumoniae**. *Master thesis.* Taiwan R.O.C.: National Tsing Hua University; 2011.