National Chiao Tung University

國立交通大學

生物科技學系

碩士論文

Comparative characterization of the ferrous iron transport systems FeoABC, SitABCD and EfeUOB in *Klebsiella pneumoniae* CG43

克雷白氏肺炎桿菌 CG43 中二價鐵螯合系統 FeoABC、SitABCD 和 EfeUOB 之特性探討與比較

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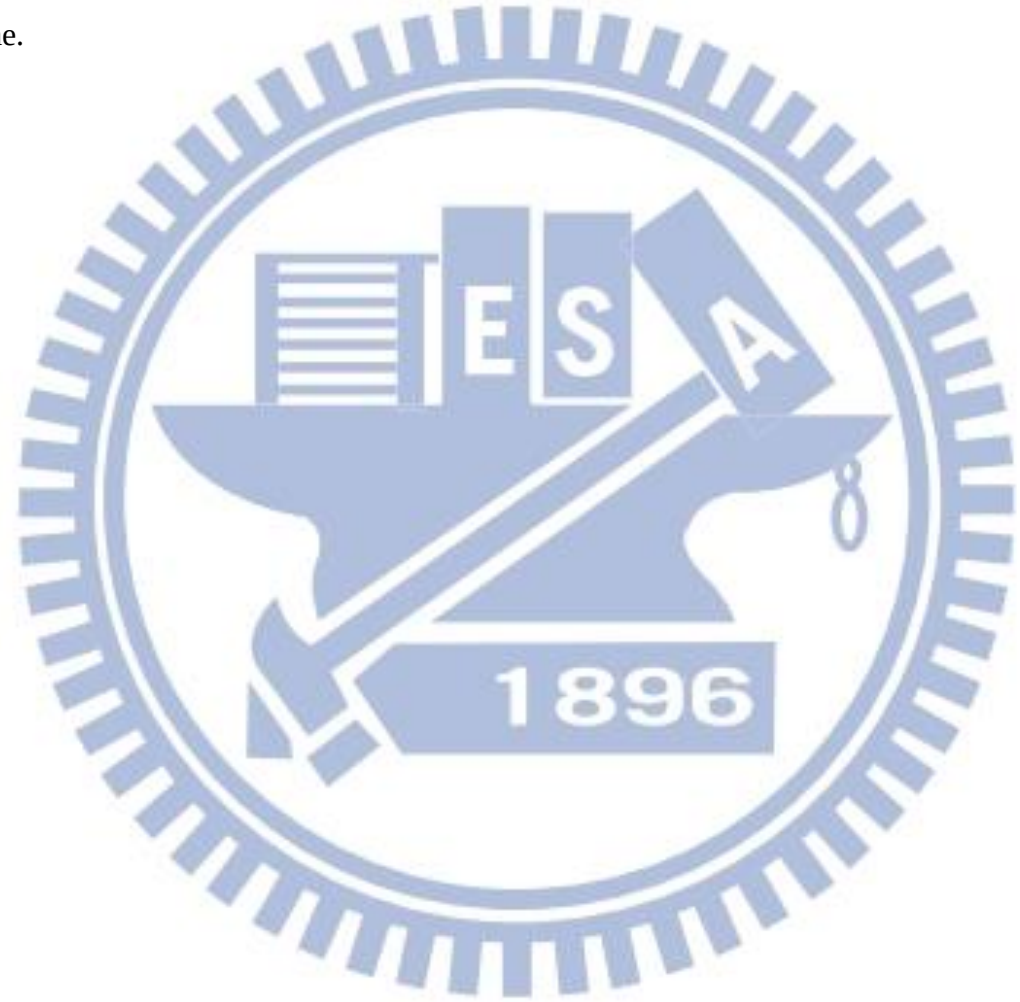
論文摘要

如同其他致病細菌，克雷白氏肺炎桿菌在宿主環境中需要克服缺鐵的考驗。克雷白氏肺炎桿菌的二價鐵的螯合系統基因組 *feoABC*、*sitABCD* 和 *efeUO*B 在含鐵量豐富的培養條件中，會受轉錄因子 Fur 抑制其表現。本研究以克雷白氏肺炎桿菌 CG43S3 為親本株分別建構了Δ*feoA*、Δ*feoC*、Δ*feoA*Δ*fur*、Δ*feoB*Δ*fur*、Δ*feoC*Δ*fur*、Δ*sitCD*、Δ*sitCD*Δ*fur*、Δ*efeUOB*、Δ*efeUOB*Δ*fur*、Δ*feoB*Δ*sitCD*、Δ*feoB*Δ*efeUOB*、Δ*sitCD*Δ*efeUOB*、Δ*efeUOB*Δ*sitCD*Δ*feoB* 和Δ*efeUOB*Δ*sitCD*Δ*feoB*Δfur 等基因缺損突變株，並比較分析這些基因缺損後對生長及致病性相關的表現型之影響；同時，這三套螯鐵系統預測的啟動子片段也被轉殖至啟動子報導質體 placZ15 中，再藉由 β-半乳糖苷酶（β-galactosidase）活性評估其基因表現。結果顯示這三套螯鐵系統的啟動子活性皆可因 *fur* 基因缺損或缺鐵環境所誘導；在缺鐵、微氧條件下，*feo* 的啟動子活性會被 FeoC 負向調控；而 *sitABCD* 活性最高。SitABCD 系統能幫助細菌對抗氧化壓力；而 *sit* 及 *feo* 基因都剔除結果會使細菌的抗氧化能力明顯下降；EfeUOB 表現可受酸性誘導，並被雙分子訊息系統的反應蛋白 CpxR 負向調控，此結果顯示 EfeUOB 可能在有氧、缺鐵及弱酸的特定環境中扮演重要角色。莢膜多醣體的生成與鐵的多寡有關，然而 FeoABC、SitABCD 或 EfeUOB 基因剔除並不影響克雷白氏肺炎桿菌 CG43 的莢膜多醣體產生；而除了Δ*sitCD*Δ*efeU* 和Δ*feoC* 之外，其他的基因缺損株都會降低生物膜的生成，在添加了三價鐵螯合劑 Deferoxamine 後，這些缺損株的生物膜減少更為顯著；有趣的是，受鐵濃度影響表現、而且是決定生物膜生成的主要因子第三型線毛，其主要單位蛋白 MrkA 並沒有受到這些基因缺損的影響，這結果暗示這些螯鐵系統並不是透過影響 MrkA 表現來影響生物膜的生成。

Thesis Abstract

As the other pathogenic bacteria, *Klebsiella pneumoniae* often encounters the challenge of iron depletion surroundings in the host cells. The iron transport system encoding operon *feoABC*, *sitABCD*, and *efeUOB* are present in the genome of *K. pneumoniae* CG43. Under iron rich cultured condition such as LB medium, the expression of *feoABC*, *efeUOB* and *sitABCD* are repressed by the global regulator Fur (ferric uptake regulator). In the study, Δ*feoA*Δ*fur*, Δ*feoB*Δ*fur*, Δ*feoC*Δ*fur*, Δ*sitCD*, Δ*sitCD*Δ*fur*, Δ*efeUOB*, Δ*efeUOB*Δ*fur*, Δ*feoB*Δ*sitCD*, Δ*feoB*Δ*efeUOB*, Δ*sitCD*Δ*efeUOB*, Δ*efeoUOB*Δ*sitCD*Δ*feoB* and Δ*efeoUOB*Δ*sitCD*Δ*feoB*Δ*fur* derived from *K. pneumoniae* CG43S3 have been generated. The mutation effects on the bacterial growth and virulence-related properties such as capsular polysaccharide (CPS) production, oxidative stress responses, type 3 fimbriae expression, and biofilm formation were analyzed and compared. The putative promoter of the three iron transport systems were isolated and individually cloned in front of the promoterless *lacZ* on the reporter plasmid pLacZ15 for the optimal expression and condition analysis using β-galactosidase activity. All three iron transport systems could be induced by the absence of Fur repressor and depleting iron from the culture medium. The *feo* promoter was negatively influenced under iron-depleted and microaerobic condition by FeoC, a predicted Fe-S sensor regulator. The microaerobically-induced *sitABCD* showed the highest promoter activity among these three systems. SitABCD plays important role in protecting bacteria from oxidative stress, with combined loss of *sit* and *feo* further decreased bacterial oxidative-resistance. EfeUOB is induced by weak acid and negatively regulated by the two-component regulator CpxR. This indicates its specific role in aerobic, iron-depleted and low pH environment. Although iron-availability affects CPS biosynthesis, FeoABC, SitABCD and EfeUOB transport systems do not involved in regulation of CPS biosynthesis. Except Δ*sitCD*Δ*efeUOB* and Δ*feoC*, deletion of genes from these acquisition

systems decreased biofilm formation of *K. pneumoniae* CG43S3, wherein iron chelators deferoxamine exerted more decreasing effects of biofilm formation. Interestingly, these deletion mutants did not decrease the expression of MrkA, the major pilin of type3 fimbriae which is iron dependent and the major determinant of biofilm formation. These results imply that these ferrous iron transport systems regulate biofilm formation independently of the expression of type 3 fimbriae.



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時間過得很快，還來不及將路上的風景看個盡興，就到達目的地。不知不覺，兩年的光陰過去，兩年的碩士班生涯也終告一段落。要我為這兩年的生活做一個總結，那就是計劃永遠趕不上變化：從一開始尋找指導教授、研究題目、說不盡的生活點滴、到畢業口試的口試委員，結果永遠不是開始的那個A計劃，也因為如此，這兩年有如坐雲霄飛車的般的生活非常充實。2012 年夏末，匆匆交接工作上的事項，隻身從屬於熱帶國家的馬來西亞飛到有四季的台灣來深造，在身邊許多人的幫助下從不同的氣候、食物、文化以及習慣中學習和適應，當中的感謝之情無法在短短的一文中傾訴。

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感謝從一開始一步一步教導我這什麼都不懂的新手、溫柔體貼的靜柔學姐，使我的實驗技術越來越熟練，在許多 try and error 中學習得更深入。學姐在我碰到問題總會放下手上的事情來協助我，囑咐我在實驗上要注意的小細節；在冬天的時候體貼我怕冷的體質

送我一條小毯子保暖，回想起來總有暖暖的感動。謝謝哲充學長在我碩班兩年來無數的幫助，實驗上有什麼疑難雜症，學長都有不一樣的解決方案，揮走我頭上的烏雲，為實驗重新找到光明。感謝同樣來自馬來西亞、外表內涵皆優的正妹燕曦，因為你的相助，我才有緣和你們認識並加入彭家的家庭。雖然冠男總是'嫉妒'我們倆同聲同氣，可是我們在很多想法和興趣上總是不謀而合，和你的幾次長談讓我對你的認識更深一層，可惜之後我們都各忙各的，希望還有機會和你一起 hang out！記得第一天加入彭家實驗室時，趕上了中秋節的烤肉活動，坐我旁邊、一直為不小心將烤肉用的用具戳到我而道歉的蕙瑜，讓我見識到原來還有人比我更迷糊、更粗神經。蕙瑜永遠有一股認真和拼命三郎的狠勁，有不到黃河心不死的決心，生活上不拘小節，力氣大得驚人，只要她用力一扭，瓶瓶罐罐沒有打不開的。我們兩個像機車衝鋒隊一樣，只要當下有興致就借機車往外衝，鬱悶的時候去散散心、開心的時候去逛逛，非常開心有一個陪我瘋的好夥伴；在我搞不清一些生活細節時，感謝你幫了我許多，你應該都不記得了，可我會一直記得的^_^ 希望你保持這積極樂觀的態度，繼續用你賢惠的一面和笑聲感染身邊的人吧！同在那次的中秋烤肉活動中，熱情的冠男手法嫻熟地為大家烤肉，親切的態度讓初來報到的我很容易融入新環境；實驗上有很多小撇步讓我的實驗進行得比較順利，至今我還記得 WesternBlot 的黑膠概念 XD 非常懷念那些空閒時我們聊得不亦樂乎的日子，希望你的補習中心越做越旺，成了彭董後要回來關照一下我們哦～在休息室和酷酷的子祥做了鄰居一年多，對你一開始冷酷的印象到後來的貓控熱血青年是怎麼一回事呢？你讓我見識到交大學生厲害之處：運動好、頭腦好、還多才多藝，質優生就是如此吧！在碩班的第一年，和同為生科系的珍儀林一起修課，幾乎每一餐都在可愛老闆的素食店用餐，還同寢了一學期，和活潑開朗的你一起時，我們會互相鼓勵，給對方打氣，總覺得自己充滿正能量。在很多人對我都只有內向安靜的印象時，

你卻觀察到我另一面，且不吝給我讚美，實在太讓我感動和開心，被了解的感覺太棒了！修課最多、進度最快、腿最長的你發展得也最快，希望你在工作上繼續勇往直前，再創佳績～和珍儀同姓又同月同日生日的小博士偉豐林，關於你的傳說已經很多了，簡短的介紹就是彭家花園的小園丁兼外交官、大潤發忠實顧客、PTT 常駐鄉民、無論大小事都略懂的豐哥，非常幸運能認識你。身為我們這屆碩班四位研究生中唯一的男生，在我們的話題圍繞著女生話題時還能和我們聊的很開心，對大家的要求都不會拒絕的好好先生，從你身上學習到很多知識和常識，感謝你聆聽我面對瓶頸時的訴苦，互相討論結果後都有茅塞頓開的 feel, 期待你接下來的發光發熱！碩班這兩年有你們三人的陪伴一起成長，是我畢生都會回味無窮的日子，謝謝你們，別忘了保持聯絡喔！

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知我在忙碌的時候忘了和你們聯絡，可是我總會想起你們，讓我恢復元氣。最後感謝我最親愛的家人，感謝無條件給予寬容和支持的爸爸媽媽，從來不求我有什麼大成就，只盼我過得平安喜樂就好，也讓我無後顧之憂地追逐我的夢想！還要感謝我的弟妹們，在你們面前我可以毫無保留地表達自己的喜怒哀樂，這些年有你們的陪伴太幸福了！感身邊的一切人、事與物，不管好壞，都讓我的視野和心境變得更寬闊更豁達！感恩！

俐君　謹致於

交通大學生物科技學系　　中華明國一零三年八月

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Abbreviations

BCIP	5-bromo-4-chloro-3-indolylphosphate
CPS	capsular polysaccharide
DIP	2‘2’-dipyridyl
DFX	deferoxamine mesylate salt
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetic acid
Fe	iron
Fe(II)	ferrous iron
Fe(III)	ferric iron
IPTG	isopropyl-1-thio-β-D-galactopyranoside
kb	kilobase(s)
KCl	potassium chloride
kDa	kilodalton(s)
LB	Luria-Bertani
$MgSO_4$	magnesium sulfate
Mn	manganese
$MnCl_2$	manganese chloride
Na_2CO_3	sodium carbonate
Na_2HPO_4	sodium phosphate dibasic
NaH_2PO_4	sodium phosphate monobasic
NBT	nitro blue tetrazolium chloride

ONPG	ortho-nitrophenyl-β-D-galactopyranoside
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PVDF	polyvinylidene difluoride
qRT-PCR	quantitative reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
sRNA	small RNA
X-gal	5-bromo-4-chloro-3-inodolyl-β-D-galactopyranoside

1.0 Introduction

1.1. Iron is essential for bacterial growth and virulence

Iron is needed for many important cellular functions and processes, such as the transport and storage of oxygen and as catalyst of many enzymes. Enzymes that utilize iron are involved in many major biochemical processes such as photosynthesis, N_2 fixation, respiration, methanogenesis, trichloroacetic acid (TCA) cycle, gene regulation and DNA biosynthesis [1]. These enzymes include ribotide reductase, nitrogenase, peroxidase, catalase, cytochromes, and succinic dehydrogenase [2]. Iron biological function is depends on its incorporation with proteins, either as a mono- or binuclear or as a part of iron-sulfur clusters [3] or heme [1, 4].

In addition, iron is also an important factor in cellular events such as virulence, biofilm formation and quorum sensing [4-7]. Low iron in the environment can trigger the pathogens to induce virulence genes such as toxins or adhesins, although their activities are not linked to iron. For instance, *Pseudomonas aeruginos*a produces an extracellular toxin that inhibits protein synthesis in eukaryotic cells [8]. A number of studies have shown higher resistance to infection in animals in which level of iron in serum have been reduced by an iron-deficient diet or iron-deficient mutants [2, 9-13]. The role of iron in many system indicates that virtually all living organisms require iron to survive and grow.

Iron is present in either an oxidized ferric [Fe(III)] or a reduced ferrous [Fe(II)] form. For most microorganism, iron is not easily available under aerobic conditions, due to the poor solubility of the oxidized Fe(III) (as low as 10^{-18} M at pH 7.0 [1]). For this reason, environmental microorganisms living in aerobic life-styles are often confronted with the shortages of bioavailability of iron. For pathogens, the iron-restriction problem is even more extreme since the

host limits iron availability by holding it within intracellular proteins. Most intracellular iron is found as hemoglobin, heme, ferritin, and hemosiderin. While the extracellular trace amount of iron is bound by the high affinity iron binding glycoproteins transferrin and lactoferrin [2, 14].

While reduced Fe(II) is more favored by bacteria in term of solubility, it can be hazardous to bacteria because Fe(II) participates in Fenton/Haber-Weiss reaction that lead to formation of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the highly destructive hydroxyl radical (•OH), which in turn causing harmful effects on fatty acids and other biological macromolecules [4, 15, 16].

1.2. Regulation of iron homeostasis in bacteria

1.2.1 Fur

To balance these dual aspects, bacteria must tightly regulate the cytoplasmic iron concentration, which is primarily mediated by the key regulator ferric uptake regulator (Fur) [1, 17, 18]. Under iron replete conditions, by using Fe(II) as a co-repressor, the Fe(II)-associated Fur functions as a transcriptional repressor of iron uptake-related genes by binding to its specific 19-bp consensus DNA sequences, called Fur box (GATAATGATwATCATTATC, w=A or T) [17], on the target promoters. The binding of Fur at the promoters impedes the binding of RNA polymerase thereby preventing transcription from these genes. We previously demonstrated several iron acquisition systems including *iro*, *iuc*, *sit* and *feo* were induced by *fur* deletion [19, 20].

On the other hand, the regulation by Fur is now known to be more complex, since Fe(II)-Fur can also activate genes in an indirect manner via the derepression of a small regulatory RNAs such as *ryhB, nrrF* and *fsrA* [4, 21-24] . In addition, regulation of Fur was revealed having

interesting link between redox stress management, acid tolerance management, adhesins factors, capsular polysaccharide (CPS) biosynthesis, some metabolic pathways with iron homeostasis [1, 17, 19, 20, 25, 26]. Finally, in some case, in the absence of iron, Fur functions as an activator on target gene promoter in *apo*-Fur form [4, 27].

1.2.2 RyhB

In *E. coli*, small RNAs (sRNA, non-coding RNA) is found involving in a variety of cellular functions such as a modulation of RNA polymerase activity (6S RNA) [28] protein tagging for degradation (SsrA and tmRNA) [29] and regulation of translation [30-33]. Some of these RNA use sequence specific RNA-RNA interaction to regulate mRNA synthesis. RyhB (also named as Sral) is a sRNA that initially discovered to be complementary to a portion of *sdhCDAB* operon encoding succinate dehydrogenase, affecting the cells growth in media containing succinate as a sole carbon source [34]. Later discovery explained this disability of growing in media lacks of specific carbon source is closely related to Fur.

The first step of sRNA RyhB mechanism of action initiated by pairing in an antisense manner with its mRNA targets. Then the pairing is further enhanced and stabled by the RNA chaperon protein Hfq [35, 36]. Following that, RyhB rapid degrades the sRNA with the mRNA target by RNase E, which is a part of complex called RNA degradasome [37, 38]. A double strand ribonuclease RNase III, is also involved in co-degradation of RyhB and its target mRNA [39]. Interestingly, the degradation leaves the promoter expression of the targeted genes intact while the mRNA is degraded [33]. This mechanism enables rapid shutdown of RyhB expression when condition returns to normal Recent discoveries suggested and revealed RyhB is involved in the positive regulation of Fur in certain genes [33, 40, 41]. Under iron-repletion, Fur exerts its

repression on *ryhB* transcription. However, in the absence of Fur or when iron becomes scarce in the cell, the repression of *ryhB* is relieved. RyhB targets only the non-essential genes for degradation [42, 43], enabling only essential proteins to have access to the free iron. The regulation is very critical during iron-deprivation, when Fur inactivation enables iron-acquisition genes and RyhB, which in turn reduces its usage to crucial proteins. Therefore, the interplay between Fur and RyhB balance the intracellular pool of available iron [44].

Previous study in *K. pneumoniae* CG43 suggested that RyhB plays a role downstream of Fur in the regulation of iron-acquisition systems and CPS biosynthesis. A qRT-PCR analysis implied that RyhB activates the expression of *iucA, fepA, fepB, entC, fecA* and *fecE,* but repress the expression of *fhuA* and *sitA* (Appendix 2). However, no apparent base pairing was found in the 5'-untranslated region of the *iuc* or *fec* operons a bioinformatics application (RNAhybrid) analysis showed no apparent base pairing was found, suggesting that the activation of *iucA* and *fecA* by RyhB is not a result of direct interaction. Yet, a possible pairing between RyhB with the adjacent sequence of translational start site of *sitA* was predicted [23].

1.3. Bacterial iron acquisition systems

Bacteria have evolved several iron uptake systems to both the ferric and ferrous form of elemental iron to meet their demands. In *Escherichia coli*, depending on the strain investigated, more than 10 iron-uptake routes known. In the presence of oxygen and at neutral pH environment, oxidized iron [Fe(III)] is more dominant, bacteria chelate iron by producing strong extracellular Fe(III) chelators, called siderophore. For Gram-negative bacteria, this transporter generally consisting of a Ton-ExbBD dependent outer-membrane receptor, an intermediate ferrisiderophore-binding protein presents in the periplasmic space and an inner-membrane ATP-binding cassette

(ABC) permease. Once ferrisiderophore enters the cytoplasm, it is either degraded by an esterase, or undergo a reducing process leading to the release of ferrous iron and allowing the *apo*-siderophore to be recycled and reused [1, 25].

In contrast, at reducing or anaerobic conditions, the equilibrium of iron shift to the soluble Fe(II) iron form. The first bacterial ferrous iron (Feo) transport system was discovered in *E. coli* K-12 [45]. This type of iron transport system is quite different from the siderophore-dependent systems, and so, this transport system is also characterized as TonExbBD-independent transport system. Another type of bacterial iron transporter is the metal type ABC transport system that have specificity for iron but not necessarily require outer-membrane receptors or siderophores. Such systems include SfuABC, SitABCD, YfeABCD, FbpABC, FutABC and EfeUOB of *Serratia marcescens*, *Salmonella typhimurium*, *Yersinia pestis*, *Neisseria gonorrhoeae*, *Synechocystis* PCC 6803 and *E. coli* [1, 46]. YfeABCD is part of the SitABCD family that regulate manganese and iron levels within the cell. The EfeUOB transport system is specific for ferrous iron transport, however, it only presents in certain pathogenic species [46-48].

1.3.1 The Feo transporter system

The Feo iron transport system is widespread among bacteria and thus appears to be a major route of ferrous iron acquisition [49, 50]. The *feo* operon in *K. pneumoniae* is constituted by three genes: *feoA*, *feoB* and *feoC* (also called as *yhgG*). In some *Acidithiobacilli*, it is preceded by a putative dedicated permease-encoding gene, *feoP*. FeoP is not contiguous with the *feoABC* cluster but its upstream predicted Fur box suggests its involvement in iron metabolism [51].

FeoA is a small Src-homology 3 (SH3)-like hydrophilic protein located in cytoplasm, predicted to act as a GTPase-activating protein (GAP) and/ or an Fe(II)-dependent repressor [49].

Recently, FeoA was shown to interact with FeoB in *S. enterica* [52] and is required for Fe(II) uptake in both *S. enterica* and *Vibrio cholera in vivo* [52, 53]. Further on, the ^{31}P nuclear magnetic resonance (NMR) study in *E. coli* proposed that FeoA may function by interacting with the highly conserved core region in the transmembrane domain of FeoB, instead of a GAP as suggested earlier [48]. An unpublished NMR structure of *K. pneumoniae* FeoA, which shares 90% sequence identity with *E. coli* FeoA protein, possess all secondary structure element same with the *E. coli* FeoA [48], suggesting they both may function similarly.

FeoB is thought to be the main Fe(II) transporter in the cytoplasmic membrane. It is a large protein containing a cytosolic N-terminal domain (NFeoB) that can divided into a Ras-like G domain and a helical S domain [54]. The G domain of NFeoB is thought to provide energy for the transport process or to regulate the transport by sensing the energy state of the cell and relays to the transmembrane [26, 48]. While the S domain may act as an open or close switch [55, 56]. The C-terminal region of FeoB is a helical transmembrane domain that likely form the porin functions as a Fe(II) permease [57, 58].

FeoC is a small, hydrophilic, winged-helix protein which is usually involved in DNA (sometime RNA) binding [49, 59]. In comparison with FeoA and FeoB, FeoC is not well conserved between specie and found only in γ-proteobacteria [49]. Multiple alignment of various bacteria, including *K. pneumoniae*, FeoC amino acid sequence shows that they possess four conserved cysteine residues with a consensus sequence $\underline{C}x_4\underline{C}xx\underline{C}x_{5\text{-}8}\underline{C}$, that likely provides binding sites for iron in the form of an iron-sulfur ([Fe-S]) cluster [49, 60]. Therefore, FeoC is suggested to act as a [Fe-S]-dependent transcriptional regulator, directly controlling *feo* expression. However, a study in *Y. pestis* suggested that FeoC did not regulate *feo* promoter [13] and no report has confirmed the DNA binding activity of FeoC.

Up until recently, two studies proposed that FeoC may function at post-translational level [53, 61]. In *S. enterica*, FeoC protein was showed contributing an increased FeoB protein level by protecting NFeoB from FtsH protease-mediated proteolysis [61]. Another evidence was discovered in *Vibrio cheolerae*, *feoC* encodes a protein that interacts with the cytoplasmic domain of FeoB, as determined using BACTH bacterial two-hybrid system [53]. Furthermore, structural study in *K. pneumoniae* showed FeoC protein binds to the NFeoB with high affinity and may coordinate the [4Fe-4S] cluster to regulate Fe(II) by modulating G protein activity [3, 55, 62].

Since the first description of the Feo system in *E. coli* [45], the importance of the Feo system has been confirmed by several bacterial systems. In both *E. coli* K12 and *Salmonella*, *feoB* mutant was reported to be deficient in uptake of ferrous iron and their colonization ability in mouse intestine [63, 64]. Besides, FeoB appears to be the main ferrous iron transporter and is required for *Helicobacter pylori* colonization of mouse gastric mucosa under Fe(II)-restricted conditions. FeoB is also required for the virulence of *Porphyromonas gingivalis* [55, 65]. Therefore, various studies have clearly established a role of the Feo system in bacterial colonization of the gut and in virulence. However, a deletion *feoB* mutant in *K. pneumoniae* CG43 was not attenuated in an 50% lethal dose (LD_{50}) assay [19].

Anaerobiosis has been shown to influence persistence and virulence of enteric pathogen such as *E. coli* [66, 67], *Salmonella* spp. [68-70], *V. cholera* [71, 72] and *Y. enterocolita* [73]. The anaerobiosis response regulators Fnr and ArcA are the primary redox regulators responsible for the activation or repression of genes associated with the transition to anaerobiosis. Fnr has been shown to stimulate transcription of *feoABC* in *E. coli* and *S. enterica* under anaerobic condition [61, 74]. As well in *Shigella* spp., both Fnr and ArcA induced promoter activity of *feo* anaerobically [75].

RstA/RstB is a member of the OmpR subfamily of two-component regulatory system, consisting of the membrane sensor RstB and its response regulator RstA [76]. A DNA microaaray analysis in *Salmonella* revealed that under iron-replete conditions, expression of RstA protein from a plasmid lowered transcription levels of various genes involved in iron acquisition, including *fhuACDEF*, *sitA*, *entAB*, *exbD*, *cirA* and *tonB* through Fur-Fe(II) protein. By contrast, the RstA protein activated transcription of the *feo* operon by binding directly to the *feoABC* promoter [77].

1.3.2 The Sit transporter system

Many microorganisms have secondary Fe(II) transporters whose primary function is the uptake of other divalent metals such as Mn(II), Mg(II) and Zn, but that can also import Fe(II) in certain circumstances. This include the Nramp-like transporter, such as MntH [78] and metal ABC permease such as SitABCD [79].

The *Salmonella* iron transporter SitABCD was first identified in *S. enterica* Serovar Typhimurium as a homolog of the *Y. pestis* YfeABCD transporter [80]. This transporter is thought to be involved in iron uptake, although its specificity between iron and manganese is not clear. SitABCD resembles a typical ABC transporter with a periplasmic binding protein (SitA), an ATP-binding protein (SitB), and two inner membrane permease (SitC and SitD). The location of *Salmonella sit* locus in the pathogenicity island SPI-1 might implicitly reveal its importance in virulence. Inactivation of the genes encoding for Sit transporter resulted in decreased virulence in murine experimental infection model [81].

A variety studies show combining mutations in the Sit and Feo system or/ and other metal transporter MntH resulted in completely avirulent or more sensitive to oxidative stress. Such

observation is found in avian pathogenic (APEC*) E. coli* χ7122 [82], *S. enterica* Serovar Typhimurium [11, 12], *S. flexneri* 2a [83] and *Y. pestis* [84].

Recently, the association of *sit* with virulence was also observed in *K. pneumoniae* NTUH-K2044 [85]. When the bacterial cells was cultured in a nutrient- and iron-limited mammalian cell culture condition (DMEM), SitA protein was found significantly increased. Similar to the forementioned observation, deletion of *sitA* showed higher sensitivity to the stress of peroxide oxidation. Additionally, virulence of *sitA* deletion mutant, comparing to the wild-type strain, was attenuated in a mouse intraperitoneal infection model [85].

In *S. enterica* Serovar Typhimurium, transcription of *sit* is regulated by Fur and MntR. Both Fur and MntR are metallo-regulatory proteins that respond primarily to Fe(II) and manganese (Mn) respectively [86]. The presence of Mn and Fe in medium caused transcriptional repression of the *sit* promoter. In contrast, increased expression of *sit* was observed under high oxygen conditions and higher cell densities. However, *sit* did not appear to be controlled by either redox regulator Fnr and ArcA or the alternative sigma factor, RpoS [87]. Hence, the increased expression affected by oxygen level and growth phase might be caused by decreased availability of metals required for repression by the metallo-regulatory proteins [87].

1.3.3 The Efe system

Many bacteria possess a particular ferrous iron transport system with functional similarities to the Fet3p/Ftr1p system in yeast, fungi and algae [88-91]. *E. coli* O157:H7 encodes an elemental ferrous iron (EfeUOB) transport system that was recently identified as a highly specific ferrous iron transport system [46, 92, 93].

Only a few experimental studies on the function of EfeUOB proteins have been reported. So the large part of understanding about *efeUOB* comes from the sequence analysis. The EfeU protein is a homologue protein of yeast iron permease Ftr1p with seven transmembrane helices, two of these helices, contain an conserved REXXE motif [47], which was initially suggested to be involved in ferrous iron transport. However, later studies suggested that Fe(II) is taken up upon Fe(II) oxidation by an oxidoreductase mechanism that possibly involves the periplasmic EfeO and EfeB protein [46, 94].

EfeO possess an N-terminal cupredoxin-type domain (Cup domain) and two distinct iron binding sites, suggesting that this protein might be involved in ferrous iron oxidation [94]. It was further suggested that ferric iron could be subsequently be transferred to the EfeU permease, and that the oxidized state of the Cup domain could be regenerated by electron transfer to the third component EfeB. EfeB is a periplasmic homodimeric heme belonged to dye-decolorizing peroxidase (DyP) protein family, which is secreted by the twin-arginine translocation (Tat) pathway [95, 96]. Recent study showed that this protein can extract iron from heme without breaking the tetrapyrrole ring [97].

The latest proposed mechanism for EfeUOB transport system was suggested by Marcus *et al.* [93] based on Gram-positive *Bacillus subtilis* bacterial model. Briefly, the binding protein EfeO and the permease EfeU form a minimal complex for ferric iron uptake. The third component EfeB is a hemoprotein that oxidizes ferrous iron to ferric iron for uptake by EfeUO. In conclusion, the EfeUOB system contributes to high-affinity uptake of iron that is available in both oxidized and reduced state. Furthermore, in *B. subtilis*, the EfeB protein is able to promote growth under microaerobic conditions where ferrous iron is more abundant. More importantly, EfeB can provide

cell envelope stress protection by eliminating reactive oxygen species that accumulate in the presence of ferrous iron [93].

In *E. coli*, transcription of *efeUOB* operon is known to be induced under iron-depleted, low pH or in the presence of exogenous copper [46]. The interesting finding is, the expression of *efeUOB* occurs under aerobic condition [46], wherein the amount of ferrous iron is relatively low. The transcriptional factor Fur and the two component system CpxAR are involved in the iron- and pH-dependent expression, respectively. The OmpR-like response regulator CpxR appears to preferentially bind and repress the *efeUOB* promoter in its deactivated (de-phosphorylated) form. However, in respond to low pH, the sensor kinase CpxA phosphorylates CpxR and causes derepression of *efeUOB*.

1.4. *K. pneumoniae* and the related studies

K. pneumoniae is a Gram-negative, non-motile, facultative anaerobic, heavily-encapsulated rod-shaped bacterium of the family Enterobacteriaceae. Its complex acidic polysaccharide capsule makes it distinct from other Enterobacteria, which is a major virulence factor avoiding phagocytosis [98]. It is an opportunistic pathogen, causing community acquired and nosocomial infections, including septicemia, pneumonia, urinary tract infection, meningitis and purulent abscess at various body sites particularly in immunocompromised patients [99]. Since 1980s, reported case of pyogenic liver abscess patients infected by *K. pneumoniae* started to be increasing [100, 101]. In Asian countries, especially Taiwan and Korea, *K. pneumoniae* is the predominant pathogen found in pyogenic liver abscess in diabetes patients [102-104]. Other than capsular antigen, lipopolysaccharide (LPS), adhesion, iron-acquisition system and serum resistance factors are also main virulence factors involved in *K. pneumoniae* infection [105].

K. pneumoniae CG43 used in this study is a clinical isolate belonged to K2 serotype with a LD_{50} of 10 CFU for laboratory mice [106, 107], isolated from diabetes mellitus patients of Chang Gung Memorial Hospital. Highly mucoid phenotype of *K. pneumoniae* CG43 has been correlated with the large virulence plasmid pLVPK [108]. The *rmpA2* on pLVPK was shown to act as a transcriptional activator for the *cps* gene cluster expression, by binding to the putative promoters, P_{orf1-2} and $P_{orf3-15}$ [109]. Interestingly, recent study revealed Fur represses the expression of *rmpA2* in an Fe(II)-dependent manner [20], which implying the availability of iron affects CPS biosynthesis.

In addition to the CPS biosynthesis, expression of the major adhesion determinant type 3 fimbriae is also iron dependent. A previous study in *K. pneomonniae* CG43 showed that the major pilin of type 3 fimbriae MrkA and biofilm formation is depend on extracellular iron availability. MrkA expression and biofilm formation was reduced gradually by the increasing concentration of iron chelator 2,2'-dipyridyl (DIP) [26]. Most *K. pneumoniae* isolates expressed two types of fimbriae adhesins, type 1 and type 3 fimbriae [105]. Type 1 fimbria are virtually found in all *Enterobacteriaceae* members, they mediate adhesion to mannose-containing structure on host cells and their expression is phase variable, which is mediated by an invertible DNA element (*fim* switch) [110]. Type 3 fimbria are present in nearly all *K. pneumoniae* isolates and mediate adhesion to several cell types *in vitro* [111, 112]. However, the specific receptor for type 3 fimbriae has not been identified yet [113]. The adhesion filaments encoded by the *mrkABCDF* operon [114] in which MrkA and MrkF are the major and minor pilin protein, respectively [115, 116].

Iron affects biofilm formation in a variety of bacteria [117-120]. The role of type 3 fimbriae in biofilm of *K. pneumoniae* has been continually demonstrated [26, 113, 121-124]. Biofilm is recognized as surface-attached bacteria embedded in a self-produced matrix, composed mainly of

polysaccharide, but also containing proteins and nucleic acids [125]. Therefore, its strong coating provide protection to bacteria from hydrodynamic forces of urine flow, host defense and antibiotic [126]. The ability of bacteria to form biofilm on medical devices is believed to play a major role in development of nosocomial infections, including the *K. pneumoniae* caused catheter-associated urinary tract infections [126].

As analyzed by Lin *et al.* [20], homologues of ten genes encode for eight different iron-acquisition systems in *K. pneumoniae* NTUH-K2044 were identified in *K. pneumoniae* CG43 using Blast search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), as listed in Appendix 1. Increased expression of these genes in *fur* deletion background further revealed the negative regulation of Fur on them.

1.5 Specific aims

The main objective of this study is to characterize the individual role of the ferrous iron transport system FeoABC, SitABCD and EfeUOB in *K. pneumoniae* CG43. The deletion mutants of the presumed important ferrous iron acquisition genes *feoB, sitCD*, and *efeUOB* are generated. The phenotype of these bacterial mutants are determined by examining the gene deleting effects on the expression of iron-related virulence factors such as CPS, resistance to oxidative stress, biofilm, and type 3 fimbriae are analyzed. In addition, different combinations of the gene deletion of the three ferrous iron transporters are included to investigate their cumulative impact. Moreover, deletion *feoA* and *feoC* mutants are also generated for their regulation in *feo* system.

2.0 Materials and Methods

2. 1. Plasmid, primers, bacterial strains and growth conditions

The bacterial strains, plasmids and primers used and constructed in present study are describe in Table 1, Table 2 and Table 3 respectively. *K. pneumoniae* CG43 is clinical isolated strains from Chang Gung Memorial Hospital Linkou branch. *E. coli* and *K. pneumoniae* strains were generally cultured aerobically at 37 °C in Luria-Bertani (LB) broth or on M9 minimal medium supplemented with appropriate antibiotics. For static culture, 1: 20 dilution of overnight culture was added to fresh media and incubated for 20 hr. Otherwise indicated, the concentrations of antibiotics used include streptomycin (500 μg/ml), ampicillin (100 μg/ml), chloramphenicol (35 μg/ml), and kanamycin (25 μg/ml). The iron chelators added in media include deferoxamine mesylate salt (DFX, Sigma Aldrich), 2, 2'-dipyridy (DIP, Sigma Aldrich) and disodium salt dehydrate (EDTA, USB cooperation) with final concentration of 200 μM.

2. 2. DNA manipulation

Plasmids was purified by using High-Speed Mini kit (Geneaid). All DNA-modifying and –restriction enzymes was recommended by the manufacture (Fermentas). PCR amplifications were performed with Blend Taq DNA polymerase (TOYOBO) or Taq DNA polymerase (MDbio Inc), PCR products and DNA fragments were purified using the Gel/ PCR DNA Fragments Extraction it (Geneaid). The primers used in this study were synthesized by MDBio, Inc or Integrated DNA technology (IDT).

2. 3. Bioinformatics analysis

Homology search analysis and gene annotation were performed with the BLAST program provided by NCBI (http://www.ncbi.nlm.nih.gov) or vector NTI (Invitrogen Vector NTI^{M} advance). Promoter prediction was carried out by SofBerry provider (http://www.softberry.com).

2. 4. Construction of gene-deletion mutants

The deletion strains constructed in this study are listed in Table 1. All the strains were made by allelic exchanged. Approximately 1000-bp sequences flanking both sides of the deleted region *(feoA, feoC, sitCD,* and *efeUOB*) were PCR amplified with respective primer pairs (Table 3). The amplified DNA fragments were then cloned into plasmid pKAS46 [127], a suicide vector containing *rpsL*, which allows positive selection with streptomycin for loss of the vector, to generate an in frame deletion plasmid. The resulting plasmid was then mobilized to *K. pneumoniae* CG43S3, *K. pneumoniae* CG43S3 Δ*lacZ* [128], *K. pneumoniae* CG43S33 Δ*fur* [19] or *K. pneumoniae* CG43S3-derived strains, through conjugation from *E. coli* S17-1 *λpir*. The transconjugants, which carried constructed plasmid integrated in the chromosome via homologous recombination, were selected by ampicillin and kanamycin on minimal media (M9). Several of the colonies was grown in LB at 37 °C for 8 hours and then spread onto a LB plate containing streptomycin. The streptomycin-resistant and kanamycin-sensitive colonies were selected and verified by PCR and deletion of gene verified by PCR by designed primer sets described in Table 3.

2.5. Construction of a pLacZ reporter system

The putative promoter regions of *feoABC* (named as P_{feo1} and P_{feo2}), *sitABCD* (named as P_{sit}) and *efeUOB* (named as P_{efe}) were PCR amplified from CG43S3 by the designed primer pairs (Table 3) and subcloned into p*lacZ*15 [128] to fuse them with promoterless *lac*Z reporter system. The promoter-reporter plasmids placZ$_{feo1}$, placZ$_{feo2}$, placZ$_{sit}$, and placZ$_{efe}$, were individually mobilized into *K. pneumoniae* strains by conjugations from *E. coli* S17-1 λ*pir*.

2.6. Measurement of bacterial growth in iron depletion or repletion conditions

Cultures of the parental strains CG43S3, along with deletion mutant strains were grown overnight in LB. 1:200 of diluted overnight cultures were inoculated into LB or LB with iron-depleted or iron-replete conditions. Iron-depletion condition was created by adding DFX or DIP to a final concentration of 200 μM; iron-repletion condition was created by adding ferric sulfate [$(Fe)_2(SO_4)_3$] and ascorbic acid to a final concentration of 50 μM into LB broth. The cultures were incubated at 37 °C agitatedly and the optical density was recorded every hour as the absorbance at 600 nm (OD_{600}).

2.7. Measurement of promoter activity through β-galactosidase activity assay

β-galactosidase activity was determined according to the method of Miller [129]. In brief, overnight culture was diluted 1 : 20 in LB broth supplemented with appropriate antibiotic and incubated at 37 °C until it reached the lag phase (OD_{600} = 0.2), early (OD_{600} = 0.5-0.6) logarithmic, late logarithmic (OD_{600} = 0.8-0.9) or stationary growth phase (OD_{600} >1.0). 100 μl of the bacterial culture was added with 900 μl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol), 17 μl of 0.1% SDS and 35 μl chloroform, followed by

vigorous shaking and incubated for 10 min at 30 °C. Subsequently, 200 µl of 4 mg/ml *o*-nitrophenyl-β-galactopyranoside (ONPG) was added and mixture was mixed thoroughly with vortex for 3 s, then incubated at 30 °C until yellow color was apparent. Finally, the reaction was stopped by adding 500 µl of stop solution (1 M Na_2CO_3) and the absorbance the supernatant was measure at OD_{420}.The activity was expressed as Miller units in which one unit of β-galactosidase is defined as the hydrolysis of 1 nmol ONPG per min per mg or protein. Each sample was assayed in triplicate, and at least 3 independent experiments were conducted. The data shown were calculated from one representative experiment, and are presented as the means and standard deviations from triplicate samples.

2.8. Sedimentation test

The capsular polysaccharide biosynthesis was analyzed by sedimentation test. The bacterial strains were cultured overnight in LB broth at 37ºC and subjected to centrifugation at 4,000 x g for 5 min. Pictures of bacterial cells centrifuged by low speed sedimentation of were taken.

2.9. Biofilm formation assay

Overnight grown bacteria were diluted 1: 100 in LB broth supplemented with appropriate antibiotic and then inoculated 150 µl into each well of a 96-well microtiter dish (DPP), and propagated statically at 37°C for 24 hr. After removal of the bacteria, each well was washed by de-ionized water twice before added with150 µl of 1% (w/v) crystal violet and placing on an orbital shaker for 50 min at 60 rpm. After washing each well three times with de-ionized water, 200 µl

95% ethanol was added to solubilize the dye for 1 hr. The capability of biofilm formation was quantified by the absorbance at 595 nm (ELx800, BIO-TEK).

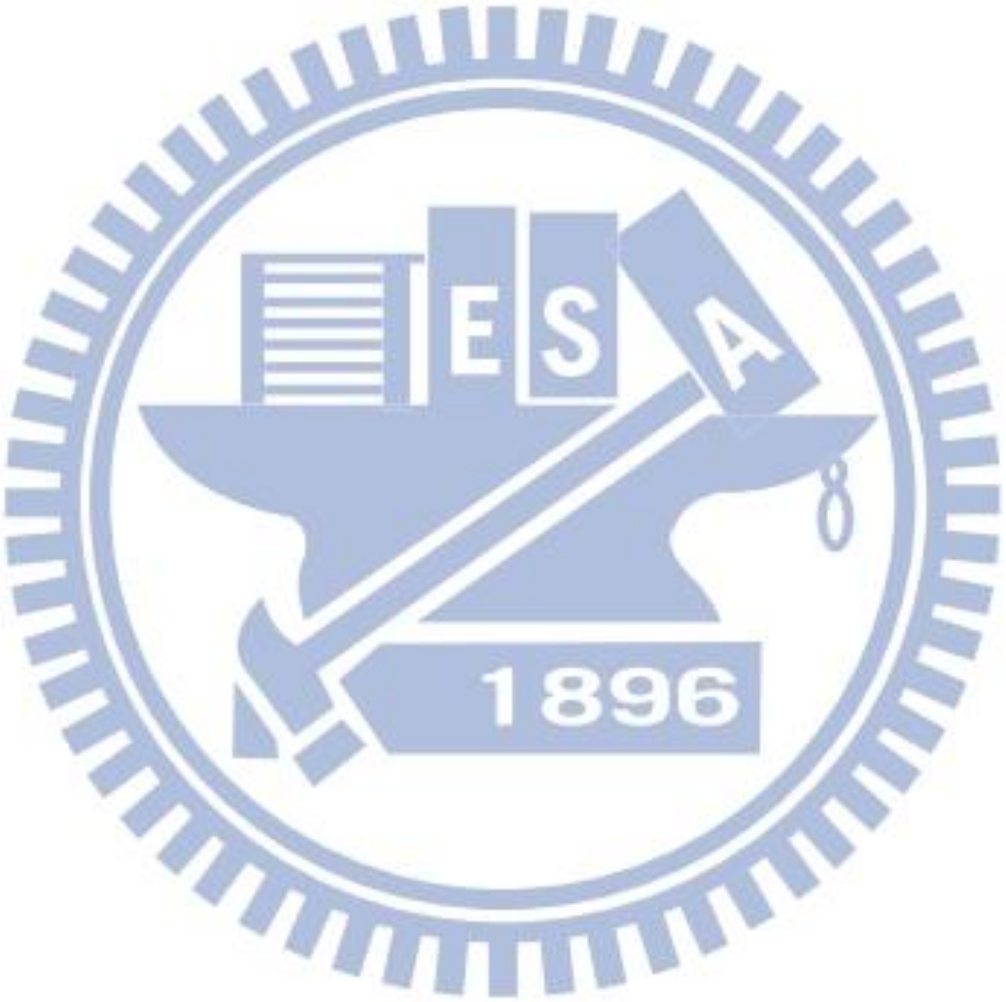
2.10. H_2O_2 sensitivity test

Sensitivity of deletion strains to H_2O_2 stress was determined by disk diffusion method on LB plate and LB plate added with 0.2 mM of different iron chelators (DFX, DIP or EDTA). Overnight cultures were diluted 1:20 to a fresh LB grown agitatedly to an OD_{600} of 0.6-0.7. The cultures were then supplemented with or without iron chelators for another 1 hr. Filter paper disks (6-mm diameter; Becton Dickinson) spotted with 5 µl hydrogen peroxide (30 %) were added onto the disks. The plates were then incubated at 37 °C for 16 hr. The diameters of inhibition zones were measured. The effect of iron chelators (deferoxamine, DIP and EDTA) was determined by its addition 1 hr prior to that H_2O_2.

2.11. Western blot analysis of the expression of type 3 fimbriae

Total cellular lysate from the bacteria were resolved by 12 % SDS-PAGE to determine the expression of type 3 fimbriae in CG43S3. The electrophoretic proteins were then transferred onto polyvinylidene difluoride (PVDF) membrane (Milipore, Billerica, MA, USA). After incubation with 5 % skimmed at 4 °C overnight, the membrane was washed three times with 1x Tris-buffered saline (TBS). Subsequently, the membrane was incubated at room temperature for 2 h with 25,000 fold diluted anti-MrkA serum. After washed three times with 1x TBS, a 5,000 fold diluted alkaline phosphate-conjugated immunoglobulin G (IgG) was added and the incubation continued for 1h. The bound antibodies were detected by chromogenic reagent BCIP (5-bromo-4-chloro-3-indolyl

phosphate), NBT (Nitro blue tetrazolium) and alkaline phosphatase butter (100mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl pH 9.5).



3.0 Results

3.1. Generation of the specific gene deletion mutants

The coding sequence of *feoA* (290-bp), *feoB* (2297-bp), and *feoC* (266-bp) were individually deleted by allelic exchange method, and the deletions were confirmed by PCR analysis. Besides Δ*feoA*, Δ*feoB*, and Δ*feoC*, the double mutation mutants including Δ*feoA*Δ*fur*, Δ*feoB*Δ*fur*, and Δ*feoC*Δ*fur*, were also obtained since Fur negatively regulates the iron uptake systems. As shown in Fig. 1A, the specific primer pairs are delFeoA-check(+)/delFeoA-check(-) for the deletion of *feoA*, delFeoB-check(+)/delFeoB-check(-) for *feoB* deletion, and delFeoC-check(+)/delFeoC-check(-) for *feoC* deletion, respectively. The expected sizes of the PCR products are respectively 590-bp for *feoA* deletion (Fig.1B), 386-bp for *feoB* deletion (Fig.1C), and 870-bp for *feoC* deletion (Fig. 1D).

Fig. 2 and 3 respectively show the position of the designed primer pairs delSitCD-check(+)/delSitCD-check(-) and delEfeUOB-check (+)/delEfeUOB-check (-), and PCR analysis employed to confirm the *sitCD* and *efeUOB* deletion. A total of 1437-bp *sitCD*- and 1640-bp *efeUOB*-containing DNA was deleted. The derived mutants are Δ*sitCD*Δ*fur*, Δ*sitCD*Δ*feoB (*Δ*SB)*, Δ*efeUOB*Δ*sitCD (*Δ*SE),* Δ*efeUOB*Δ*fur*, Δ*efeUOB*Δ*feoB (*Δ*BE)*, and Δ*efeUOB*Δ*sitCD*Δ*feoB* (Δ*ESB*). As shown in Fig. 2(B) and Fig. 3(B), the amplicon of 546-bp for *sitCD* deletion and 759-bp for *efeUOB* deletion were obtained.

3.2. Effect of iron availability on the growth of the specific gene deletion strains

To investigate the effect of iron availability on these iron-acquisition systems, the growth of CG43S3 along with Δ*feoA,* Δ*feoB,* Δ*feoC,* Δ*sitCD,* Δ*efeUOB*, Δ*sitCD*Δ*feoB (*Δ*SB)*, Δ*sitCD*Δ*efeUOB (*Δ*SE)*, Δ*efeUOB*Δ*feoB (*ΔBE*)* and Δ*efeUOB*Δ*sitCD*Δ*feoB* (ΔESB) mutant strains

in LB, iron-supplemented or iron-depleted medium was investigated. LB medium adding with 50 μM $Fe_2(SO_4)_3$ and 50 μM ascorbic acid is the iron-supplemented condition, while iron-depleted condition was created by adding 200 μM ferrous iron chelator DIP or 200 μM ferric iron chelator DFX. All the strains grown in LB medium reached OD_{600} of 1.2 in 6 h (Fig. 4A) and all appeared to grow faster when supplemented with iron (Fig. 4B). As shown in Fig. 4(C) and 4(D), the bacteria grown in medium containing DIP or DFX grew slower compared to the growth in LB or LB loaded with iron. Interestingly, the deletion mutants grew slightly slower than the parental strain CG43S3 only in the LB medium added with DIP. Otherwise, they exhibited similar growth pattern.

As shown in Fig. 5, the growth of double deletion strains Δ*feoA*Δ*fur*, Δ*feoB*Δ*fur*, Δ*feoC*Δ*fur*, Δ*sitCD*Δ*fur*, Δ*efeUOB*Δ*fur and* Δ*feoB*Δ*sitCD*Δ*efeUOB*Δ*fur* (ΔESBΔ*fur*) had similar growth pattern as Δ*fur* but grew slower than CG43S3 in LB medium. The Δ*fur*-derived strains reached OD_{600} of 0.25 while CG43S3 of 0.5 after 2 h growth.

3.3. Analysis of *feoABC, sitABCD* and *efeUOB* promoter region

As shown in Fig. 6(A), 6(B), and 6(C), a Fur box-like sequence could be identified in the putative promoter regions of *feoABC, sitCD* and *efeUOB.* Besides, other regulatory sequences predicted using Softberry are respectively RstA- and Fnr-box within P_{feo}, ArcA- and MntR-box in P_{sit}, and CpxR box in P_{efe}. The presence of Fnr-box in *feoABC* and ArcA-box in *sitABCD* promoter region suggests oxygen availability affects the expression of *feoABC and sitABCD.* However, whether Fnr regulates expression of *feoABC* remains unknown since the Fnr binding sequence overlaps with the Fur box in P_{feo}. The manganese repressor MntR binding box (AAACATAGCN_4GCTATGTTT) is located 81-bp upstream of the start codon of SitA. The

predicted CpxR box (GTAAAN$_{4-8}$GTAAA) identified in *efeUOB* promoter region implies a negative control by CpxR as reported in *E. coli* [46].

3.4. Effect of growth phase and oxygen availability on the expression of *feo*, *sit* and *efe*

To examine the promoter activity, the DNA fragments encompassing the putative promoter regions of *feoABC*, *sitABCD* and *efeUOB* were individually cloned in front of the promoterless *lac*Z to generate pP$_{feo1}$-*lac*Z, pP$_{feo2}$-*lac*Z, pP$_{sit}$-*lac*Z and pP$_{efe}$-*lac*Z.

As shown in Fig. 7A, the activities of P$_{feo}$, P$_{sit}$ and P$_{efe}$ gradually increased following their growth. The activity of P$_{feo}$, P$_{sit}$ and P$_{efe}$ in stationary phase showed approximately 1.5-fold higher than in late log phase. Among these three Fe(II) uptake systems, *sit* exhibits the highest expression, following by *efe* and *feo*. It is interesting to note that P$_{feo1}$ which contains two predicted -10 and -35 promoters had a much lower activity than P$_{feo2}$ implying the presence of repressor binding element in the region only contained by P$_{feo1}$.

Since Fnr and ArcA binding box are found respectively in promoter region of *feo* and *sit*, activity of P$_{feo}$, P$_{sit}$ and P$_{efe}$ was assessed under static condition (Fig. 7B). In compared to shaking condition, static condition did not apparently increase or decrease the activity of both P$_{feo1}$ and P$_{feo3}$. Interestingly, P$_{sit}$::*lac*Z expression under static culture showed as high as 2000 Miller units in compared with 1000 Miller unit observed under shaking culture (stationary phase). In contrast, P$_{efe}$::*lac*Z expression was decreased under the microaerobic condition, showing merely 250 Miller unit of activity. These results supported the possibility that oxygen availability may affect the transcriptional level of *sit and efe* in adverse manner.

3.5. Effect of *fur* deletion and iron-depletion on expression of *feo, sit,* and *efe*

The effect of *fur* deletion on the expression of the *feo, sit* and *efe* was further verified by promoter activity assay. As shown in Fig. 8A, the activity of P_{feo1}, $P_{feo2,}$ P_{sit} and P_{efe} were apparently induced in Z01Δ*fur* strain. In the meanwhile, P_{feo1}, $P_{feo2,}$ P_{sit} and P_{efe} activity were increased in response to addition of iron chelators (Fig. 8B). Interestingly, the inducible expression of *feo* was more effective by DFX than by DIP. On the other hand, both *sit* and *efe* promoters were more responsive to DIP than to DFX.

3.6. Effect of *feoC* deletion and *rstA* deletion and on expression of *feo, sit,* and *efe*

To investigate whether the two-component system response regulator RstA regulates *feo* expression, deletion effect of *rstA* on activity of P_{feo1} and P_{feo2} was examined. The result from left panel of Fig. 9(A) showed no apparent effect on P_{feo1} and P_{feo2} by *rstA* deletion. RstA has been shown to be involved in the acid stress response by regulating *asr* (acid shock RNA) expression in *Salmonella* [77], weak acid was treated to examine whether P_{feo} is also acid-inducible. However, as shown in right panel of Fig. 9A, both P_{feo1} and P_{feo2} activity were not affected by acid.

Feo system is the first ferrous iron transport system identified in *Enterobacteriae*, and FeoB is well known as the major ferrous iron transport system in many bacteria, but the role of FeoA and FeoC are not well studied thoroughly. FeoC was proposed to regulate Fe(II) uptake of FeoB by coordinating its iron-sulfur cluster [3, 55, 62]. In order to clarify the regulatory role of FeoC in *feo* expression, activities of P_{feo1} and P_{feo2} were studied in Z01Δ*feoC* strain under different environmental conditions (shaking, static, iron-depleted and iron-supplemented culture). As shown in Fig. 9B (right panel), the promoter activity of P_{feo1} and P_{feo2} was induced by *feoC* deletion

when bacterial strains were cultured in LB supplemented with DFX under static condition. By contrast, the increased activity was not observed under shaking condition (left panel).

3.7. Effect of manganese on expression of *feo, sit* and *efe*.

MntR is a manganese repressor using Mn^{2+} as a cofactor to regulate Mn^{2+}-related uptake systems. To investigate whether P_{sit} is negatively regulated by MntR, the culture of CG43S3Δ*lacZ* (Z01) was treated by 0.1mM $MnCl_2$ and the activity of P_{feo}, P_{sit} and P_{efe} were examined. Interestingly, no suppressing effect was observed in P_{feo}, P_{sit} or P_{efe}, instead, their activities were induced by manganese (Fig.10).

3.8. Effect of weak acid and *cpxR* deletion effect on the *efe* expression

In *E. coli* O157:H7, expression of *efeU* was low-pH induced while repressed by CpxAR at high pH. The CpxR box (GTAAAnnnnnnnnGTAAA) found in promoter region of *efeUOB* suggests a possible role of CpxR in regulation of *efeUOB* in CG43. To investigate if *efeUOB* expression in CG43 works similar with O157:H7, Z01 and Z01Δ*cpxAR* carrying P_{efe}-*lacZ* were grown in pH 7 or pH 5 LB media (Fig. 11). The expression of P_{efe} in Z01 at pH 5 was 4-fold higher than at pH 7. Deletion of *cpxAR* increased P_{efe}-lacZ expression under both pH 7 and pH 5 media, suggesting CpxR negatively regulates the expression of *efeUOB* under both acid and neutral pH conditions.

3.9. Analysis of cumulative effect of acid and iron-depletion or iron-supplemented on growth

From the result of promoter activity, acid and iron play a regulatory role on the expression of these specific iron-acquisition genes. Growth curve analysis of all the mutants cultured in pH 5

LB with additional Fe or Fe-depleted condition was performed. As shown in Fig. 12 (A) and (B), all growth curve of the mutant strains appeared similarly as the parental strain CG43S3 in pH 5 LB or with additional Fe. However, an apparent growth defect was observed when the mutants grown in pH 5 LB supplemented with DIP. As Fig. 12(C) shows the mutant strains displayed more acute slow-growing phenomenon of which the OD_{600} could only reach 0.8 at stationary phase. This may be explained by no sufficient ferrous iron Fe(II) for the bacterial growth. No growth change was observed when Fe(III) iron chelator DFX was added to the cultures (Fig. 12D).

3.10. Analysis of ferrous iron transporter regulation in CPS biosynthesis

The Δ*fur* mutant strain exhibited profound CPS biosynthesis and hence the bacterial culture was precipitated much slower than CG43S3 [19]. The Fe(II)-associated Fur metalloprotein requires iron as cofactor to regulate expression of various genes. To investigate whether Feo, Sit or Efe system acts as the major source of Fe(II) for Fur, CPS production of the strains were analyzed by subjecting the overnight-grown bacteria to low-speed centrifugation. As shown in Fig. 13(A), the CPS formed by the mutant strains showed no apparent change compared to that of the parental strain CG43S3 and CG43S3Δ*fur*, respectively. Fig. 13(B) shows that the mutant strains grown in LB with DFX displayed more viscous phenotype than those grown in LB media and hence could not be easily precipitated down. On the other hand, the addition of DIP resulted in poor growth of all the bacteria and the bacteria could be readily precipitated (Fig. 15C). Nevertheless, no apparent of change of the phenotype in comparing with that of the parental strain CG43S3 suggesting FeoABC, SitABCD or EfeUOB transport systems does not directly influence the CPS biosynthesis.

3.11. Analysis of deletion effects on oxidative stress response

Fe(II) could be hazardous to bacteria because of the Fenton reaction leading to ROS formation. As reported in many other bacteria, iron transport systems play a role in oxidative stress responses [12, 83, 85]. To investigate if any of the three iron transport systems is also involved, disk diffusion assay was employed with the disc immersed with 30% hydrogen peroxide (H_2O_2). The effect of iron chelators (DFX, DIP and EDTA) on the oxidative stress response was also tested.

By measuring the diameters of inhibition zones of the bacteria grown agitatedly in LB, Δ*sitCD*Δ*efeUOB (*ΔSE), Δ*sitCD*Δ*feoB* (ΔSB), and Δ*efeUOB*Δ*sitCD*Δ*feoB* (ΔESB) showed increased sensitivity to H2O2 compared to CG43S3 (Fig. 14A). When comparing the H_2O_2 inhibition zone with that of CG43S3, only Δ*feoC* exhibited more susceptibility when the culture added with DIP (Fig. 14B) while Δ*feoB,* Δ*sitCD* and Δ*efeUOB* were more sensitive by the addition of DFX (Fig. 14C) than double deletion and triple deletion mutant strains Δ*feoB*Δ*efeUOB* (ΔBE), Δ*sitCD*Δ*feoB* (ΔSB), Δ*sitCD*Δ*efeUOB* (ΔSE) and Δ*efeUOB*Δ*sitCD*Δ*feoB* (ΔESB) (Fig. 14C). Fig. 14(D) showed the bacteria Δ*sitCD*, Δ*sitCD*Δ*feoB* (ΔSB) and Δ*efeUOB*Δ*sitCD*Δ*feoB* (ΔESB) which carrying deletion of *sitCD* displayed a more sensitive phenotype in the presence of the divalent-cation chelator EDTA.

Since FeoABC, SitABCD, and EfeUOB are repressed by Fur under LB medium, the derepressing effect of Fur on these system towards oxidative stress response was also tested. As Fig. 15 that Δ*efeUOB*Δ*sitCD*Δ*feoB*Δ*fur* (ΔESBΔ*fur*) is more sensitive while Δ*feoB*Δ*fur*, Δ*sitCD*Δ*fur*, or Δ*efeUOB*Δ*fur* is slightly resistant than Δ*fur*.

3.12. Analysis of deletion effects on the biofilm formation under different culture conditions

Iron plays an important role in bacterial biofilm formation. As shown in Fig. 16, all the deletion mutants except Δ*sitCD*Δ*efeUOB* (ΔSE) exhibited decreased biofilm forming activity compared to CG43S3. It is interesting to note that an apparent defect of biofilm formation was found by the deletion of *fur*.

Addition of DIP or DFX to the medium conferred different effects on the biofilm formation activity. As shown in Fig. 17, DIP negatively influenced the biofilm formation for CG43S3 as well as all the derived mutants. By contrast, addition of DFX allows identifying the deletion effect on the biofilm formation. Compared to CG43S3, only Δ*feoC* exhibited an increase of biofilm formation while the other mutants showed reduced biofilm formation in the presence of DFX. The *feoC* deletion may induce the expression of *feoAB* thereby promote iron-uptake and increase biofilm formation.

3.13. Analysis of the deletion effects on the expression of type 3 fimbriae

As described by Wu *et. al*., expression of the major pilin MrkA of type 3 fimbriae depends on the extracellular iron availability [26]. Consistent with their findings, Fig. 18(A) shows MrkA production is reduced by adding DIP or DFX to the culture, in which DIP almost abolished the MrkA expression. To investigate the role of ferrous iron uptake system on type 3 fimbriae expression, the deletion mutants along with wild-type strain were treated with different conditions and the MrkA production assessed by Western blot analysis. As shown in Fig. 18(B), Δ*feoB* and Δ*efeUOB*Δ*sitCD*Δ*feoB* (ΔESB) exhibited a slightly decreased production of MrkA compared with CG43S3. However, *feoB* deletion caused a slightly increased production of MrkA under iron

depletion condition (Fig. 18C). The iron depletion condition had no effect on the MrkA production of the other mutants.

We have shown that Fur positively regulates the expression of type 3 fimbriae. Whether FeoABC, SitABCD or EfeUOB is involved in this regulatory pathway is investigated. As shown in Fig. 19, Δ*fur* exhibited no MrkA production and further removal of either of *feo*, *sit*, or *efe* iron transport system could not rescue the deficiency caused by *fur* deletion. This implies that Fur-dependent expression is independent to the iron-mediated expression of type 3 fimbriae.

MrkA expression was increased under microaerobic condition in CG43S3 and the derived mutant strains (Fig. 20A). In the presence of DFX, MrkA production was slightly decreased by the deletion of *feoA*, *feoB*, *feoC*, or *efeUOB* (Fig. 20B). Interestingly, Δ*sitCD* and ΔESB revealed an increase of MrkA expression compared to CG43S3 (Fig. 20B).

4.0 Discussion

Iron is important factor for bacterial survival and many biological functions. Iron presents in one of two states: ferric [Fe(III)] or ferrous [Fe(II)] iron. In aerobic environment, iron usually present in an oxidized and relatively insoluble Fe(III) form. Less is understood that the facts ferrous iron should be predominant in niches within the host that is oxygen limited. The study of ferrous iron acquisition system should provide knowledge and insight on bacterial strategy in competing limited iron source within host cell. Sequence analysis of *K. pneumoniae* CG43 genome revealed three major ferrous iron uptake transporter-encoding gene clusters: *feoABC*, *sitABCD* and *efeUOB*. In this study, we analyzed the preferential expression of individual ferrous iron acquisition systems in response to different environment stimuli and their possible effects on *K. pneumoniae* growth and virulence-related properties such as oxidative stress response, biofilm formation and type 3 fimbriae expression.

Generally, individual or cumulative loss of Feo, Sit and Efe transporter proteins do not affect the growth of CG43S3. DIP, 2, 2'-dipyridy, is a high-affinity chelating agent for ferrous iron while DFX, deferoxamine is a siderophore produced from *Stretomyces pilosus* that function to chelate ferric iron [130-132]. The expression of *feo* is induced by DFX but not DIP suggesting Fe(II) transport system could be stimulated by the scarcity of Fe(III). As described in *H. pylori*, when Fe(III) supplied in the environment, uptake of Fe(II) by FeoB involved a Fe(III) reductase. This was further verified by a study in *Leptospira* spp, *feoB* mutant unable to transport Fe when iron sources were Fe(III)-dicitrate and iron sulfate. Thus, FeoB is involved in the uptake of Fe(III) as well as Fe(II) [133, 134].

In the promoter activity analysis, FeoC displayed a negative regulation on the *feo* expression under iron-depleted and static cultured condition. This suggests that FeoC

represses *feo* expression when the environmental oxygen contents and iron concentration are low. However, the regulatory role on *feo* expression was not observed in *S. enterica* or *V. cholerae* [53, 61]. The activity of P_{feo1} is much lower than P_{feo2} implies a negative regulatory element is located in the region. Sequence analysis of the region reveals a possibility of NagC repressor binding element. NagC which participates in regulating the *E. coli* phosphotransferase system is N-acetylglucosamine regulator. The regulatory role of NagC on *feo* expression is pending for verification.

In either of P_{feo}, P_{sit} or P_{efe}, a relatively conserved Fur box could be found. The promoter activity analysis further supports a negative role of Fur in the expression of *feo*, *sit* and *efe*. A recent report in CG43 suggested an involvement of small RNA, RyhB in SitA transcription. If RyhB plays a negative role in *sit* expression remained to be investigated for P_{sit} activity in Δ*ryhB* or Δ*ryhB*Δ*fur* background. The level of promoter activity is ranked P_{sit}> P_{efe}> P_{feo2}>P_{feo1}. An ArcA box located in P_{sit} might explain the microaeribic induction of *sit* expression. However, the study in *S. enterica* showed transcriptional activity of *sitABCD* was not affected by the deletion of *arcA* or *fnr*. Highest expression of *feoABC*, *sitABCD* and *efeUOB* occurred in stationary phase, whether the involvement of growth-regulated elements (e.g. sigma S factor, *rpoS*) requires further study.

Fe(II) is known to predominant under acidic and reducing environment. It's reasonable to speculate these Fe(II) transporter would have a potential role under acidic media. EfeUOB system is an acid inducible and CpxR-regulated Fe(II) transporter identified in *E. coli* O157:H7 [46]. A single-base-pair deletion in *efeU* caused *efeUOB* cryptic in *E. coli* K12. In another word, EfeUOB was particularly function in *E. coli* O157:H7. To investigate whether EfeUOB is cryptic in KPCG43, sequence alignment as shown in Appendix 3

revealing that the amino acid similarities of EfeU, EfeO and EfeB between CG43 and O157:H7 are 82.9 %, 86.9 % and 82.9 % respectively. The gene organization analysis showed the flanking genes are the phosphate starvation gene *phoH* (56.8% amino acid similarity) and *pgaABCD* in *E. coli* [135]. However, *pgaABCD* is located somewhere in CG43 genome. Consistent with *E. coli*, P_{efe} of CG43S3 is induced by weak acid (pH 5) and *cpxR* deletion. The microaerobic-repressed P_{efe} expression implies EfeUOB plays an important role in the presence of oxygen. Unlike result from promoter activity of this study, contrasted observation was found in a comparative transcriptome analysis of CG43S3 which using RNA-sequence approach to identify the genes responded to acid stress (pH 3) [136]. Among the significantly down-regulated genes, the expression of D364_05435 (EfeO) was 6.26-fold decreased. This could be resulted from different pH treatment (pH 5 versus pH 3) or different roles of EfeO between the two bacteria.

In summary, all three systems are induced by iron-depletion (low Fe) but negatively regulated by Fur-Fe^{2+}. FeoC possibly represses expression of *feo* via the iron-sulfur cluster when both iron and oxygen are depleted (low Fe and low O_2). CpxAR represses *efe* expression when CG43S3 cultured in a rather alkaline environment (OH^-), i.e., when the environment switches to acidic, CpxAR repression on *efe* expression is released, leading to the uptake of ferrous iron. Increased expression of *feo*, *sit* and *efe* were observed by addition of Mn^{2+}. The de-repression of Fur is controlled by a manganese-dependent regulator, PerR [137]. When Mn^{2+} becomes excess, Mn^{2+}-PerR represses the expression of *fur*, subsequently releases the repressing effect of Fur on these iron-acquisition systems (Fig.21).

Accumulation of excess Fe(II) is hazardous to bacteria since it lead to formation of ROS when participating in Fenton reaction. Mn is required for some enzymes involved in

oxidative stress response proteins, such as superoxide dismutase SodA and the non-heme catalase KatN. [138, 139]. The potential of Mn/Fe transporter SitABCD in protection against oxidative stress was studied by H_2O_2 stress response. From the oxidative stress response study, SitABCD may be more important in protecting from H_2O_2 stress, especially in the depletion of divalent transition metal cations. As reported in *Salmonella* [12], the loss of *sit* and *feo* resulted in increased-sensitivity to oxidative stress. The interruption of Mn and Fe hemostasis triggered by loss of multiple Fe(II) transporter proteins might be the major reason in causing an oxidative-sensitive phenotype. On the other hand, aerobically grown Δ*feoC* loaded with DIP showed decreased bacterial oxidative stress resistance further supports a negative role of FeoC for Feo system, which is induced by iron-depletion and oxygen-depletion. In this study, the inhibition zone formed in disk assay did not show significant difference from each other. A quantitative assay such as measurement of survival rate between treated bacteria versus untreated bacteria might provide more convincing evidence of their role in oxidative resistance.

Biofilm formation in bacteria was found very much dependent on iron availability. Our study showed deletion of these iron acquisition genes decrease CG43S3 biofilm formation ability. Yet, the combined deletion of these genes did not show gradient decrement of biofilm formation. Nevertheless, deletion of *sitCD* and *efeUOB* rendered an increase of biofilm formation. FeoB may also be the sole Fe(II) uptake system under iron depletion condition. Increasing intracellular Fe levels by FeoB permease leads to escalate biofilm formation. Under iron- and oxygen-depleted condition, Δ*feoC* exhibits increasing *feoAB* expression which may in turn increase Fe(II) uptake by *feoB* and consequently increase biofilm formation.

The availability of iron could activate expression of type 3 fimbriae and also the biofilm formation in CG43S3 [26]. However, none of the Fe(II) transporter systems had significant effect on the MrkA expression. It is interesting that Δ*feoB* in iron-depleted condition enhanced MrkA expression. The loss of *feoB* may induce iron-acquisition related element(s) to enhance MrkA expression. It is possible that the regulation of these iron acquisition system-mediated biofilm formation is independent to the iron-regulated MrkA expression. Moreover, manganese may also play an important role in regulating type 3 fimbriae since the loss *sit* (Δ*sitCD* and Δ*ESB*) increased MrkA expression.

From the results above, a proposed functional model of these systems is suggested (Fig. 22). The ferrous iron-acquisition is mediated by FeoB which contains a cytoplasmic GTPase domain to hydrolysis GTP to GDP; cytoplasmic FeoC senses environmental oxygen and iron to regulate *feo* system; the role of FeoA is unclear yet (this model is based on a model proposed by Cartron *et al.* [49]). Transport of ferrous iron or/ and manganese by permease SitC and SitD is activated by the periplasmic SitA which in turn facilitated by SitB that hydrolyze ATP to ADP (this model is based on their structural analysis). The acid-inducible EfeU uptakes ferrous iron with the assistance of EfeO and EfeB (the real mechanism is little known in Gram-negative bacteria). All three systems affect the formation of biofilm in CG43S3 while SitABCD protects CG43S3 against H_2O_2. In a Western blot analysis, FeoB and Sit system negatively regulate the production of major pilin of type 3 fimbriae in iron-depleted (low Fe) or / and microaerobic condition (low O_2)

In most of the assessments performed, the addition of DIP caused slower growth rate in compared to general cultured condition. Hence, the toxic effect of DIP toward bacterial growth has to be taken into consideration. The divalent-cations chelator EDTA or Fe(II)-

chelating agent ferrozine may be better agent to create an iron-depleted condition. Besides, an alternative reporter system to determine ferrous iron level in cell could provide a better understand of these system. The quinone antibiotic, streptonigrin (SNG) binds specifically to iron and promotes formation of hydroxyl radicals [140]. Hence, it has been used to select mutant of defective in iron-uptake in *E. coli* [141]. SNG could serve as an indicator of Fe level in deletion mutants, where deletion mutants will grow better than wild-type strain when treated with SNG. However, the thick mucoid capsule formed by CG43 protects it from harsh oxidative stress, a higher concentration of SNG might be needed.

Iron availability affects bacterial biological function in many ways. Environmental iron availability apparently affects the expression of bacterial iron-acquisition system. On the other hand, the intracellular Fe(II) level in bacteria may affecting their biological functions as well. The homeostasis of Fe in bacteria is tightly regulated and hence the intracellular Fe deficiency caused by deletion of the ferrous iron transport system may very likely be compensated by other ferric iron acquisition systems.

5.0 References

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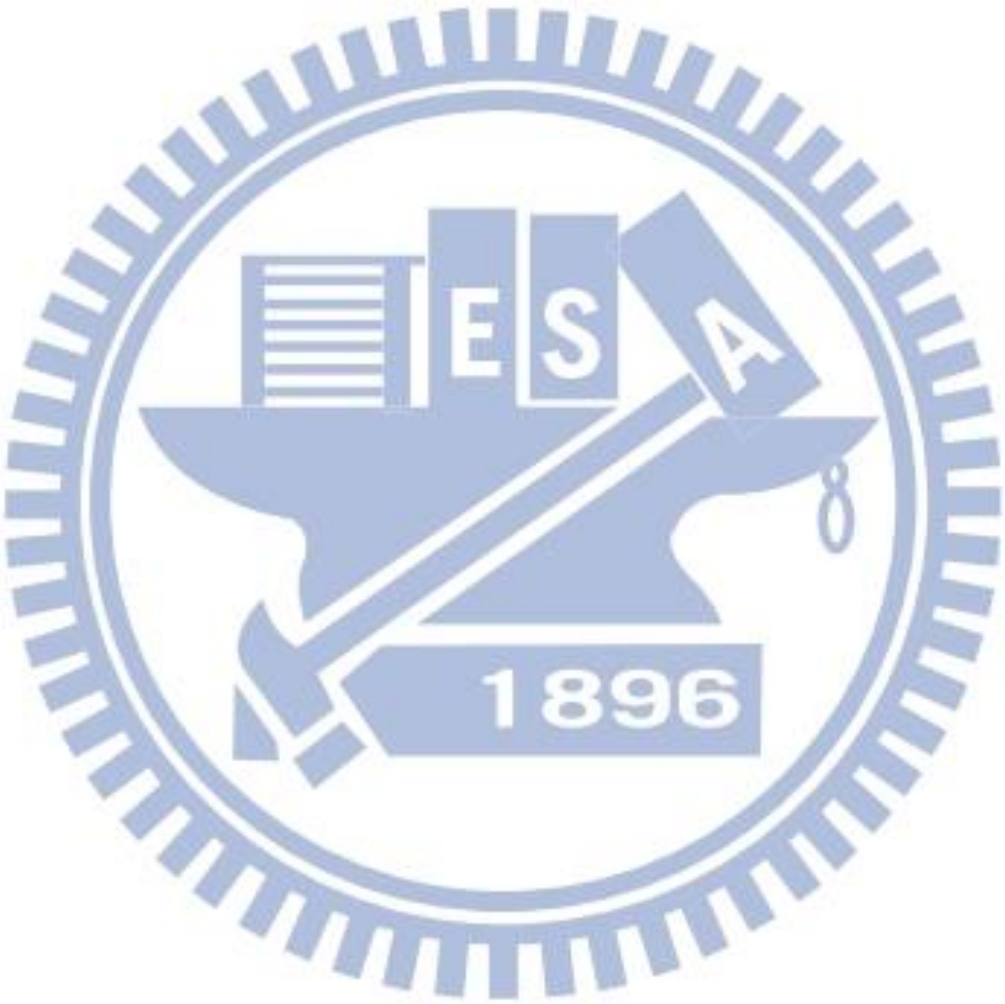


6.0 Tables

Table 1. Bacterial strains used and constructed in this study

Strain	Genotype or relevant property	Reference or source
***E. coli*:**		
JM109	*RecA1 supE44 endA1 hsdR17 gyrA96 rolA1 thi* Δ(*lac-proAB*)	Laboratory stock
S17-1 λpir	Tp^r Sm^r *recA*, *thi*, *pro*, *hsdR*$^-$*M*$^+$ [PR4-2 Tc::Mu:Km^rTn7](*pir*)	Laboratory stock
***K. pneumoniae*:**		
CG43	Clinical isolate belonged to K2 serotype	Laboratory stock
CG43S3	Δ*rspl*, Sm^r	Laboratory stock
Δ*feoA*	*feoA* deletion mutant in CG43S3	This study
Δ*feoB*	*feoB* deletion mutant in CG43S3	Laboratory stock
Δ*feoC*	*feoC* deletion mutant in CG43S3	This study
Δ*sitCD*	*sitCD* deletion mutant in CG43S3	This study
Δ*efeUOB*	*efeUOB* deletion mutant in CG43S3	This study
Δ*sitCD*Δ*feoB (*Δ*SB)*	*sitCD* and *feoB* deletion mutant in CG43S3	This study
Δ*sitCD*Δ*efeUOB (*ΔSE*)*	*sitCD* and *efeUOB* deletion mutant in CG43S3	This study
Δ*feoB*Δ*efeUOB (*ΔBE*)*	*feoB* and *efeUOB* deletion mutant in CG43S3	This study
Δ*efeUOB*Δ*sitCD*Δ*feoB (*ΔESB*)*	*feoB, sitCD* and e*feUOB* triple deletion mutant in CG43S3	This study
Δ*fur*	*fur* deletion mutant in CG43S3	Laboratory stock
Δ*feoA*Δ*fur*	*fur* and *feoA* double deletion mutant in CG43S3	This study

Strain	Genotype or relevant property	Reference or source
Δ*feoB*Δ*fur*	*fur* and *feoB* double deletion mutant in CG43S3	This study
*feoC*Δ*fur*	*fur* and *feoC* double deletion mutant in CG43S3	This study
Δ*sitCD*Δ*fur*	*fur* and *sitCD* double deletion mutant in CG43S3	This study
Δ*efeUOB*Δ*fur*	*fur* and *efeUOB* double deletion mutant in CG43S3	This study
Z01	CG43S3Δ*lacZ*	Laboratory stock
Z01Δ*feoC*	*feoC* deletion mutant in Z01	This study
Z01Δ*cpxRA*	*cpxRA deletion mutant in Z01*	Laboratory stock
Z01Δ*rstA*	*rstA* deletion mutant in Z01	Laboratory stock
Z01Δ*fur*	*fur* deletion mutant in Z01	Laboratory stock

Table 2. Plasmids used and constructed in this study

Plasmid	Descriptions	Reference or source
yT&A	Ap^r, TA cloning vector	Yeastern
pKAS46	Ap^r, Km^r, positive selection suicide vector, *rpsL*	Laboratory stock
pKAS46-*feoA*	1.74-kb fragment containing a 290-bp deletion in *feoA* locus cloned into pKAS46	This study
pKAS46-*feoB*	2.15-kb fragment containing a 2297-bp deletion in *feoB* locus cloned into pKAS46	Laboratory stock
pKAS46-*feoC*	2.08-kb fragment containing a 266-bp deletion in *feoC* locus cloned into pKAS46	This study
pKAS46-*sitCD*	2.05-kb fragment containing a 1438-bp deletion in *sitCD* locus cloned into pKAS46	This study
pKAS46-*efeUOB*	2.23-kb fragment containing a 1675-bp deletion in *efeUOB* locus cloned into pKAS46	This study
pLacZ15	A derivative of PYC016 [128], containing *lacZ* as a reporter, Cm^r	Laboratory stock
P_{feo1}	562-bp fragment of the upstream region of *feo* cloned into placZ15	This study
P_{feo2}	322-bp fragment of the upstream region of *feo* cloned into placZ15	This study
P_{sit}	613-bp fragment of the upstream region of s*it* cloned into placZ15	This study
P_{efe}	613-bp fragment of the upstream region of *efe* cloned into placZ15	This study

Table 3. Primers used in this study

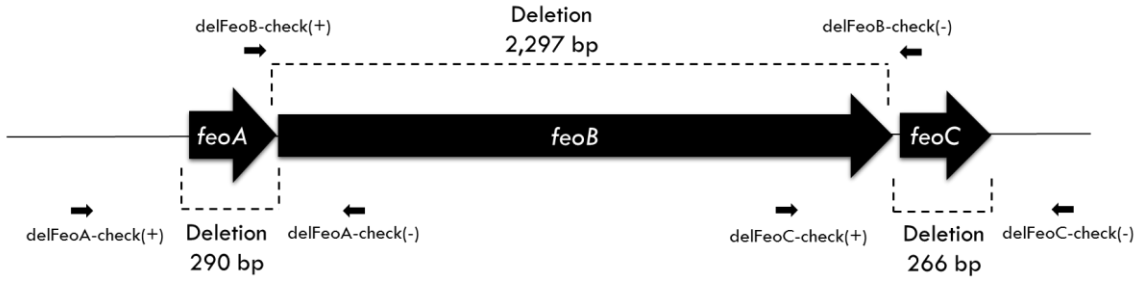
Function	Primer's name	Sequence (5'→ 3')
Gene deletion		
feoA	feoA-delA(+) [*XbalI*]	ATCTAGAGAAGTTTGGCGTCCCGA
	feoA-delA(-) [*KpnI*]	ATGGTACCGACGCCTGGAGGCA
	feoA-delB(+) [*KpnI*]	ATGGTACCTTGCACAATAACGGCTG
	feoA-delB(-) [*Eco*RI]	TGAATTCGACTGAGCCGGCAT
feoC	feoC-delA(+) [*XbalI*]	ATTCTAGATTCGTGATGGAGCTGCCG
	feoC-delA(-) [*KpnI*]	ATGGTACCGACTTCCATTAACGACGC
	feoC-delB(+) [*KpnI*]	ATGGTACCGGCGCTG CGATGAAAG
	feoC-delB(-) [*EcoRI*]	ATGAATTCTCGCGGC TCGTGACG
efeUOB	delEfeUOB-A(+) [*Eco*RI]	AGAATTCATTCCAGCTTACCCATGC
	delEfeUOB-A(-) [*KpnI*]	ATGGTACCTCCATTTGAAGAACACGC
	delEfeUOB-B(+) [*KpnI*]	AGGTACCATTCTATGGCCGGCATC
	delEfeUOB-B(-) [*Eco*RV]	AGATATCGCCTCGCCGTTAAGTCGT
Gene deletion check		
feoA	delFeoA-check(+)	GCCATCGCCATCACACACAAA
	delFeoA-check(-)	CGTCG CCGCTCAGGATGTAGT
feoB	delFeoB-check(+)	AGCCCAGCATACCGTCAGAA ACT
	delFeoB-check(-)	TCAAGCATGGCGTCAATCAGCG
feoC	delFeoC-check(+)	GCAGCGGCGGCATACAGC
	delFeoC-check(-)	GATGGGAAACAAACGGCGCA
sitCD	delSitCD-check(+)	ACAGAAACCACCTTCACCGC CGA
	delSitCD-check(-)	ATCCTCACTGTTCGCCATCT GCTG
efeUOB	delEfeUOB-check(+)	AGCGTGAACAGGAACTGTTC GAAG
	delEfeUOB-check(-)	ACCGTATCCTGAGTATTGGC GCAGA

Function	Primer's name	Sequence (5'→ 3')
Promoter construct		
P_{feo1}	Prom-feo01(+) [*Bam*HI]	GGATCCCAACAGCGCGATGATG
	Prom-feo02(-) [*Bgl*II]	AGATCTCAGCATGCCGAGGGAGA
P_{feo2}	Prom-feo03(+) [*Bam*HI]	GGATCCCGCGCATTTTTGATTACAG
	Prom-feo02(-) [*Bgl*II]	AGATCTCAGCATGCCGAGGGAGA
P_{sit}	Promoter_Psit(+) [*Bgl*II]	AGATCTTGCCAGCCGTCAGATGCT
	Promoter_Psit(-) [*Bgl*II]	AGATCTGCTGGCCAGCAGCAATG
P_{efe}	Promoter_Pefe(+) [*Bam*HI]	ATGGATCCGGAAAATAAAGGCTAAGC
	Promoter_Pefe(-) [*Bgl*II]	ATAGATCTGGCGATCAGGCTGACAAT

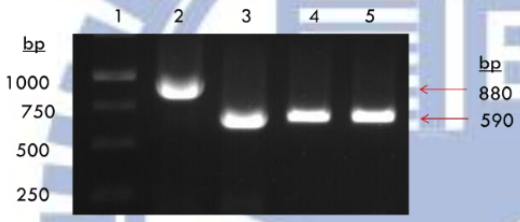
Underlined nucleotides indicate the restriction enzymes cleavage sites [square bracket]

7.0 Figures

(A)

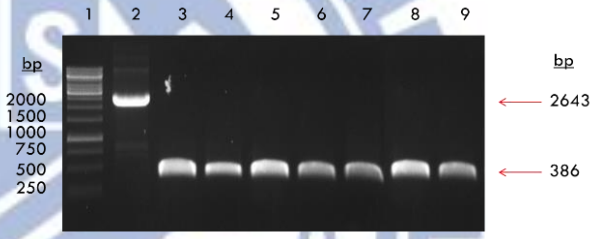


(B)



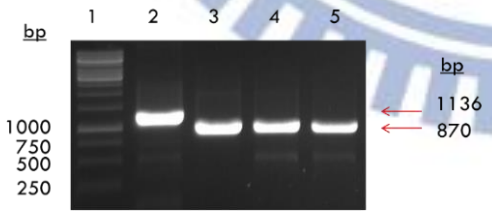
Lane 1 : Marker
Lane 2 : Wild-type
Lane 3 : pKAS46-gene flanking sequence
Lane 4 : ΔfeoA
Lane 5 : ΔfeoA Δfur

(C)



Lane 1 : Marker
Lane 2 : Wild-type
Lane 3 : pKAS46-gene flanking sequence
Lane 4 : Δ*feoB*
Lane 5 : Δ*feoB*Δ*fur*
Lane 6 : Δ*feoB*Δ*sitCD* (Δ*SB*)
Lane 7 : Δ*feoB*Δ*efeUOB* (Δ*BE*)
Lane 8 : Δ*feoB*Δ*efeUOB*Δ*sitCD* (Δ*ESB*)

(D)

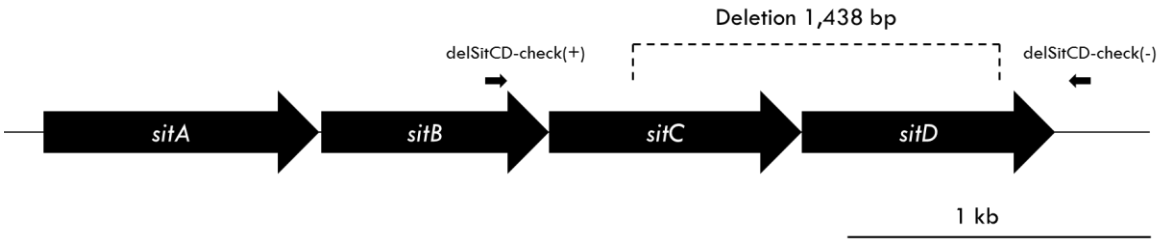


Lane 1 : Marker
Lane 2 : Wild-type
Lane 3 : pKAS46-gene flanking sequence
Lane 4 : ΔfeoC
Lane 5 : ΔfeoC Δfur

Figure 1. Schematic representation of the *feoA*, *feoB* and *feoC* deletions and the verification of deletions by PCR.

(A) The mutants were confirmed using PCR with specific primer pairs (small black arrow) located upstream and downstream of the target genes; the dashed lines represent deleted regions of target genes. PCR verification for deletion strains of (B) Δ*feoA* and Δ*feoA*Δ*fur* (B) Δ*feoB*, Δ*feoB*Δ*fur*, Δ*feoB*Δ*sitCD (*ΔSB*)*, Δ*feoB*Δ*efeUOB (*ΔBE*)* and Δ*feoB*Δ*sitCD*Δ*efeUOB* (ΔESB) (C) Δ*feoC* and Δ*feoC*Δ*fur*; small arrows denote the size of PCR product in bp of wild-type and deletion strains

(A)



(B)

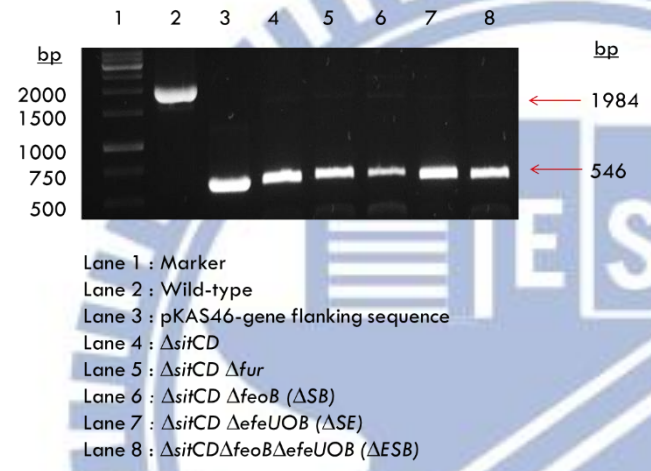
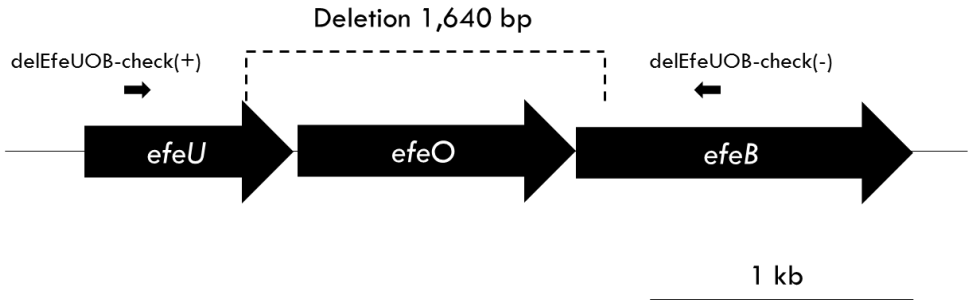


Figure 2. Schematic representation of the *sitCD* deletions and the verification of deletions by PCR.

The mutants were confirmed using PCR with specific primer pairs (small black arrow) located upstream and downstream of the target genes; the dashed lines represent deleted regions of target genes. PCR verification for deletion strains of (B) Δ*sitCD*, Δ*sitCD*Δ*fur*, Δ*sitCD*Δ*efeUOB (*ΔSE*)* and Δ*sitCD*Δ*efeUOB*Δ*feoB (*ΔESB); small arrows denote the size of PCR product in bp of wild-type and deletion strains.

(A)



(B)

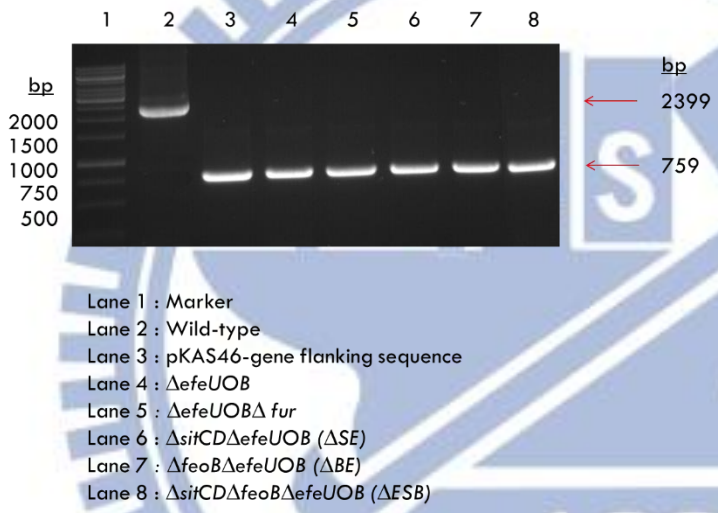
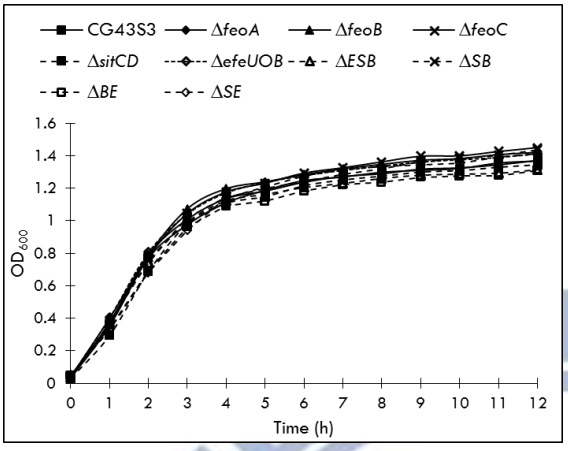


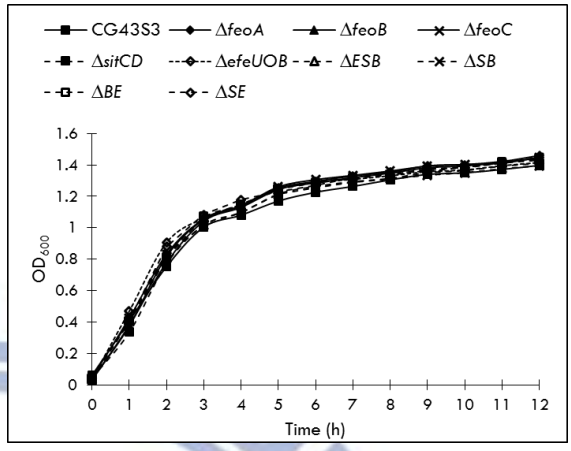
Figure 3. Schematic representation of the *efeUOB* deletions and the verification of deletions by PCR.

(A) The mutants were confirmed using PCR with specific primer pairs (small black arrow) located upstream and downstream of the target genes; the dashed lines represent deleted regions of target genes. PCR verification for deletion strains of (B) Δ*efeUOB*, Δ*efeUB*Δ*fur*, Δ*sitCD*Δ*efeUOB* (ΔSE), Δ*feoB*Δ*efeUOB* (ΔBE), *and* Δ*efeUOB*Δ*sitCD*Δ*feoB* (ΔESB)*;* small arrows denote the size of PCR product in bp of wild-type and deletion strains

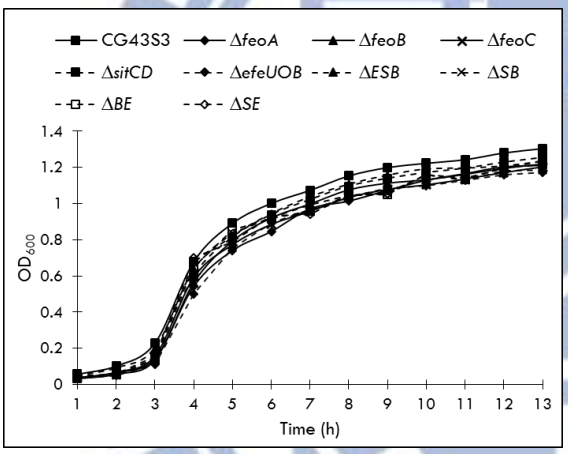
(A) LB



(B) LB + Fe



(C) LB + DIP



(D) LB + DFX

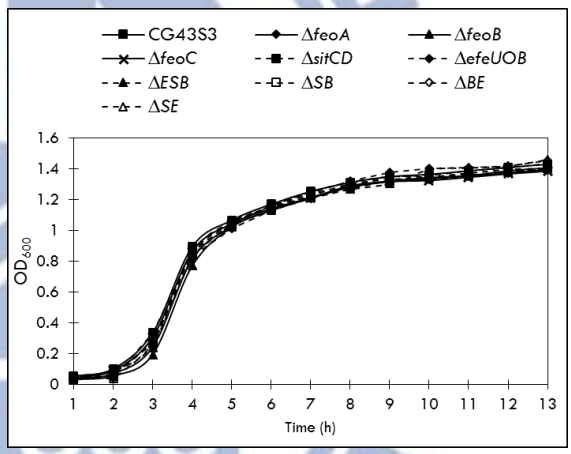


Figure 4. Growth analysis of *K. pneumoniae* strains under iron-replete or iron-depleted media.

The optical density at 600 nm of CG43S3 and deletion mutant strains grown in (A) LB or (B) LB loaded with 50 μM ferric sulfate [$Fe_2(SO_4)_3$] and 50 μM ascorbic acid or (C) LB loaded with 200 μM DIP or (D) LB loaded with 200 μM DFX was recorded every hour and plotted.

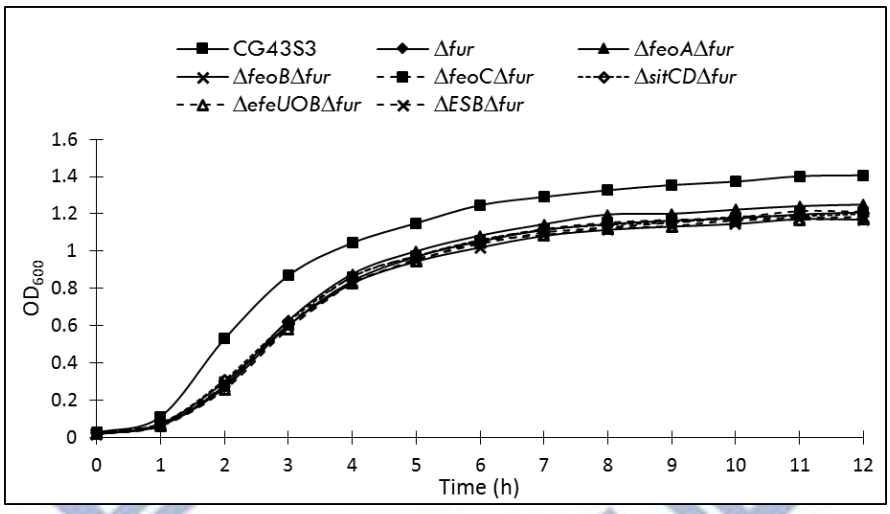
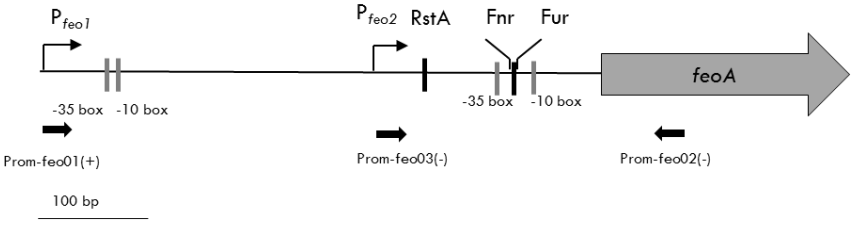


Figure 5. Growth analysis of *K. pneumoniae* Δ*fur*-derived strains.

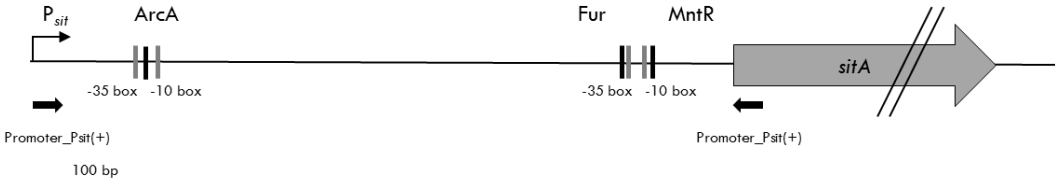
The optical density at 600 nm of CG43S3, Δ*fur* and Δ*fur*-derived double deletion mutants grown in LB at 37 °C was recorded every hour and plotted.

(A)



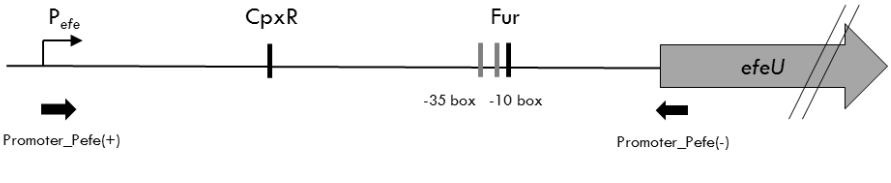
		TACA $(N)_6$ TACA			**RstA Box**
-216	TGAT	TACA GGCTGA CTTTG	CGGC	-193	*feoA*
		TTGAT $(N)_4$ ATCAA			**Fnr Box**
-138	ATTG	ATGAT AAAA ACCAT	TCTC	-116	*feoA*
		GATA ATGAT A/T ATCATT ATC			**Fur Box**
-131	TATT	GATG ATAAA A ACCATT CTC	ATTA	-117	*feoA*

(B)



		TG TTAA TTAA			**ArcA Box**
-498	GACC	TTAA TTAA	CAGC	-483	*sitA*
		GATA ATGAT A/T ATCATT ATC			**Fur Box**
-104	GCAT	GCAA ATAAG A ATTATT TTC	ATTT	-78	*sitA*
		AAACA TAGC $(N)_4$ GCTATGTTT			**MntR Box**
-81	ATTT	ATAAA TAGC TTGT GCTATATAA	CATA	-52	*sitA*

(C)

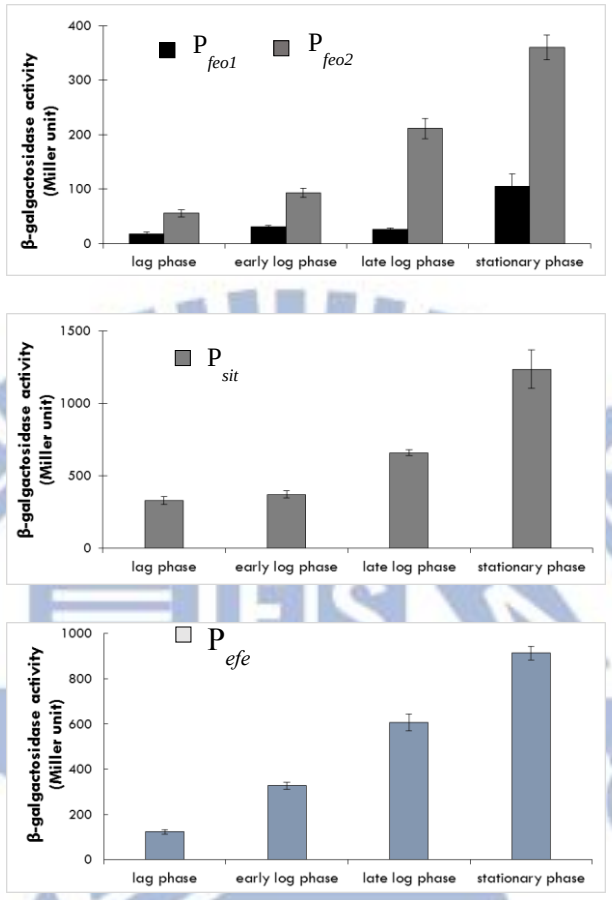


		GTAAA $(N)_{4-8}$ GTAAAT			**CpxR Box**
-220	GCGG	GTAAA TATCTGGC GTAAA	AAAT	-195	*efeU*
		GATA ATGAT A/T ATCATT ATC			**Fur Box**
-79	ATTG	TGTA ATGAT A ATCACT ATC	ATAA	-55	*efeU*

Figure 6. Diagrammatic representation of K. pneumoniae CG43 *feoABC, sitABCD* and *efeUOB* promoter region and P_{feo}::*lacZ*, P_{sit}::*lacZ* and P_{efe}::*lacZ* fusion construction.

The upstream, non-coding sequence of *feo, sitABCD and efeUOB,* along with predicted transcriptional factor are shown. The open reading frame are shown in large grey arrows, the predicted position of -10 and -35 box are indicated in gray vertical line and putative transcriptional factors are indicated in black vertical line. The PCR primers (small black arrow) used to amplify DNA fragments harboring (A) P_{feo}, (B) P_{sit} and (C) P_{efe} regions that cloned in the *lac*Z fusion constructs are depicted. The dashed boxes indicate the putative RstA, Fnr, Fur, MntR, CpxR binding sequences aligned with the promoters and identical nucleotides are underlined.

(A) Shaking culture



(B) Static culture

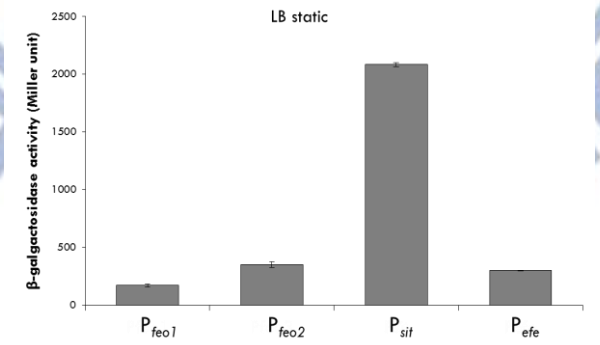
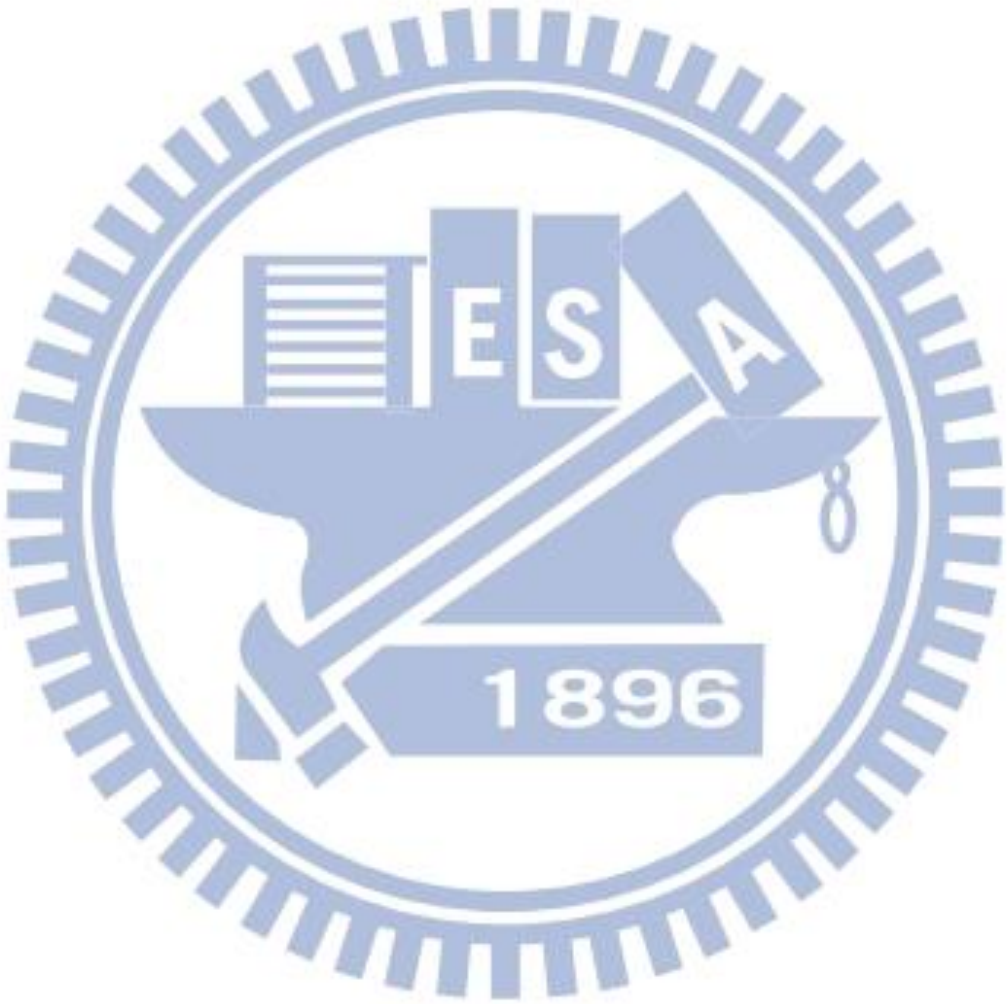
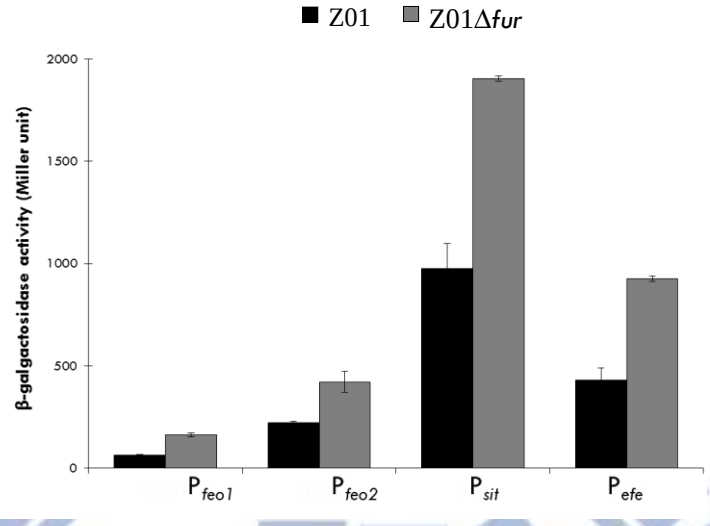


Figure 7. Analysis of growth phase and static-cultured effects on activity of P_{feo}, P_{sit} and P_{efe} in *K. pneumoniae* CG43S3 Δ*lacZ* (Z01)

(A) The β-galactosidase actitvities of Z01 carrying placZ15-P_{feo1}, P_{feo2}, P_{sit} and P_{efe} were determined at different growth phase: lag phase, early log phase, late log phase and stationary phase. (B) The effect of anaerobic condition on the β-galactosidase actitvities of P_{feo1}, P_{feo2}, P_{sit} and P_{efe} were determined after statically growing until reaching approximately of OD_{600} 0.6.



(A)



(B) Z01

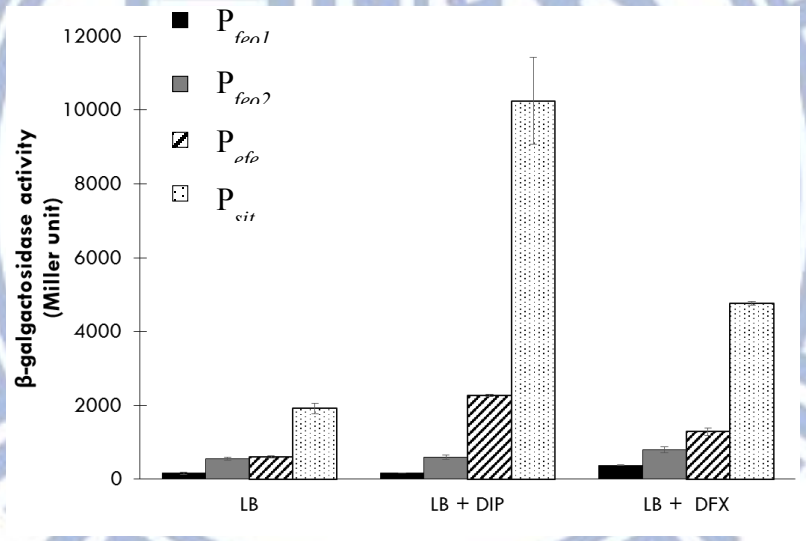
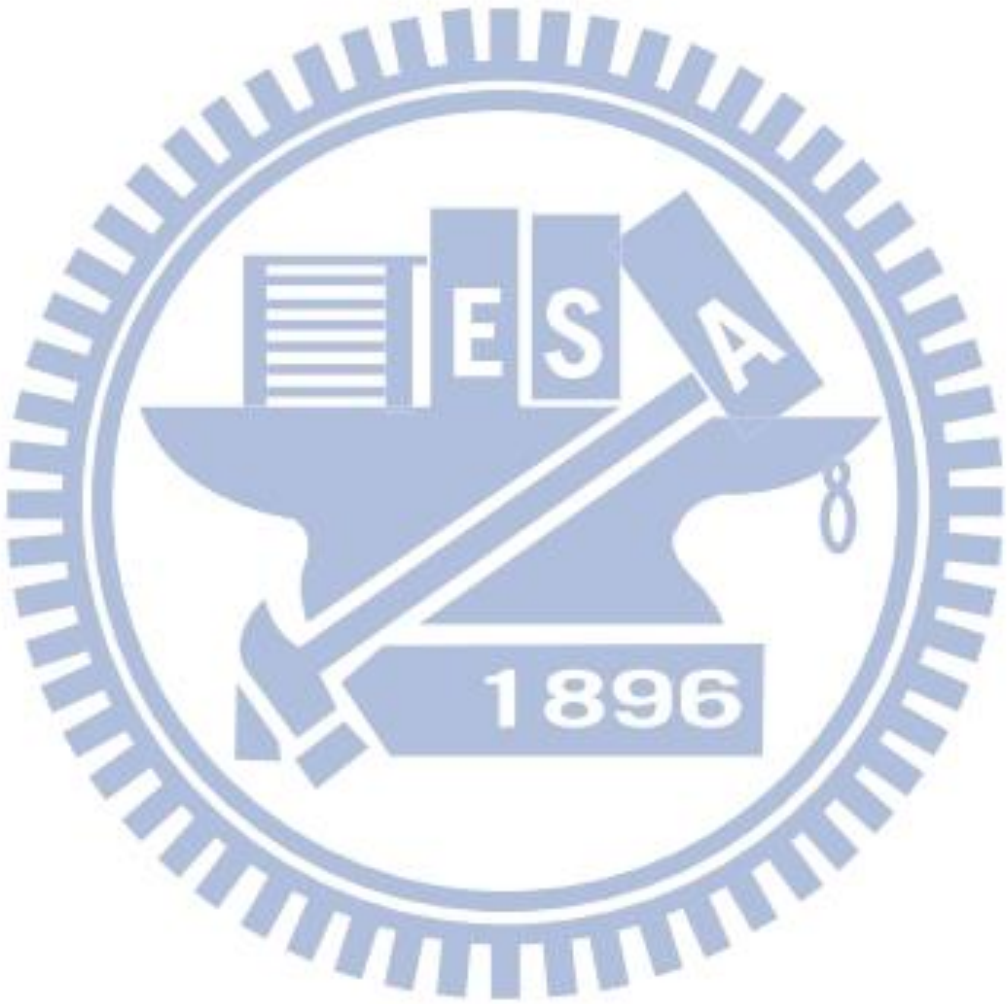


Figure 8. Analysis of *fur* deletion effect and iron-depletion effect on P_{feo}, P_{sit} and P_{efe} activity

(A) The β-galactosidase activities of late log-phased (OD_{600} 0.8-0.9) cultures of Z01 and Z01Δ*fur* carrying P_{feo1}, P_{feo2}, P_{sit} and P_{efe} grown in LB medium agitatedly were determined and expressed as Miller unts. (B) The response of P_{feo1}, P_{feo2}, P_{sit} and P_{efe} to iron chelators were tested by growing the cells agitatedly in LB medium, LB medium with DIP or DFX

unil late log phase and harvested. Their β-galactosidase activities were then determined as Miller units.



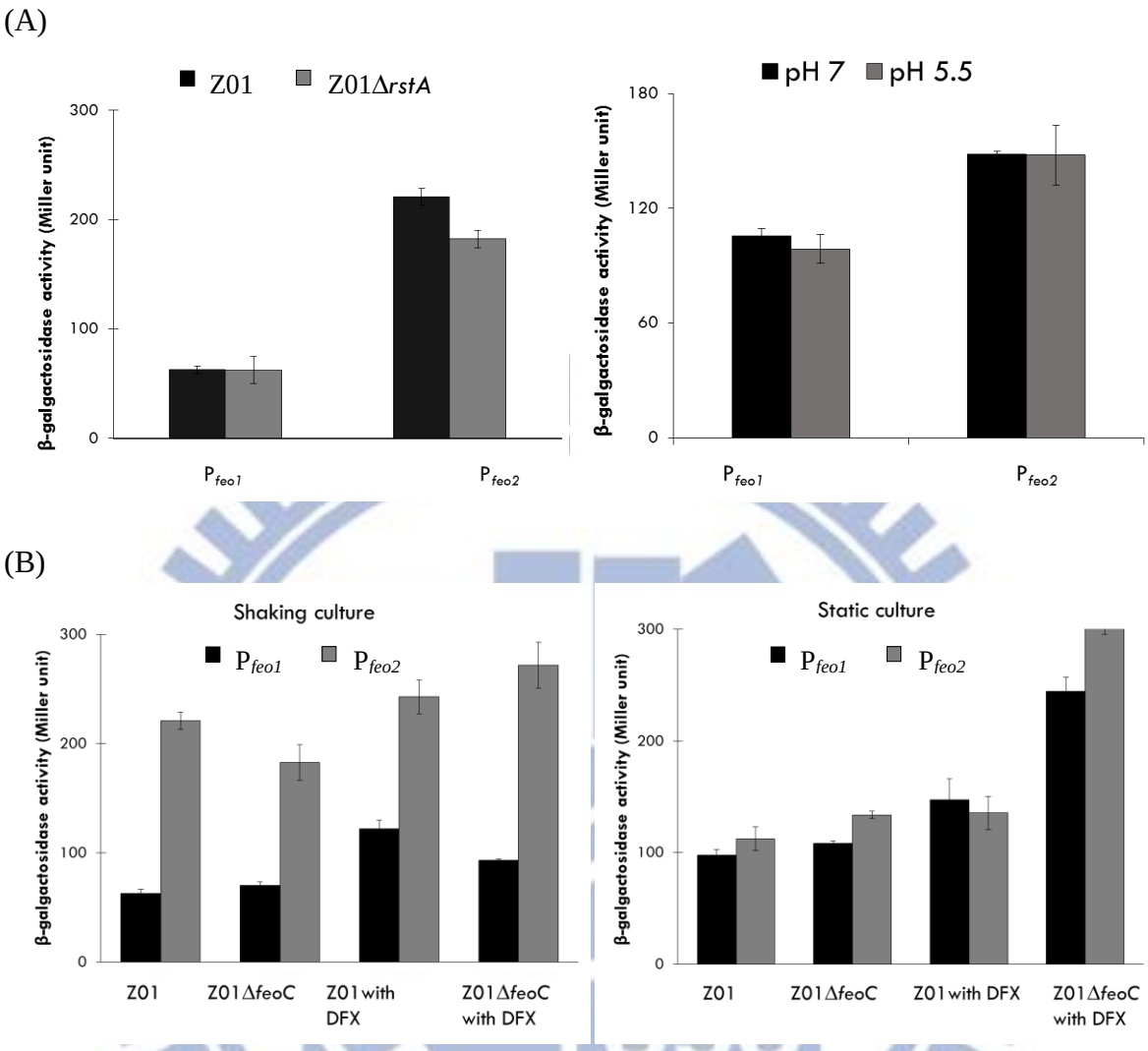
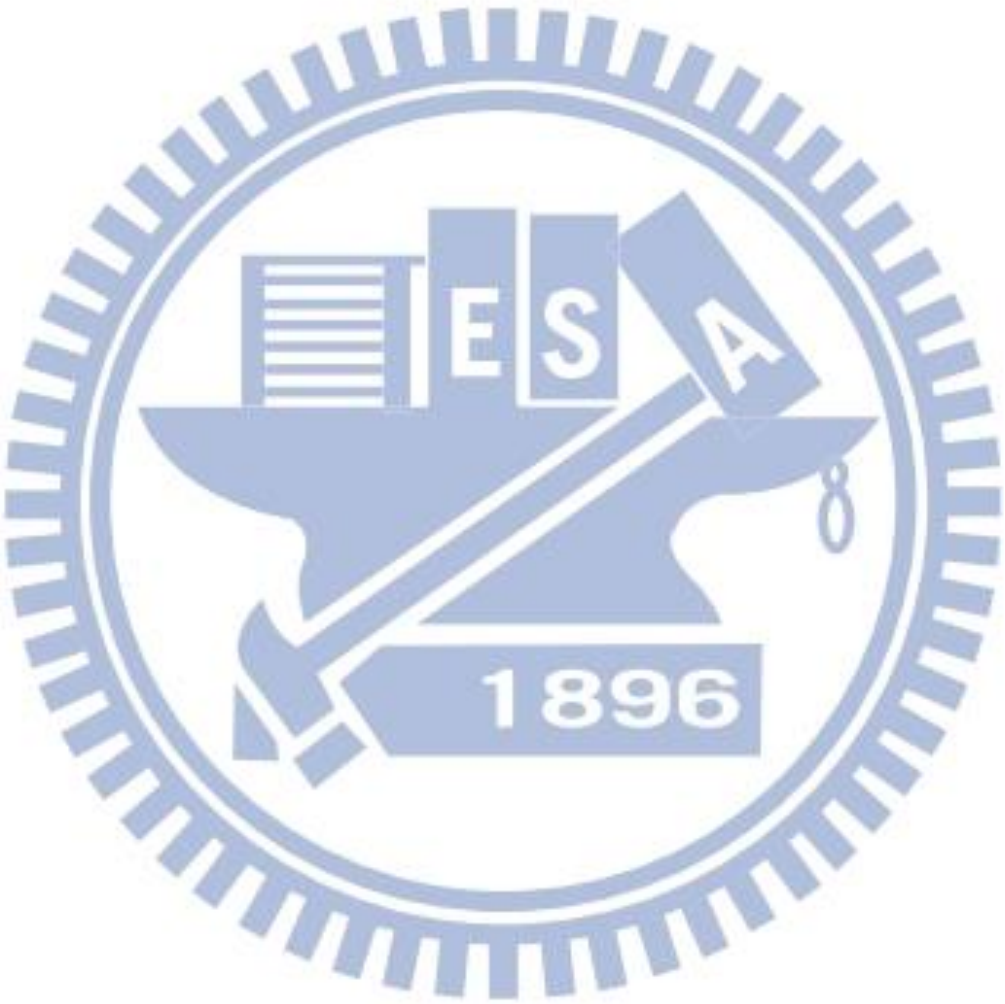


Figure 9. Analysis *rstA*, low pH and *feoC* deletion effect on P_{feo} activity in different culture conditions.

(A) The left panel showed the determined β-galactosidase activities of late log-phased (OD_{600}0.8-0.9) cultures of Z01 and Z01Δ*rstA* (The left panel) carrying P_{feo1} and P_{feo2} grown in LB medium agitatedly. The right panel showed the β-galactosidase activities of Z01 carrying P_{feo1} and P_{feo2} growing in pH 7 and pH 5.5 LB (B) The deletion effect of *feoC* on acitivity of P_{feo1} and P_{feo2} coupled with O_2 availability (Left panel – shaking; right panel –

static) and iron-depleted effect were determined by β-galaoctosidase activity and expressed as Miller unit.



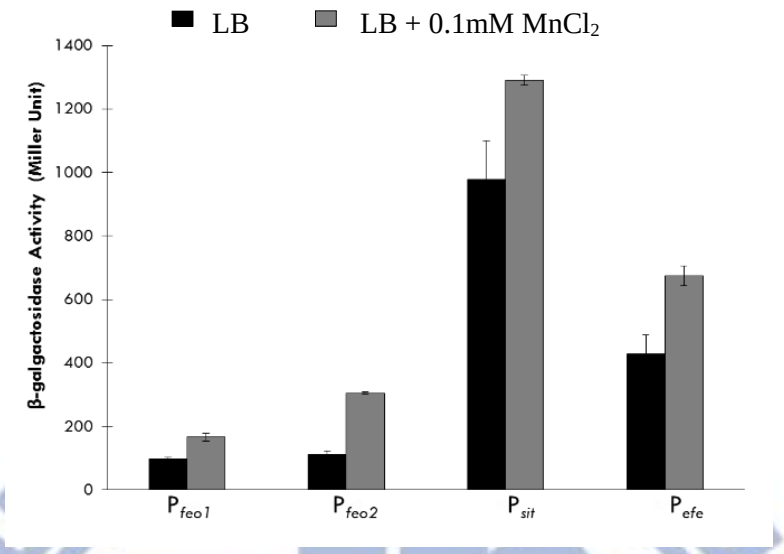


Figure 10. Analysis of effect of manganese on activity of P_{feo}, P_{sit} and P_{efe}.

Z01 carrying P_{feo1}, P_{feo2}, P_{sit} and P_{efe} grown agitatedly in LB medium suplemented with or without 0.1mM $MnCl_2$ until reaching OD_{600} of 0.8 to 0.9. Then, β-galactosidase activities were determined as Miller units.

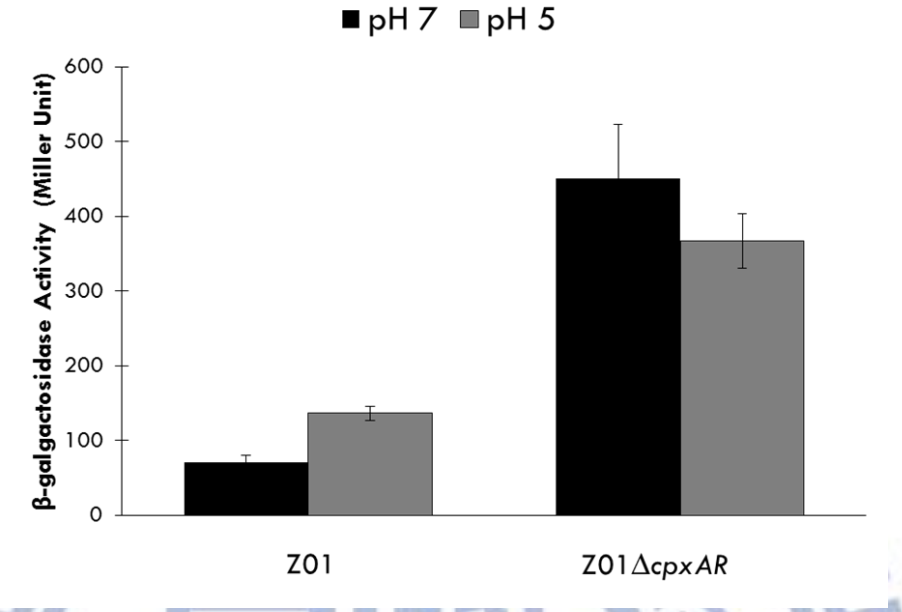
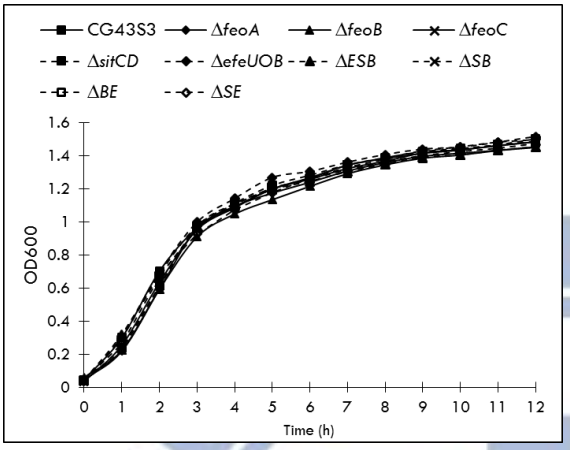


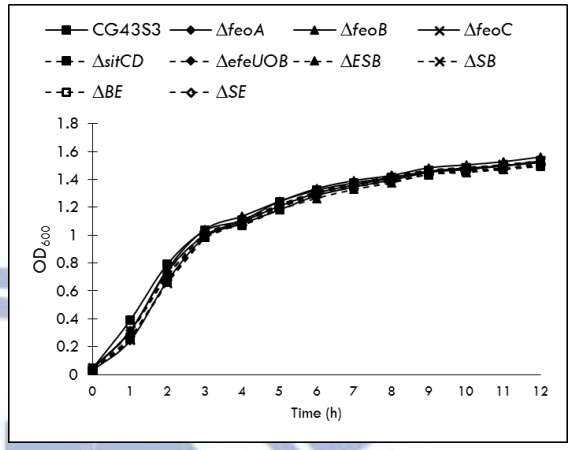
Figure 11. Analysis of weak acid and *cpxAR* effects on activity of P_{efe}

The β-galactosidase activities of late log-phased (OD_{600}0.8-0.9) cultures of Z01 and Z01Δ*cpxAR* carrying P_{efe} grown in pH 7 or pH 5 LB media agitatedly were determined and expressed as Miller unts

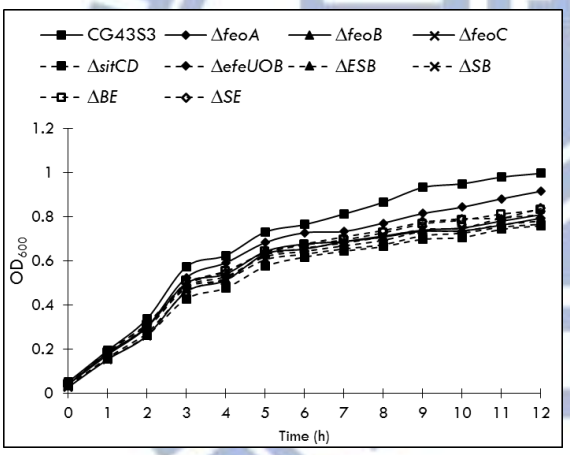
(A) LB pH 5



(B) LB pH 5 + Fe



(C) LB pH 5 + DIP



(D) LB pH 5 + DFX

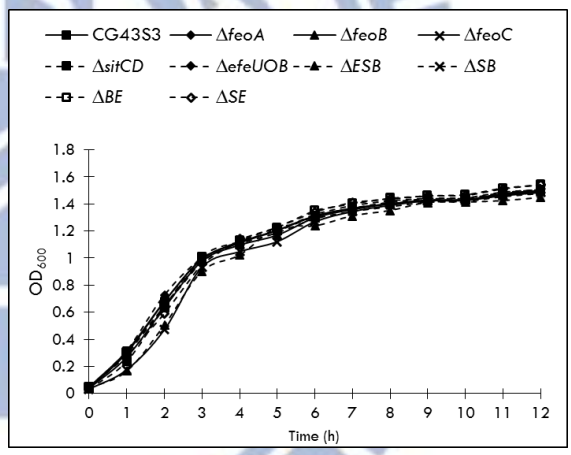
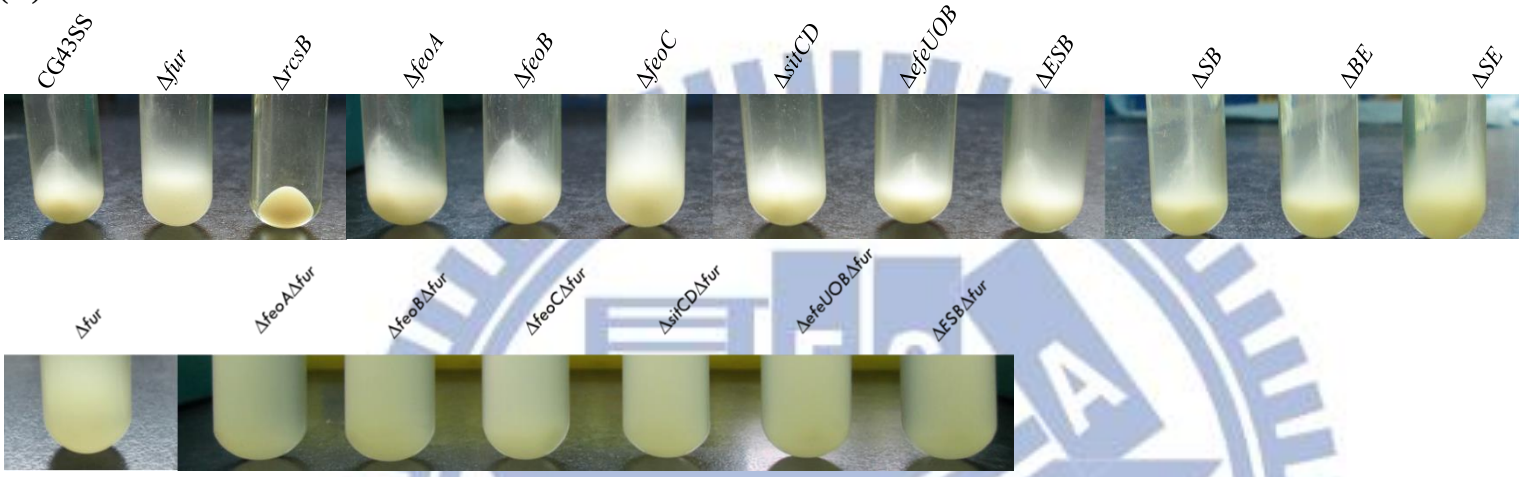
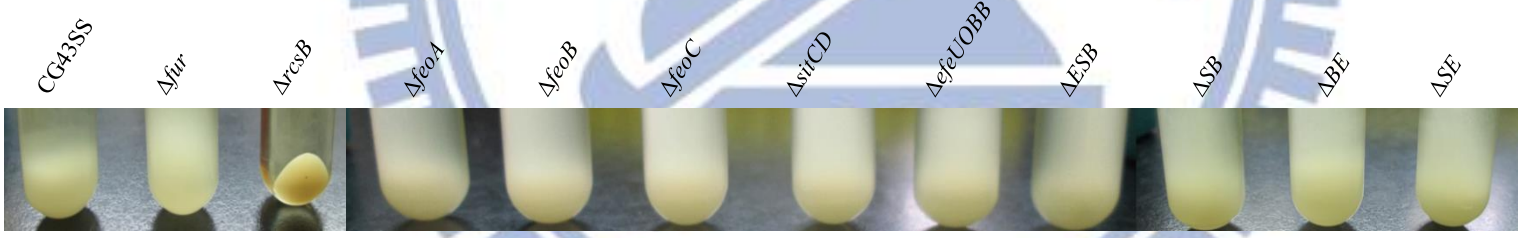


Figure 12. Effect of iron availability on growth of *K. pneumoniae* strains in acidic media. The optical density at 600 nm was recorded and plotted for CG43S3 and deletion mutant strains that grown in pH 5 LB broth (A) alone or (B) loaded with 50 μM ferric sulfate [$Fe_2(SO_4)_3$] and 50 μM ascorbic acid or (C) loaded with 200 μM DIP or (D) loaded with 200 μM DFX was every hour.

(A) LB



(B) LB + DFX



(C) LB + DIP

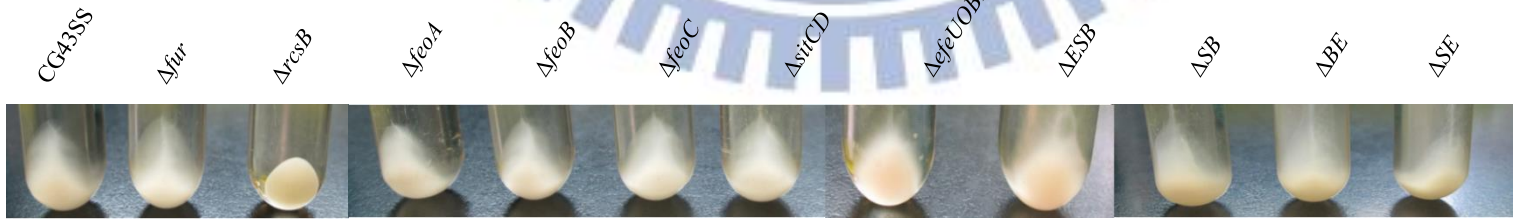
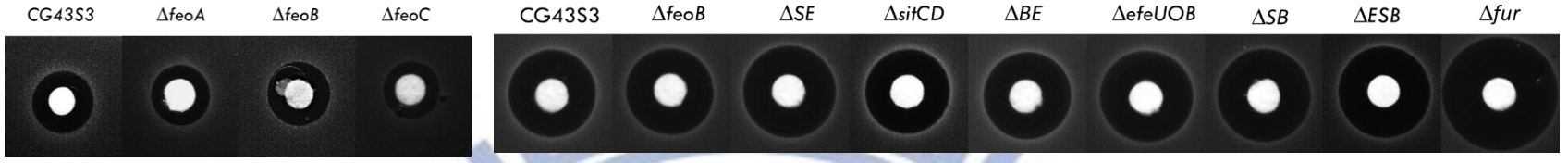


Figure 13. Analysis of capsular polysaccharide (CPS) biosynthesis in of *K. pneumoniae* strains in different media.

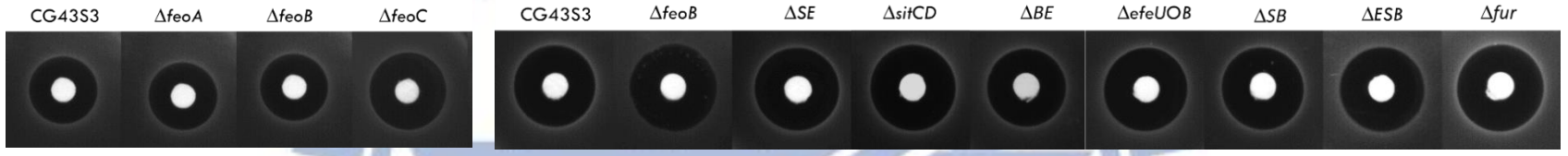
Comparison of CPS production between CG43S3 and deletion mutant strains was done by sedimentation test. The bacterial strains were grown 16 hr in LB (A), LB with 0.2 mM DFX (B) or LB with 0.2 mM DIP (C) at 37°C and subjected to centrifugation at 4,000 rpm for 5 min. Pellet formation after centrifugation was observed and photographed.

(A) LB



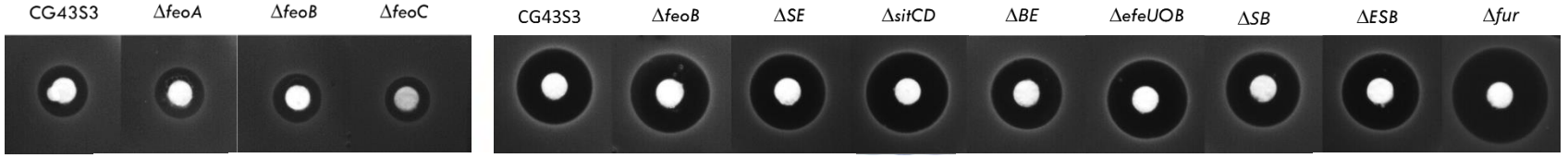
1.21 ± 0.03 | 1.21 ± 0.03 | 1.33 ± 0.03 | 1.21 ± 0.03 | 1.6 ± 0.003 | 1.63 ± 0.02 | 1.73 ± 0.03 ▲ | 1.56 ± 0.03 | 1.56 ± 0.03 | 1.75 ± 0.003 ▲ | 1.82 ± 0.03 ▲ | 1.7 ± 0.07 ▲ | 2.2 ± 0.06

(B) LB + DIP



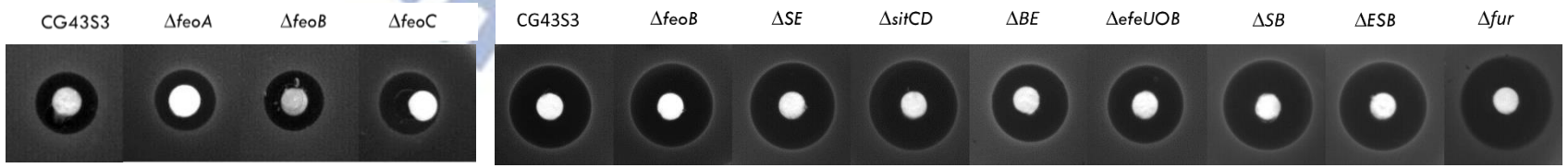
1.71 ± 0.03 | 1.65 ± 0.03 | 1.7 ± 0.03 | 1.9 ± 0.03 ▲ | 1. 8 ± 0.02 | 1.8 ± 0.05 | 1.8 ± 0.05 | 1.8 ± 0.05 | 1.76 ± 0.05 | 1.86 ± 0.02 | 1.8 ± 0.02 | 1. 93 ± 0.02 | 1. 9 ± 0.02

(C) LB + DFX



1.13 ± 0.03 | 1.12 ± 0.03 | 1. 13 ± 0.03 | 1. 12 ± 0.03 | 1. 78 ± 0.03 | 1. 83 ± 0.05 ▲ | 1. 75 ± 0.05 | 1. 88 ± 0.02 ▲ | 1. 7 ± 0.05 | 1. 82 ± 0.02 ▲ | 1. 7 ± 0.05 | 1. 78 ± 0.05 | 2.23 ± 0.05

(D) LB + EDTA



1.22 ± 0.003 | 1.2 ± 0.03 | 1.22 ± 0.03 | 1.2 ± 0.03 | 1. 91 ± 0.03 | 1. 91 ± 0.07 | 1.91 + 0.15 | 2.08 ± 0.07 ▲ | 1.78 ± 0.07 | 1.82 ± 0.02 | 2.15 ± 0.05 ▲ | 2.16 ± 0.02 ▲ | 2.06 ± 0.02

Figure 14. Effect of deletion mutants and iron chelators on the oxidative stress response

Overnight bacterial culture was inoculated into fresh LB broth and incubated at 37°C agitatedly until reaching OD_{600} of 0.6-0.7. Following that, 1 ml of each culture was suspended in pre-warmed liquefied agar and puored over the LB plates supplementd with or without iron chelators before placing filter paper disks spotted with 30% of H_2O_2 onto the surface culture. After 16 hr incubation at 37 °C, the inhibition zones of each plate were measured in cm (indicated as the average of triplicate samples with stanard deviations). The effect of iron chelators on oxidative stress response was tested by adding (B) 0.2 mM DIP or (C) 0.2 mM DFX or (D) 0.2 mM EDTA 1 h prior pouring onto agar. The small black triangles marked the larger inhibition zone in compared with CG43S3.

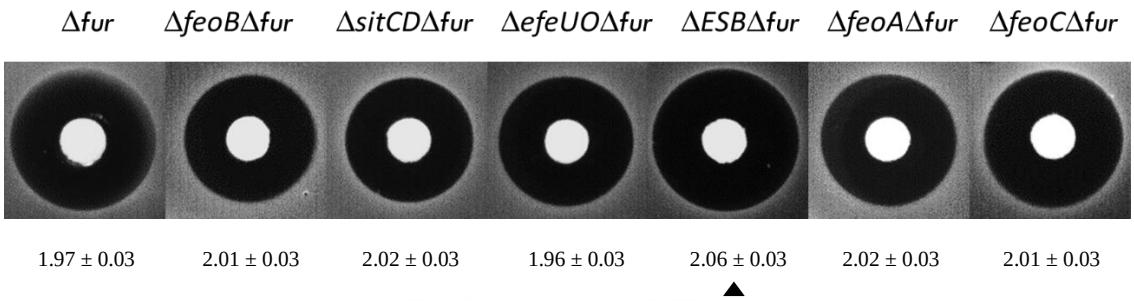


Figure 15. Effect of Δ*fur*-derived deletion mutants on the oxidative stress response

Overnight bacterial culture was inoculated into fresh LB broth and incubated at 37˚C agitatedly until reaching OD_{600} of 0.6-0.7. Following that, 1 ml of each culture was suspended in pre-warmed liquefied agar and puored over the plates supplementd with iron chelators before placing filter paper disks spotted with 30% of H_2O_2 onto the surface culture. After 16 hr incubation at 37 ℃, the inhibition zones of each plate were measured (indicated as the average of triplicate samples with stanard deviations). The small black triangles marked the larger inhibition zone in compared with CG43S3Δ*fur*.

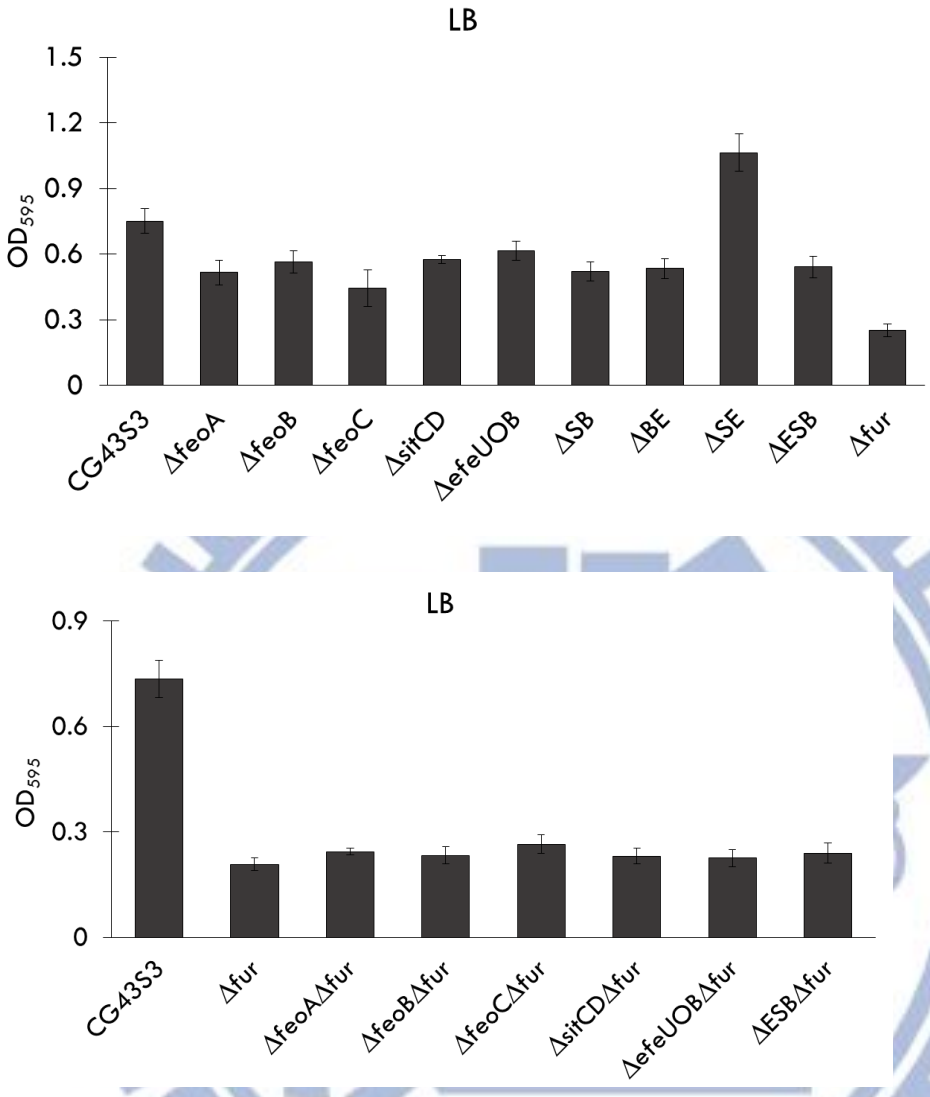


Figure 16. Biofilm formation analysis of deletion mutant strains.

The abilities of deletion mutants and Δ*fur*-derived mutants form biofilm were tested by growing cultures statically in plastic 96-well supplemented with LB medium at 37°C for 24 hr. The biofilm was visualized by staining with 1% crystal violet and quantified by absorbance at 595 nm.

(B)

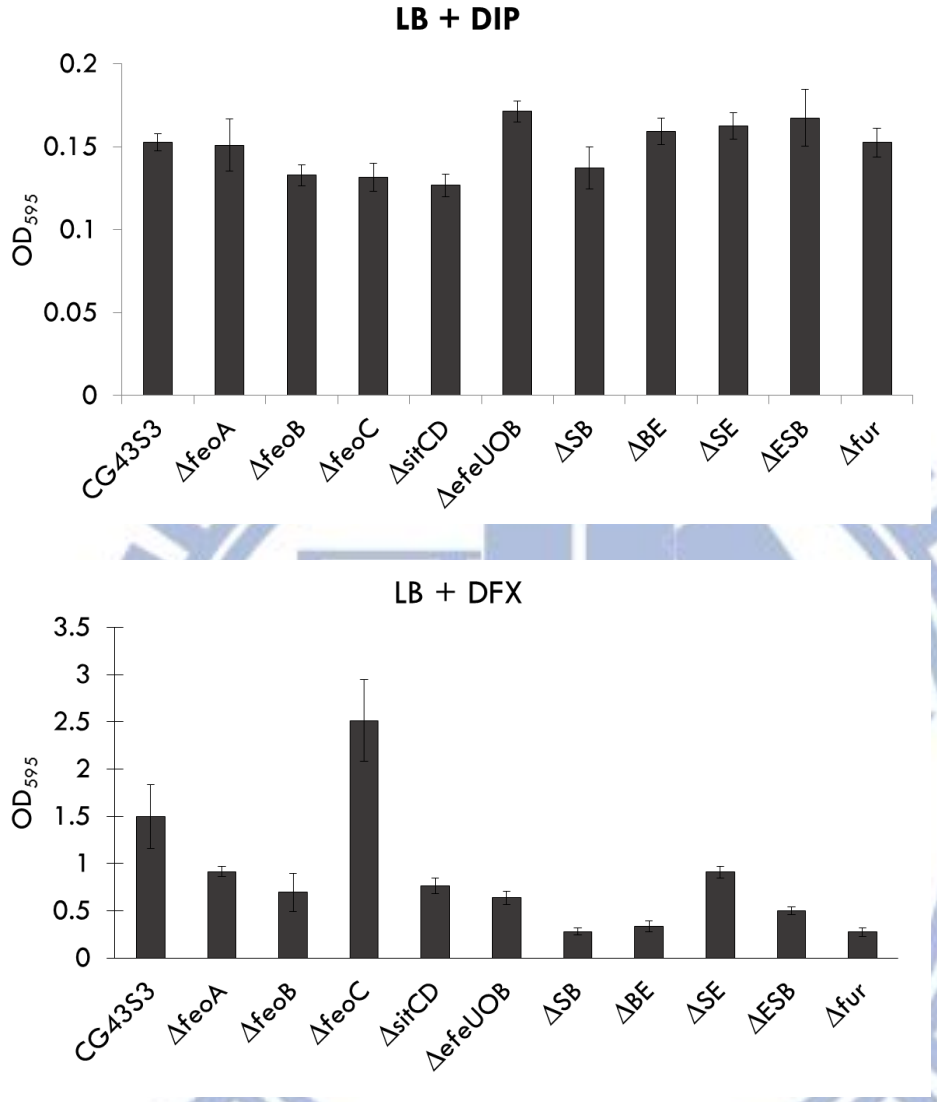
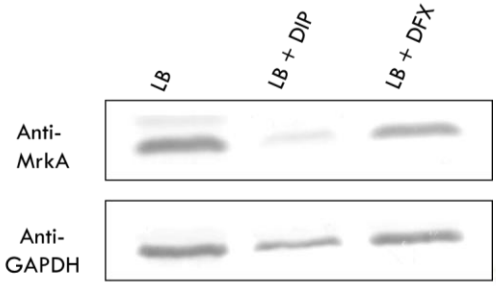


Figure 17. Biofilm formation analysis of deletion strains under iron-depletion

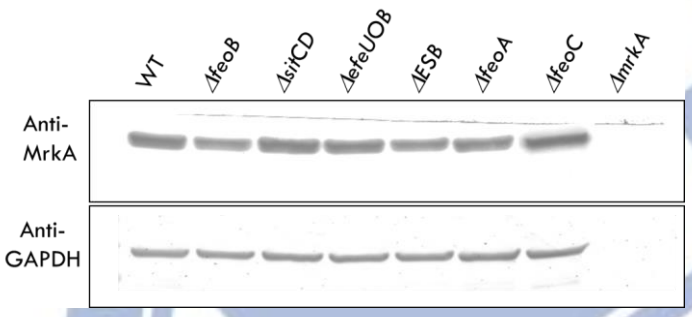
Biofilm formation assay was tested for bacterial culture that grown in LB medium supplemented with DIP (upper panel) or DFX (lower panel) with static incubation on plastic 96-well at 37°C for 24 hr. The biofilm were visualized by staining with 1% crystal violet and quantified by absorbance at 595 nm.

Shaking culture

(A) LB



(B) LB



(C) LB + DFX

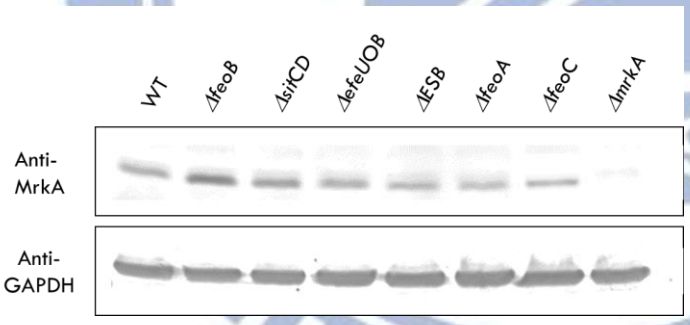


Figure 18. Iron-depleted effect on the iron-dependent MrkA expression under shaking condition

Total protein of bacterial cultures, approximately 10 μg per lane, were separated by SDS-PAGE, transferred to PVDF membrane and subjected to hybridization against the antibodies of anti-MrkA and anti-GAPDH (as an internal control). Proteins were collected from

bacterial culture treated with different growing conditions. (A) CG43S3 (WT) treated with or without iron chelator (DIP or DFX); WT and deletion mutant strains grown agitatedly at 37 °C for 16 hr in (B) LB or (C) LB added with DFX

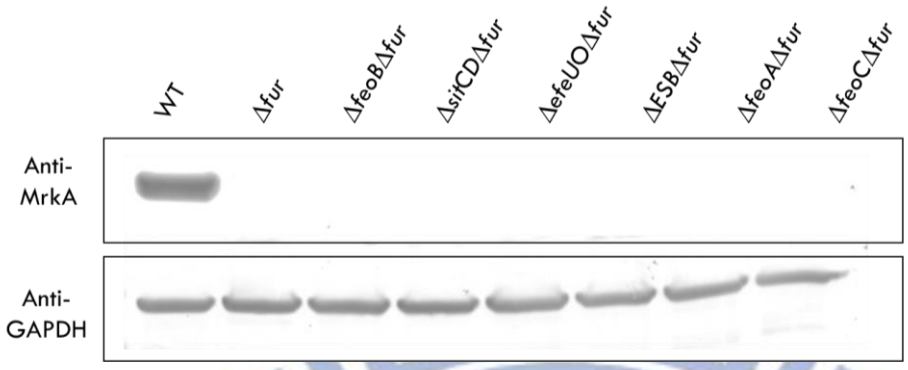
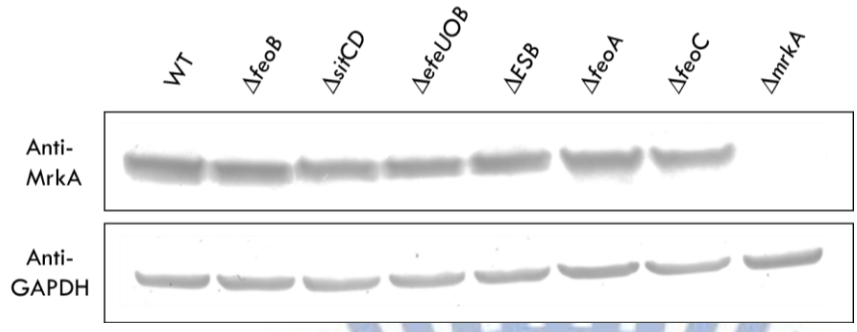


Figure 19. MrkA expression of the Δ*fur*-derived deletion mutants under shaking condition

Total protein of bacterial cultures, approximately 10 μg per lane, were separated by SDS-PAGE, transferred to PVDF membrane and subjected to hybridization against the antibodies of anti-MrkA and anti-GAPDH (as an internal control). Proteins were collected from CG43S3 and Δ*fur*-derived deletion mutants after growing agitatedly at 37 °C for 16 hr.

Static culture

(A) LB



(B) LB + DFX

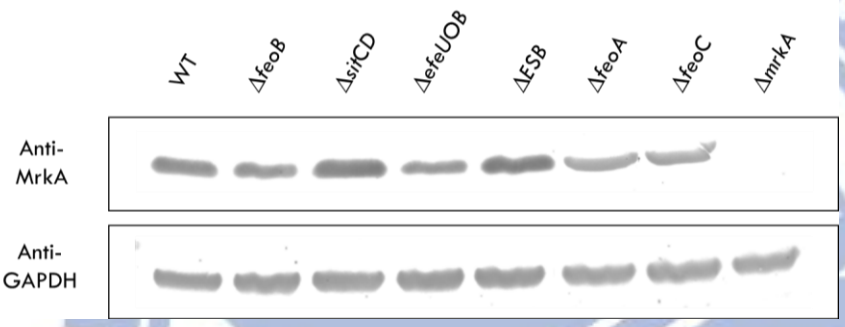


Figure 20. Iron- and oxygen-depleted effect on MrkA expression for the deletion mutants

Total protein of bacterial cultures, approximately 10 μg per lane, were separated by SDS-PAGE, transferred to PVDF membrane and subjected to hybridization against the antibodies of anti-MrkA and anti-GAPDH (as an internal control). Proteins were collected from CG43S3 and deletion mutants that grown statically at 37 °C for 24 h in (A) LB or (B) LB supplemented with DFX

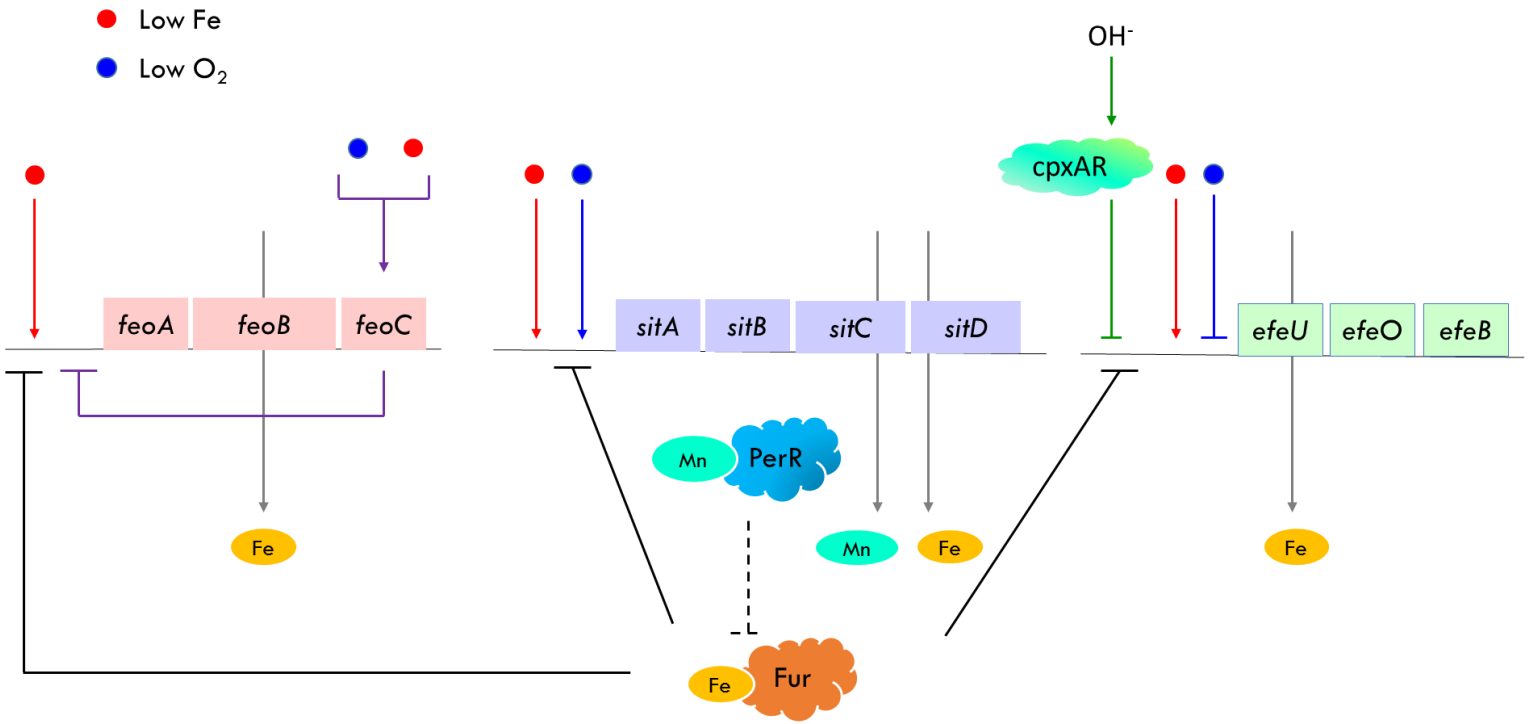


Figure 21. Schematic representation (not to scale) of the proposed regulation of *feoABC*, *sitABCD* and *efeUOB* in CG43S3.

All three systems are induced by iron-depletion (low Fe) but negatively regulated by Fur-Fe^{2+}. FeoC possibly represses expression of *feo* when both iron and oxygen are depleted (low Fe and low O_2). The repression of CpxAR on *efe* expression when CG43S3 in a rather a non-acidic environment (OH^-). The increased level of Mn^{2+} might enable repression of Fur by PerR. [→ represents positively regulates; ⊥ represents negatively regulates]

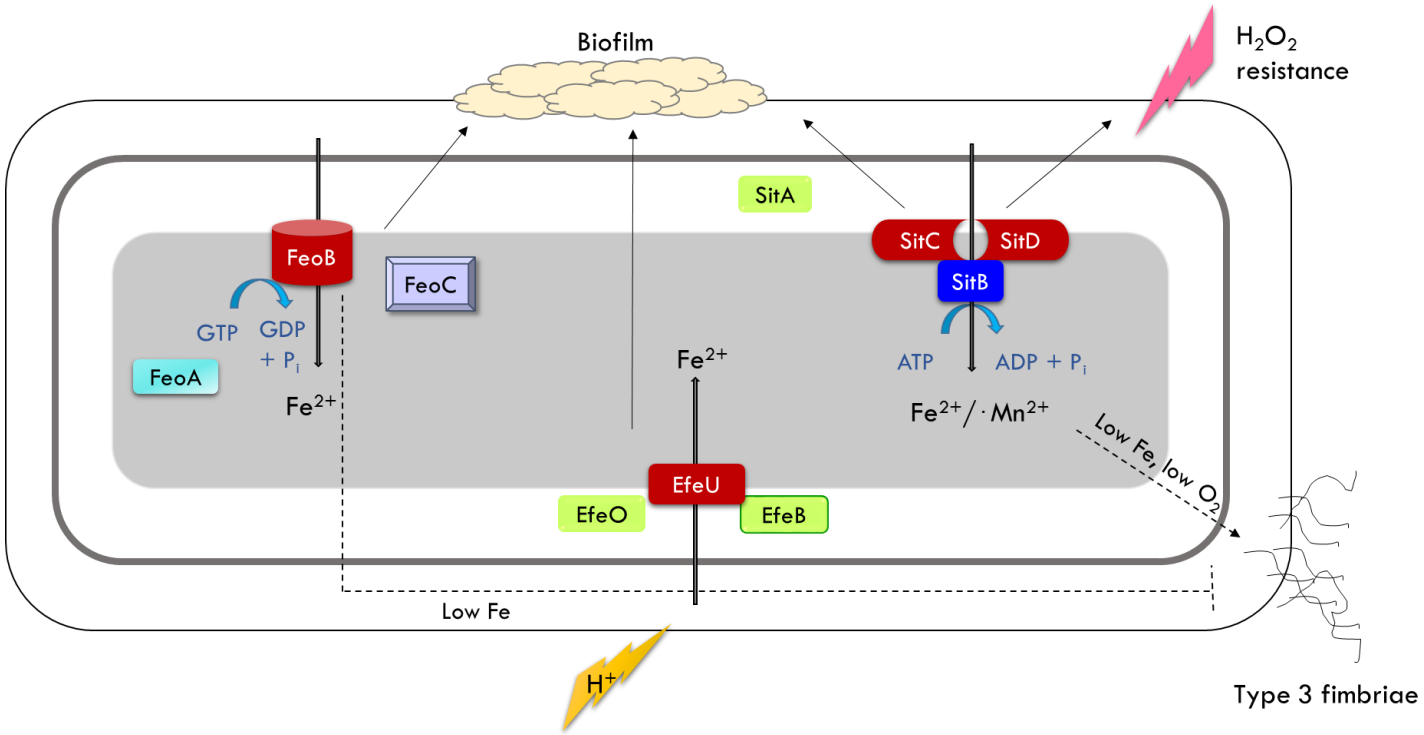


Figure 22. Schematic representation of the proposed model of functional role and location of FeoABC, SitABCD and EfeUOB in CG43S3.

The ferrous iron-acquisition is mediated by FeoB which contains a cytoplasmic GTPase domain to hydrolysis GTP to GDP; cytoplasmic FeoC senses environmental oxygen and iron to regulate *feo* system; the role of FeoA is unclear yet (this model is based on a model proposed by Cartron *et al.* [49]). Transport of ferrous iron or/ and manganese by permease SitC and SitD is activated by the periplasmic

SitA which in turn facilitated by SitB that hydrolyze ATP to ADP. The acid-inducible EfeU uptakes ferrous iron with the assistance of EfeO and EfeB (yet the mechanism is little known). All three systems affect the formation of biofilm in CG43S3. The SitABCD protects CG43S3 against H_2O_2. In a Western blot analysis, FeoB and Sit system negatively regulate the production of major pilin of type 3 fimbriae in iron-depleted (low Fe) or / and microaerobic condition (low O_2). [$\rightarrow$ represents positively regulates; $\perp$ represents negatively regulates]

8.0 Appendices

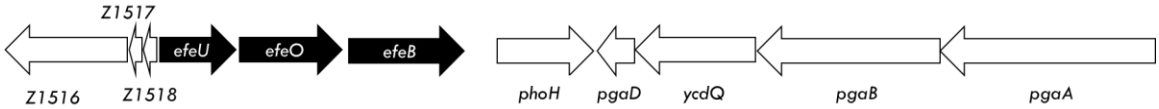
Appendix 1. Iron-acquisition genes in *K. pneumoniae* CG43 that expression were induced in Δ*fur* strain

System		Gene
Fe^{3+}		
	Ferrichrome	*fhuA*
	Aerobactin	*iucA*
	Enterobactin	*fepA*
		fepB
		entC
	Ferric citrate	*fecA*
		fecE
	Salmochelin	*iroB*
	Haem	*hmuR*
Fe^{2+}		
	Ferrous iron	*feoB*
		sitA

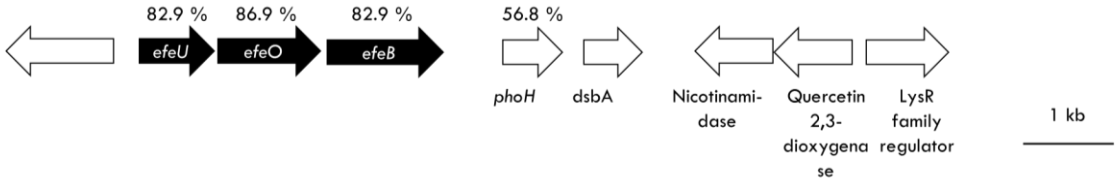
Appendix 2. qRT-PCR analyses of the expression of iron-acquisition genes in *K. pneumoniae* CG43 Δ*fur* Δ*ryhB* and Δ*fur* strains

Up-regulating			**Down-regulating**	
Fe^{3+}			Fe^{3+}	
	Ferrichrome	*fhuA*	Aerobactin	*iucA*
Fe^{2+}			Enterobactin	*fepA*
	Ferrous iron	*sitA*		*fepB*
				entC
				fecA
				fecE

E. coli O157:H7 EDL933



K. pneumoniae CG43



Appendix 3. The comparison of gene organization along with *efeUOB* operon between *E. coli* and *K. pneumoniae* CG43.

Genes located upstream and downstream (white-filled arrows) of *efeUOB* operon (black-filled arrows) in *E. coli* O157:H7 EDL933 and *K. pneumoniae* CG43. The similarity of *efeU*, *efeO*, and *efeB* amino acid sequences between two strains was indicated in percentage.