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碩士論文

Fur 蛋白質在克雷白氏肺炎桿菌 CG43 中的功能探討

Functional characterization of Fur in *Klebsiella pneumoniae* CG43S3

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中文摘要

在細菌生命週期中，具有足量的鐵離子是維持新陳代謝和生長必需的，因此多數細菌演化出同時擁有數個螯鐵系統。然而，過多鐵離子卻會造成氧化自由基形成，這對細菌本身將會造成致命的傷害，是故微生物體內的螯鐵系統都需要受到緊密的調控。在革蘭氏陰性菌中，許多參與螯鐵系統基因轉錄過程都受到 Fur 蛋白質的抑制。此外, 除了調控鐵離子濃度在細菌體內外的平衡，當細菌遭受到酸性或過氧化環境壓力 Fur 蛋白質也參與啟動保護機制。克雷白氏肺炎桿菌 CG43 是一具有毒性的臨床分離株，被 K2 血清型莢膜所包覆。為了瞭解 Fur 在克雷白氏肺炎桿菌 CG43 中扮演的角色，我們建構了 *fur* 基因缺損株，此基因缺損明顯影響細菌生長。有趣的是，*fur* 突變株菌落相較於野生株顯得比較黏稠，透過莢膜多醣定量顯示 *fur* 突變株莢膜明顯增量；進一步分析啟動子活性，我們發現莢膜多醣合成基因組 *orf3-15* 表現量因 *fur* 基因缺損而增加；而 *fur* 突變株對小白老鼠的半致死劑量較野生株減少，顯示 Fur 蛋白質可能參與調控細菌的毒性。另外，我們也發現 *fur* 缺損使克雷白氏肺炎桿菌 CG43 對於酸性(pH 3)和過氧化(H_2O_2 treatment)的環境壓力變得較為敏感。最後，*fur* 的缺損還會提高 *iro*、*iuc* 基因組(攝取三價鐵相關)以及 *feo* 基因組(攝取二價鐵相關)的啟動子活性，顯示 Fur 蛋白質在調控克雷白氏肺炎桿菌體內鐵離子平衡上扮演重要的角色。

Abstract

Bacteria have evolved several acquisition systems for sufficient quantities of iron to support their metabolism and growth. Iron overloading would lead to the formation of hydroxyl radicals and hence microorganisms have equipped a tight regulatory system for iron uptake. In Gram-negative bacteria, Fur protein represses the transcription of many genes that are involved in iron acquisition. In addition to control iron homeostasis, Fur also participates in protective responses to acid stress and oxidative stress. Herein, we report the construction of *fur* deletion mutant and found that the deletion impaired the growth of *Klebsiella pneumoniae* CG43S3, a highly virulent strain heavily encapsulated with K2 serotype. Interestingly, the deletion rendered the bacteria more mucoid phenotype which probably resulted from increasing amount of the glucuronic acid content. In addition, an increased activity of $P_{cps\text{-}orf3\text{-}15}$ was found. The deletion of *fur* slight reduced the LD_{50} using mouse lethality assay suggesting an involvement of Fur in virulence regulation. Moreover, *fur* deletion was found to increase the bacterial sensitivity to either acidic or oxidative stress. Finally, promoter activity measurement revealed that the *fur* deletion enhanced the activity of *iro* and *iuc*, respectively encoding enterobactin and aerobactin siderophore uptake systems, and *feoABC*, coding for ferrous iron uptake system. This indicated that Fur plays a major role for the regulation of iron acquisition system in the bacteria.

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如果每個人都是一顆星星，

那麼我這顆黯淡的隕石，是因為你們的碰觸而有了發光的理由。

像是從外太空被一股莫名引力牽動，我闖入了一個陌生的國度，步行了一段出口近在咫尺的漫漫長路。因為走得吃力，所以在途中的風景更顯得深刻而珍貴，由衷地感謝這兩年出現在我生命裡的所有人，沒有你們，我將不知道如何去完整這趟旅途。

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Abbreviation

BLAST	basiclocal alignment search tool
bp	base pair
CFU	colony forming unit
CPS	capsular polysaccharide
DNA	deoxyribonucleic acid
IPTG	isopropyl-lthio- β -D-galactopyranoside
LB	Luria-Bertani
ORF	open reading frame
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
PCR	polymerase chain reaction
rpm	revolutions per minute

Introduction

Iron is essential for growth

Iron is essential for the growth of nearly all organisms. It could be a cofactor of many enzymes including ribonucleotide reductase, RNA polymerase Ⅲ, various amino acid hydroylases and dioxygenases, and the enzymes, such as superoxide dismutase, catalase and peroxidase, participate in oxygen metabolism in microbial and mammalian cells (83). Iron is also necessary for the activities of cytochromes, hydrogenase, ferridoxin and succinate dehydrogenase that are involved in electron transfer. In vertebrates, T- and B-lymphocyte activity and natural killer cell function are all dependent on iron (11, 12).

Iron exists as oxidized (Fe^{3+}) and reduced form (Fe^{2+}). Ferric iron has lower solubility and ferrous iron participates in Haber-Weiss-Fenton chemistry ($H_2O_2+Fe^{2+} \rightarrow Fe^{3+}+OH^-+OH\cdot$) that causes potentially cell damage (81). Hence, higher organisms have evolved mechanisms to lower the levels of free iron. In human body, the majority of iron is intracellular, either complexed with metalloproteins such as haemoglobin (74.3%), myoglobin (3.3%), catalase (0.11%) and cytochrome C (0.08%), or stored in the iron-storage protein ferritin (16.4%) and its insoluble degradation product haemosiderin (21, 74). In blood, tissues, and tissue fluids, there are concentrations of iron sufficient to supply the needs for cellular metabolism. The

iron-binding protein including transferrin and lactoferrin take charge of the iron transport and recycling. Thus, usable iron in human host is below that required for microbial growth.

Bacteria have evolved several systems for the acquisition of sufficient quantities of iron to support their metabolism and growth. These include production of powerful iron-binding compounds (siderophores), direct utilization or uptake host iron-binding proteins, reduction of the Fe^{3+} insoluble form to soluble useful form of ferrous iron (Fe^{2+}), enzymatic degradation of iron-binding compounds, production of lethal compound (exotoxins) that may eliminate competitors for usable iron resources (77). For instance, *E. coli* strains harboring the plasmid ColV-K30 were reported to own two independent siderophore systems, enterochelin and aerobactin (82, 80). *Neisseria meningitidis* (24), *Haemophilus influenzae* (65), *Vibrio cholerae* (69) and *Campylobacter jejuni* (64) use host iron compounds, heam and heamoglobin. *Listeria monocytogenes* (16), *Pseudomonas aeruginosa* (17), *Bifidobacterium bifidus* (7) and *Streptococcus mutans* (28) were reported to reduce ferric iron at cytoplasmic membrane and subsequently transport ferrous iron into the cytoplasm.

To avoid iron overloading that would lead to the formation of hydroxyl radicals, microorganisms have tight regulation of iron uptake. In Gram-negative bacteria, iron regulation is mediated by the Fur protein, which represses the transcription of many

genes that are involved in iron acquisition across a wide range of species (29). Some bacteria have also evolved mechanism whereby gene transcription is initiated by the availability of iron-loaded siderophores on the cell surface. The best known of these systems is the ferric citrate system in *E. coli* (25). In addition, a global response to iron limitation has been observed at the level tRNA modification (36) observed in *Salmonella typhimurium*, *K. pneumoniae*, *P. aeruginosa* and *N. meningitidis* (56, 2).

Klebsiella pneumoniae

K. pneumoniae is a gram-negative, nonmotile, encapsulated rod-shaped bacterium of the family *Enterobacteriaceae*. It is an opportunistic pathogen, attacking immunocompromised and hospitalized patients suffering severe diseases. *K. pneumoniae* causes community acquired and nosocomial infections, including septicemia, pneumonia, urinary tract infection, meningitis, and purulent abscess at various sites (84).

Klebsiella usually have well developed polysaccharide capsules, which make their colonies characteristic mucoid. Capsules that form thick bundles of fibrillous structures covering the bacterial surface in massive layers is the essential virulence factor. This could protect the bacterium from phagocytosis by polymorphonuclear granulocytes, and prevents killing by bactericidal serum factors (12). Acording to the diverse structures of capsular polysaccharide, *K. pneumoniae* could be classified into

77 serological K antigen types (45). K1 and K2 strains were found to be especially virulent assessed with the mouse peritonitis model (58). Beside the capsular polysaccharide, lipopolysaccharide, adhesin, iorn-acquisition system, and serum resistance factors are also major virulence factors involved in *K. pneumoniae* infections (66).

Multiple siderophore biosynthesis systems have been reported in *K. pneumoniae*, which include enterobactin, aerobactin and yersiniabactin; respectively encoded by *iro*, *iuc* and *ybt* gene clusters (47). We have previously identified two siderophore biosynthesis gene clusters, *iro* and *iuc*, on the large virulence plasmid pLVPK in *K. pneumoniae* CG43, a highly virulent clinical isolate of K2 serotype (14). Besides the ferric iron uptake systems, two ferrous iron uptake systems, *feoABC* and *sitABCD*, could be identified in the genome of *K. pneumoniae* MGH78578 (http://www.ncbi.nlm.nih.gov/sites/entrez).

Fur

In 1978, Ernst *et al*., (26) described a mutation in *S..enterica* Typhimurium, designated *fur* (ferric uptake regulator), that resulted in constitutive expression of several high-affinity iron assimilation systems. Fur, a 17-kDa polypeptide, negatively regulates the iron acquisition systems in most gram negative bacteria. (3, 51) In iron replete condition, Fe^{+2}-Fur binds to the promoter of iron-regulated genes thereby

prevents their expression. Under low iron, Fur is present in the iron-free form, which does not bind to the regulated promoters (3). Fur not only controls iron homeostasis by regulating the promoters of iron uptake systems directly, but also represses a small RNA, RyhB, that in turn negatively regulates the expression of iron-rich enzymes such as succinate dehydrogenase, fumarase, and aconitase. This allows the production of these enzymes to be activated in response to available iron (55).

The N-terminal domain of Fur is involved in DNA binding and the C-terminal domain in dimerization (72). A consensus sequence has been derived for the Fur binding site in *E coli*, and this is referred to as an iron or Fur box (34). The palindromic sequence GATAATGATAATCATTATC has been elucidated for different bacteria (62, 85).

In addition to the role is in the regulation of iron uptake systems, its involvement in regulation of acid tolerance response, oxidative stress response, some metabolic pathways, and expression of virulence factors has been proposed (27). In some of these cases, Fur acted positively rather than negatively in the regulation of the expression of certain genes (61, 37, 23). For example, *fur* deletion resulted in decrease of oxidative stress response and increase of DNA damage in *E. coli* (75). In *S. enterica* Typhimurium, *fur* mutation rendered an acid-sensitive phenotype (30). In *H. pylori*, Fur is also involved in acid resistance (8). In response to iron, Fur is often

but not always involved in autoregulation in many Gram-negative bacteria (19, 1, 53).

FeoABC

Feo system was described for the first time in *E. coli* in 1987 (38). The ferrous iron acquisition system has been experimentally identified in seven additional microbes including *Porphyromonas gingivalis* (18), *Leptospira biflexa* (52), *Helicobacter pylori* (78), *Shigella flexneri* (68), *Salmonella enterica* serovar Typhimurium (10), *Legionella pneumophila* (67), and the cyanobacterium *Synechocystis* sp (44.). In *Campylobacter jejuni*, FeoB-mediated ferrous iron acquisition has been found to play an essential role in bacterial virulence (60).

Feo consists of FeoA, a 75-residue hydrophilic protein probably required to sense the concentrations of iron; FeoB, an integral cytoplasmic membrane protein of 773 amino acids required for ferrous iron acquisition; FeoC (YhgG), a small protein (75 amino acid) with unknown function (43, 54). In *E. coli*, Fur and Fnr binding sites were found in *feo* promoter, and both global regulators have been shown to regulate the expression of Feo system (13).

Materials and methods

■ Bacterial strains, and growth conditions

The bacterial strains and plasmids used in the present study are described in Table 1. *K. pneumoniae* CG43 is clinical isolated strains from Chang Gung Memorial Hospital Linkou branch. *E. coli* and *K. pneumoniae* were cultured aerobically at 37 ℃ in Luria-Bertani (LB) broth or on LB agar plates supplied with appropriate antibiotics. The concentrations of antibiotics added in mediums include streptomysin (500 μg/ml), ampicillin (100 μg/ml), chloramphenicol (35 μg/ml), kanamycin (25 μg/ml), and tetracycline (5 μg/ml).

■ Construction of gene-deletion mutants and complement strains

The *fur* and *feoB* deletion strains were made by allelic exchange. Approximately 1000 bp sequences flanking both sides of the deleted region were cloned into plasmid pKAS46 (49), 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 plasmids were then mobilized to *K. pnumoniae* CG43S3 through conjugation from *E. coli* S17-1 λ*pir*. The transconjugants, carrying with constructed plasmid integrated in the chromosome via homologous

recombination, were selected by ampicillin and kanamycin on minimal medium.one of colonies was grown in LB at 37 ℃ for overnight and then spread onto a LB plate containing 500 μg/ml streptomysin. The streptomysin-resistant and kanamycin sensitive colonies were selected and the deletion of gene verified by PCR. The primer pairs used for PCR amplification are listed in Table 3.

To construct the complement strains (Table2), the target gene including the promoter and coding sequences was amplified with the specific primers, the PCR product cloned to yT&A, and then subcloned to pRK415.

■ **Quantification of CPS**

CPS was extracted by using the method described (22). Briefly, bacteria were collected from 500 μl of culture medium and mixed with 100 μl of 1% Zwittergent 3-14 detergent (Sigma-Aldrich, Milwaukee, WI) in 100 mM citric acid (pH 2.0). The mixture was incubated at 50℃ for 20 min. After centrifugation, 250 μl of the supernatant was transferred to a new tube and CPS was precipitated with 1 ml of absolute ethanol. The pellet was then dissolved in 200 μl distilled water and a 1,200 μl of 12.5 mM borax (Sigma-Aldrich, Milwaukee, WI) in H_2SO_4 was added. The mixture was vigorously vortexed, boiled for 5 min, cooled, and then 20 μl 0.15% 3-hydroxydiphenol

(Sigma-Aldrich, Milwaukee, WI) was added and the absorbance at 520 nm was measured. The uronic acid content was determined from a standard curve of glucuronic acid (Sigma-Aldrich, Milwaukee, WI) and expressed as μg per 10^9 CFU.

- **Survivals under acid stress**

According to the described method with some modification (37), the bacteria were grown overnight in LB and 1/20 of the bacteria refreshed grown for 3 h. The cultures were then transferred to pH 5.8 or pH 4.4 LB broth (adjusted with HCl) for acid adaptation 1 h. Finally, the cultures were subjected to pH 3 acid stress for 1 h and then plating onto LB agar for viable counts. Data shown are from the representative experiment performed triplicately.

- **Survival rate of oxidative stress**

As described with some modification (75), the bacteria grown in LB medium at 37℃ overnight were diluted in 1/20 and refreshed grown for 3 h, and H_2O_2 from 0 to 30 mM was added. After 20 min of incubation by shaking at 37℃, the viable bacteria were determined by plating the cultures onto LB plates.

- **Streptonigrin resistance**

As the described method (67), the bacteria were grown in LB broth for 16 h and 100 μl of the bacteria in 10-fold serial dilution were plated on LB agar containing either 0.75, 1.5 or 3 μM streptonigrin.

■ β-galactosidase activity assay

β-galactosidase was assayed according to the method of Miller (57). The bacteria in the early or late logarithmic growth phase (optical density at 600 nm 0.4 or 0.7) were taken 100 μl, and mixed with 900 μl 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 and incubated for 10 min at 30℃. Subsequently, 200 μl of 4 mg/ml *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG) was added and the mixture vortexed for 10 s, then incubated at 30℃ 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 of the supernatant was measured OD_{420}. One unit of β-galactosidase is defined as the hydrolysis of 1 nmol ONPG per min per mg protein.

■ Mouse lethality assay

The 4~5-week-old female Balb/c mice were obtained from National

Laboratory Animal Center and were acclimatized in an animal house for 1 week. The tested bacteria were cultured at 37℃ for 16 h in LB broth. Four mice of a group were injected intraperitoneally with bacteria resuspended in 0.2 ml PBS. The 50% lethal doses, based on the number of survivors after 10 days, were calculated by the method of Reed and Muench (59) and expressed as colony forming units (CFU).

Results

✧ **Sequence analysis of *K. pneumoniae* Fur.**

In comparison of the Fur amino acid sequences of *K. pneumoniae* CG43, *Escherichia coli* K12, and *Shigella flexneri* 5b (http://www.ncbi.nlm.nih.gov /GenBank) as shown in Fig. 1B revealed 94% identityl feature. Analysis of the genomic location also revealed a conserved organization with *fur* gene in the middle, upstream with *fldA*, coding for a putative citrate-proton symporter, and Flavodoxin 1 encoding gene *citA* at the downstream (Fig. 1A).

✧ **Construction of *fur* deletion muatnt.**

As shown in Fig. 2A, 454 bp of the *fur* coding sequence was designed to be deleted by the allelic exchange method. The PCR products of 1743 bp and 1289 bp obtained in Fig. 2B using the same primer pair on different templates DNA from *K. pneumoniae* CG43S3 and the selected *fur* mutant demonstrated the deletion. The *fur* deletion appeared to negatively affect the growth in LB or M9 broth (Fig. 3A & 3B). Introducing the plasmid pfcpRK, pRK415 carrying *fur* gene, into the mutant was found to compensate the deficiency.

✧ **Fur is involved in the regulation of CPS biosynthesis.**

Interestingly, the mutant strain displayed larger and more glistening colony morphology than the wild type on LB agar (Fig. 4A). Introduction of pfcpRK into the *fur* mutant restored the phenotype (Fig. 4B). When the bacteria cultures were subjected to low-speed centrifugation, the mutant were precipitated much slower than its parental strain *K. pneumoniae* CG43S3 (Fig. 5A). Subsequently, the CPS production in CG43S3 and the mutant were quantified by measuring the glucuronic acid content, the core component of the K2 CPS. As shown in Fig. 5B, the deletion of *fur* enhanced the CPS synthesis. The transformation of the *fur* mutant with the plasmid pfcpRK decreased the sedimentation rate (Fig. 6A) and CPS production (Fig. 6B).

As shown in Fig. 7A, biosynthesis of *K. pneumoniae* CG43 K2 CPS has been shown to be determined by three major transcripts namely *orf 1-2*, *orf 3-15*, and *orf 16-17* (88). The promoter activity measurement as shown in Fig. 7B, deletion of *fur* had positive effect on the expression of *cps* clusters *orf 3-15* and *orf 16-17*. However, no apparent effects on the promoter activities of RmpA, RmpA2, RcsB, and KvgA, the regulators of mucoid phenotype, were noted (Fig. 8).

✧ **Effect of *fur* deletion on the acid sensitivity**

If *K. pneumoniae* Fur is involved in acid stress response as the Fur reported for many other bacteria (37, 39, 70, 73) is also investigated. As shown in Fig. 9, *fur* deletion had negative effect on the bacterial survivals at pH 3 regardless the adaptation at pH 5.8 or pH4.4. The introduction of the *fur*-expression plasmid pfcpRK appeared to restore the viability.

✧ **The *fur* deletion reduced the bacterial survival upon H_2O_2 treatment**

Hydrogen peroxide causing DNA damage via the Fenton reaction had been demonstrated. *E. coli* Fur has been associated with the bacterial survivals with H_2O_2 treatment (75). To know if Fur provides protection in *K. pneumoniae* under H_2O_2 treatment, different concentrations of H_2O_2 were added to the cultures of the wild type bacteria, △*fur* muatnt, △*fur* [pRK415], and △*fur* [pfcpRK]. While the concentration of H_2O_2 reached to 20 mM, *fur* deletion had apparent effect on the bacterial survivals. As shown in Fig. 10, an increased sensitivity to H_2O_2 of the mutant was observed and the complementation with pfcpRK was able to restored the survivals.

✧ **A decreased LD_{50} of the *fur* mutant**

In many pathogenic bacteria, deletion of *fur* causes virulence reduction (46). To know whether Fur affects the virulence of *K. pneumoniae* CG43S3, LD_{50} of the mutant using mouse lethality assay was determined. As shown in Table. 4, LD_{50} of the Fur deletion mutant slightly decreased.

✧ **Fur regulates the expression of the iron acquisition systems, *iro*, *iuc* and *feo*.**

In many bacteria, iron acquisition was regulated in part by the Fur protein (15). The effect of *fur* deletion on the expression of the iron acquisition systems including *iro*, *iuc*, and *feoABC*, all carrying a typical Fur box on the putative promoters (respectively Fig. 11A, 12A, and 13A), were examined. As shown respectively in Fig. 11B, 12B, and 13B, the putative promoter activity of *iro*, *iuc*, and *feo* were determined using LacZ as promoter reporter. As shown in Fig. 11C, 12C, and 13C, the activity of P_{iro}, P_{iuc} or P_{feo} apparently enhanced in the *fur* deletion mutant. Although a consensus Fur box could be identified upstream of the *fur* gene (Fig. 14A), the deletion of *fur* had no effect on the expression of fur using the promoter activity measurement (Fig. 14 B and C).

✧ **Sequence analysis of *feoABC* and construction of *feoB* deletion mutant**

In general, Feo system consists of at least two genes, *feoA* and *feoB*, which encode, respectively, an iron-sensing protein and an iron permease. In some organisms, an additional locus, *feoC*, is present immediately downstream (13). Amino acid sequence comparison with *E. coli* K12 FeoA, FeoB, and FeoC revealed identity of respectively 81%, 67%, and 69%.

To investigate if Feo system plays a major role in ferrous iron uptake in *K. pneumoniae*, *feoB* deletion mutant was constructed. The entire FeoB coding sequence of 2316 bp as shown in Fig. 15A was deleted and the deletion in the selected mutant verified by PCR (Fig 15B).

To investigate the *feoB* deletion effect, growth of *K. pneumoniae* CG43S3 and the *feoB* deletion mutant were monitored in LB broth and LB broth loaded with ferrous iron. As shown in Fig. 16A and B, no obvious difference could be observed between the wild type and the *feoB* mutant in LB or the medium loaded with 50 μM ferrous iron. The deletion effect on the resistance to streptonigrin was also investigated. This antibiotic, streptonigrin, possesses an iron-dependent toxicity and has been used to select *E. coli* FeoB mutant (67). As shown in Fig. 17, addition of 0.75 μM to 3 μM of streptonigrin had no apparent difference for the survivals between *K. pneumoniae* CG43S3 and the *feoB* mutant.

✧ **LD_{50} of the *feoB* mutant using mouse lethality assay**

The LD_{50} determined as shown in Table 4 indicated that the deletion of *feoB* does not cause apparent effect on the virulence of *K. pneumoniae*.

Discussion

Fur protein was named as “ferric iron uptake regulator”, which directly points out the tight correlation of this protein with iron uptake. However, more and more recent articles indicate that Fur plays roles not only in iron uptake, but also others functions including acid tolerance in *S. typhimurium* and *S. flexneri* (76, 9), and oxidative stress response in *E. coli* and *H. pylori* (75, 79).

Similar to the report in *Pseudomonas aeruginosa* (77), the deletion of *fur* appeared to retard the growth. While the changes of colony phenotype to more sparkling and transparent has not been mentioned. In addition to the reported functions, our results add to a novel role of Fur which is a negative regulator for the expression of the *cps* genes. Analysis the promoter sequences of the *cps-orf 3-15* and *cps-orf 16-17*, it revealed a consensus Fur box on the $P_{orf\ 3\text{-}15}$, but not on $P_{orf16\text{-}17}$. Although Fur box was identified for promoters of RmpA, RmpA2, and RcsB, there was no distinct difference in the LacZ activity assay between the wild type and the Fur gene mutant. Besides, there was no Fur box on P_{kvgA}, and no effect of Fur deletion on P_{kvgA} activity was observed.

pH homeostasis is the process whereby a cell maintains a relatively constant intracellular pH over a broad range of external values. The acid tolerance response

(ATR), one of mechanisms that keep pH homeostasis, has been discovered in *S. typhimurium* and *E. coli* (77). The acid tolerance response is triggered in *Salmonella* species at pH values between 6.0 and 5.5, but protects bacteria against much stronger acid (pH 3 to 4.0) (32). In *S. typhimurium*, the *fur* gene product has been implicated to contribute a key regulatory function to the acid tolerance response (32). Furthermore, Fur has been shown to participate in responses to low pH in *S. flexneri* (41) and *H. pylori* (35). In *K. pneumoniae* CG43S3, the deletion of *fur* reduced the survivals under acid treatment suggesting a positive regulatory role in the acid stress response.

The permanent derepression of iron assimilation system in Fur mutant could produce oxidative stress leading to various cell damages. *E. coli fur* mutant has been shown to be sensitive to hydrogen peroxide, increased oxidative DNA damage, and mutations under aerobic conditions (75, 86). In *H. pylori*, Fur was also proved as a key role in antioxidant systems (79). As shown in Fig. 10, following the increasing doses of hydrogen peroxide, from 10 mM to 30 mM, the survival rate of the *fur* mutant decreased more sharply than the wild type and the mutation effect could be complemented by introducing a *fur*-expression plasmid. This indicates that Fur protein probably also acts a positive role in the oxidative stress response.

Deletion of *fur* caused attenuated virulence in *S. flexneri* (63) and *A. tumefaciens* (46), and a competitive defect in colonization in *H. pylori* (35). To assess

the role of Fur in *K. pneumoniae*, LD_{50} using mouse lethality assay of the mutant was measured. Although the *fur* deletion mutant appeared to grow more slowly than the wild type, a slight reduction of LD_{50} was observed.

The *iroBCDN* gene cluster found on the virulence-associated plasmid pLVPK is responsible for enterochelin synthesis. In *S. enteric*, a typical Fur DNA binding site on the *iro* promoter region is required for the regulation of Fur on the expression of *iro* gene cluster (4, 5). The *iucABCD* involved in the biosynthesis of aerobactin is transcriptionally regulated by Fur in *E. coli* (20). In *K. pneumoniae* NTUH2044, *iucABCDiutA* was shown to be significantly prevalent in PLA-associated (pyogenic liver abcess) isolates (KP-PLA) (40). The *iucA* in *K. pneumoniae* CG43S3 has been reported as an IVE gene (48). In either P_{iro}, P_{iuc}, or P_{feo}, putative Fur box could be found. The LacZ activity measurement further supports that the regulation of *iro*, *iuc*, or *feo* by Fur is probably via direct binding to the Fur box. These indicated that Fur is a major regulator for iron acquisition in *K. pneumoniae*. Autoregulation of Fur has been described in *K. pneumoniae* (1), and a putative Fur box was predicted on the *fur* promoter. However, the LacZ activity measurement revealed no apparent regulation.

The presence of multiple iron transport system in *K. pneumoniae* suggests that iron acquisition systems are needed during infection. The PLA-associated strains were shown to be able to use wider range of iron sources than non-PLA-associated strains

(40). Whereas, obvious negative effect of *feoB* deletion suggested another ferrous iron uptake system SitABCD plays more important role in *K. pneumoniae*. The possibility could be demonstrated by generation of *sitBCD* deletion mutant. It is also possible that the activity of other ferric iron uptake system acts to compensate the deficiency of *feoB*.

References

1. Achenbach LA, Yang W. 1997. The fur gene from Klebsiella pneumoniae: characterization, genomic organization and phylogenetic analysis. *Gene* 185: 201-7

2. Ala'Aldeen DA, Borriello SP, Griffiths E. 1993. Alteration of transfer ribonucleic acid of Neisseria meningitidis during growth in the presence of human transferrin. *J Med Microbiol* 38: 442-8

3. Bagg A, Neilands JB. 1987. Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in Escherichia coli. *Biochemistry* 26: 5471-7

4. Baumler AJ, Norris TL, Lasco T, Voight W, Reissbrodt R, Rabsch W, Heffron F. 1998. IroN, a novel outer membrane siderophore receptor characteristic of Salmonella enterica. *J Bacteriol* 180: 1446-53

5. Baumler AJ, Tsolis RM, van der Velden AW, Stojiljkovic I, Anic S, Heffron F. 1996. Identification of a new iron regulated locus of Salmonella typhi. *Gene* 183: 207-13

6. Bereswill S, Greiner S, van Vliet AH, Waidner B, Fassbinder F, Schiltz E, Kusters JG, Kist M. 2000. Regulation of ferritin-mediated cytoplasmic iron

storage by the ferric uptake regulator homolog (Fur) of Helicobacter pylori. *J Bacteriol* 182: 5948-53

7. Bezkorovainy A, Topouzian N, Miller-Catchpole R. 1986. Mechanisms of ferric and ferrous iron uptake by Bifidobacterium bifidum var. pennsylvanicus. *Clin Physiol Biochem* 4: 150-8

8. Bijlsma JJ, Waidner B, Vliet AH, Hughes NJ, Hag S, Bereswill S, Kelly DJ, Vandenbroucke-Grauls CM, Kist M, Kusters JG. 2002. The Helicobacter pylori homologue of the ferric uptake regulator is involved in acid resistance. *Infect Immun* 70: 606-11

9. Boulette ML, Payne SM. 2007. Anaerobic regulation of Shigella flexneri virulence: ArcA regulates Fur and iron acquisition genes. *J Bacteriol* 189: 6957-67

10. Boyer E, Bergevin I, Malo D, Gros P, Cellier MF. 2002. Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of Salmonella enterica serovar Typhimurium. *Infect Immun* 70: 6032-42

11. Brock JH, Mainou-Fowler T. 1986. Iron and immunity. *Proc Nutr Soc* 45: 305-15

12. Bryan CF, Leech SH, Bozelka B. 1986. The immunoregulatory nature of iron. II. Lymphocyte surface marker expression. *J Leukoc Biol* 40: 589-600

13. Cartron ML, Maddocks S, Gillingham P, Craven CJ, Andrews SC. 2006. Feo--transport of ferrous iron into bacteria. *Biometals* 19: 143-57

14. Chen YT, Chang HY, Lai YC, Pan CC, Tsai SF, Peng HL. 2004. Sequencing and analysis of the large virulence plasmid pLVPK of Klebsiella pneumoniae CG43. *Gene* 337: 189-98

15. Conrad ME, Umbreit JN. 2000. Iron absorption and transport-an update. *Am J Hematol* 64: 287-98

16. Cowart RE, Foster BG. 1985. Differential effects of iron on the growth of Listeria monocytogenes: minimum requirements and mechanism of acquisition. *J Infect Dis* 151: 721-30

17. Cox CD. 1986. Role of pyocyanin in the acquisition of iron from transferrin. *Infect Immun* 52: 263-70

18. Dashper SG, Butler CA, Lissel JP, Paolini RA, Hoffmann B, Veith PD, O'Brien-Simpson NM, Snelgrove SL, Tsiros JT, Reynolds EC. 2005. A novel Porphyromonas gingivalis FeoB plays a role in manganese accumulation. *J Biol Chem* 280: 28095-102

19. De Lorenzo V, Herrero M, Giovannini F, Neilands JB. 1988. Fur (ferric uptake regulation) protein and CAP (catabolite-activator protein) modulate transcription of fur gene in Escherichia coli. *Eur J Biochem* 173: 537-46

20. de Lorenzo V, Neilands JB. 1986. Characterization of iucA and iucC genes of the aerobactin system of plasmid ColV-K30 in Escherichia coli. *J Bacteriol* 167: 350-5

21. Deiss A. 1983. Iron metabolism in reticuloendothelial cells. *Semin Hematol* 20: 81-90

22. Domenico P, Schwartz S, Cunha BA. 1989. Reduction of capsular polysaccharide production in Klebsiella pneumoniae by sodium salicylate. *Infect Immun* 57: 3778-82

23. Dubrac S, Touati D. 2000. Fur positive regulation of iron superoxide dismutase in Escherichia coli: functional analysis of the sodB promoter. *J Bacteriol* 182: 3802-8

24. Dyer DW, West EP, Sparling PF. 1987. Effects of serum carrier proteins on the growth of pathogenic neisseriae with heme-bound iron. *Infect Immun* 55: 2171-5

25. Enz S, Mahren S, Stroeher UH, Braun V. 2000. Surface signaling in ferric citrate transport gene induction: interaction of the FecA, FecR, and FecI regulatory proteins. *J Bacteriol* 182: 637-46

26. Ernst JF, Bennett RL, Rothfield LI. 1978. Constitutive expression of the iron-enterochelin and ferrichrome uptake systems in a mutant strain of

Salmonella typhimurium. *J Bacteriol* 135: 928-34

27. Escolar L, Perez-Martin J, de Lorenzo V. 1999. Opening the iron box: transcriptional metalloregulation by the Fur protein. *J Bacteriol* 181: 6223-9

28. Evans SL, Arceneaux JE, Byers BR, Martin ME, Aranha H. 1986. Ferrous iron transport in Streptococcus mutans. *J Bacteriol* 168: 1096-9

29. Faraldo-Gomez JD, Sansom MS. 2003. Acquisition of siderophores in gram-negative bacteria. *Nat Rev Mol Cell Biol* 4: 105-16

30. Foster JW. 1993. The acid tolerance response of Salmonella typhimurium involves transient synthesis of key acid shock proteins. *J Bacteriol* 175: 1981-7

31. Foster JW, Hall HK. 1990. Adaptive acidification tolerance response of Salmonella typhimurium. *J Bacteriol* 172: 771-8

32. Foster JW, Hall HK. 1991. Inducible pH homeostasis and the acid tolerance response of Salmonella typhimurium. *J Bacteriol* 173: 5129-35

33. Foster JW, Hall HK. 1992. Effect of Salmonella typhimurium ferric uptake regulator (fur) mutations on iron- and pH-regulated protein synthesis. *J Bacteriol* 174: 4317-23

34. Friedman YE, O'Brian MR. 2003. A novel DNA-binding site for the ferric uptake regulator (Fur) protein from Bradyrhizobium japonicum. *J Biol Chem*

278: 38395-401

35. Gancz H, Censini S, Merrell DS. 2006. Iron and pH homeostasis intersect at the level of Fur regulation in the gastric pathogen Helicobacter pylori. *Infect Immun* 74: 602-14

36. Griffiths E. 1991. Iron and bacterial virulence--a brief overview. *Biol Met* 4: 7-13

37. Hall HK, Foster JW. 1996. The role of fur in the acid tolerance response of Salmonella typhimurium is physiologically and genetically separable from its role in iron acquisition. *J Bacteriol* 178: 5683-91

38. Hantke K. 1987. Ferrous iron transport mutants in Escherichia coli K12 *FEMS Microbiology Letters* 44: 53-7

39. Hantke K. 1987. Selection procedure for deregulated iron transport mutants (fur) in Escherichia coli K 12: fur not only affects iron metabolism. *Mol Gen Genet* 210: 135-9

40. Hsieh PF, Lin TL, Lee CZ, Tsai SF, Wang JT. 2008. Serum-induced iron-acquisition systems and TonB contribute to virulence in Klebsiella pneumoniae causing primary pyogenic liver abscess. *J Infect Dis* 197: 1717-27

41. Jennison AV, Verma NK. 2007. The acid-resistance pathways of Shigella flexneri 2457T. *Microbiology* 153: 2593-602

42. Joseph Sambrook PMCI, Melbourne, Australia; David Russell, ed. 2001. *Molecular Cloning: A Laboratory Manual*: University of Texas Southwestern Medical Center, Dallas

43. Kammler M, Schon C, Hantke K. 1993. Characterization of the ferrous iron uptake system of Escherichia coli. *J Bacteriol* 175: 6212-9

44. Katoh H, Hagino N, Grossman AR, Ogawa T. 2001. Genes essential to iron transport in the cyanobacterium Synechocystis sp. strain PCC 6803. *J Bacteriol* 183: 2779-84

45. Keynan Y, Rubinstein E. 2007. The changing face of Klebsiella pneumoniae infections in the community. *Int J Antimicrob Agents* 30: 385-9

46. Kitphati W, Ngok-Ngam P, Suwanmaneerat S, Sukchawalit R, Mongkolsuk S. 2007. Agrobacterium tumefaciens fur has important physiological roles in iron and manganese homeostasis, the oxidative stress response, and full virulence. *Appl Environ Microbiol* 73: 4760-8

47. Koczura R, Kaznowski A. 2003. Occurrence of the Yersinia high-pathogenicity island and iron uptake systems in clinical isolates of Klebsiella pneumoniae. *Microb Pathog* 35: 197-202

48. Lai YC, Peng HL, Chang HY. 2001. Identification of genes induced in vivo during Klebsiella pneumoniae CG43 infection. *Infect Immun* 69: 7140-5

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

50. Lin MF, Clinton GM. 1988. The epidermal growth factor receptor from prostate cells is dephosphorylated by a prostate-specific phosphotyrosyl phosphatase. *Mol Cell Biol* 8: 5477-85

51. Litwin CM, Boyko SA, Calderwood SB. 1992. Cloning, sequencing, and transcriptional regulation of the Vibrio cholerae fur gene. *J Bacteriol* 174: 1897-903

52. Louvel H, Saint Girons I, Picardeau M. 2005. Isolation and characterization of FecA- and FeoB-mediated iron acquisition systems of the spirochete Leptospira biflexa by random insertional mutagenesis. *J Bacteriol* 187: 3249-54

53. Lowe CA, Asghar AH, Shalom G, Shaw JG, Thomas MS. 2001. The Burkholderia cepacia fur gene: co-localization with omlA and absence of regulation by iron. *Microbiology* 147: 1303-14

54. Marlovits TC, Haase W, Herrmann C, Aller SG, Unger VM. 2002. The membrane protein FeoB contains an intramolecular G protein essential for Fe(II) uptake in bacteria. *Proc Natl Acad Sci U S A* 99: 16243-8

55. Masse E, Arguin M. 2005. Ironing out the problem: new mechanisms of iron homeostasis. *Trends Biochem Sci* 30: 462-8

56. McLennan BD, Buck M, Humphreys J, Griffiths E. 1981. Iron-related modification of bacterial transfer RNA. *Nucleic Acids Res* 9: 2629-40

57. Miller JH. 1972. *Experiments in Molecular Genetics,Cold Spring Habor Laboratory Press*: Cold Spring Habor,HY

58. Mizuta K, Ohta M, Mori M, Hasegawa T, Nakashima I, Kato N. 1983. Virulence for mice of Klebsiella strains belonging to the O1 group: relationship to their capsular (K) types. *Infect Immun* 40: 56-61

59. Muench Ra. 1938. *A simple method of estimating the fifty percent endpoints*: Am. J. Hyg. 493-7 pp.

60. Naikare H, Palyada K, Panciera R, Marlow D, Stintzi A. 2006. Major role for FeoB in Campylobacter jejuni ferrous iron acquisition, gut colonization, and intracellular survival. *Infect Immun* 74: 5433-44

61. Niederhoffer EC, Naranjo CM, Bradley KL, Fee JA. 1990. Control of Escherichia coli superoxide dismutase (sodA and sodB) genes by the ferric uptake regulation (fur) locus. *J Bacteriol* 172: 1930-8

62. Ochsner UA, Vasil ML. 1996. Gene repression by the ferric uptake regulator in Pseudomonas aeruginosa: cycle selection of iron-regulated genes. *Proc Natl*

Acad Sci U S A 93: 4409-14

63. Payne SM, Wyckoff EE, Murphy ER, Oglesby AG, Boulette ML, Davies NM. 2006. Iron and pathogenesis of Shigella: iron acquisition in the intracellular environment. *Biometals* 19: 173-80

64. Pickett CL, Auffenberg T, Pesci EC, Sheen VL, Jusuf SS. 1992. Iron acquisition and hemolysin production by Campylobacter jejuni. *Infect Immun* 60: 3872-7

65. Pidcock KA, Wooten JA, Daley BA, Stull TL. 1988. Iron acquisition by Haemophilus influenzae. *Infect Immun* 56: 721-5

66. Podschun R, Ullmann U. 1998. Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev* 11: 589-603

67. Robey M, Cianciotto NP. 2002. Legionella pneumophila feoAB promotes ferrous iron uptake and intracellular infection. *Infect Immun* 70: 5659-69

68. Runyen-Janecky LJ, Reeves SA, Gonzales EG, Payne SM. 2003. Contribution of the Shigella flexneri Sit, Iuc, and Feo iron acquisition systems to iron acquisition in vitro and in cultured cells. *Infect Immun* 71: 1919-28

69. Stoebner JA, Payne SM. 1988. Iron-regulated hemolysin production and utilization of heme and hemoglobin by Vibrio cholerae. *Infect Immun* 56:

2891-5

70. Stojiljkovic I, Baumler AJ, Hantke K. 1994. Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated Escherichia coli genes by a fur titration assay. *J Mol Biol* 236: 531-45

71. Stojiljkovic I, Cobeljic M, Hantke K. 1993. Escherichia coli K-12 ferrous iron uptake mutants are impaired in their ability to colonize the mouse intestine. *FEMS Microbiol Lett* 108: 111-5

72. Stojiljkovic I, Hantke K. 1995. Functional domains of the Escherichia coli ferric uptake regulator protein (Fur). *Mol Gen Genet* 247: 199-205

73. Tardat B, Touati D. 1991. Two global regulators repress the anaerobic expression of MnSOD in Escherichia coli::Fur (ferric uptake regulation) and Arc (aerobic respiration control). *Mol Microbiol* 5: 455-65

74. Theil EC. 1987. Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annu Rev Biochem* 56: 289-315

75. Touati D, Jacques M, Tardat B, Bouchard L, Despied S. 1995. Lethal oxidative damage and mutagenesis are generated by iron in delta fur mutants of Escherichia coli: protective role of superoxide dismutase. *J Bacteriol* 177: 2305-14

76. Tsolis RM, Baumler AJ, Stojiljkovic I, Heffron F. 1995. Fur regulon of

Salmonella typhimurium: identification of new iron-regulated genes. *J Bacteriol* 177: 4628-37

77. Vasil ML, Ochsner UA. 1999. The response of Pseudomonas aeruginosa to iron: genetics, biochemistry and virulence. *Mol Microbiol* 34: 399-413

78. Velayudhan J, Hughes NJ, McColm AA, Bagshaw J, Clayton CL, Andrews SC, Kelly DJ. 2000. Iron acquisition and virulence in Helicobacter pylori: a major role for FeoB, a high-affinity ferrous iron transporter. *Mol Microbiol* 37: 274-86

79. Wang G, Alamuri P, Maier RJ. 2006. The diverse antioxidant systems of Helicobacter pylori. *Mol Microbiol* 61: 847-60

80. Warner PJ, Williams PH, Bindereif A, Neilands JB. 1981. ColV plasmid-specific aerobactin synthesis by invasive strains of Escherichia coli. *Infect Immun* 33: 540-5

81. Weinberg ED. 1989. Cellular regulation of iron assimilation. *Q Rev Biol* 64: 261-90

82. Williams PH. 1979. Novel iron uptake system specified by ColV plasmids: an important component in the virulence of invasive strains of Escherichia coli. *Infect Immun* 26: 925-32

83. Wooldridge KG, Williams PH. 1993. Iron uptake mechanisms of pathogenic

bacteria. *FEMS Microbiol Rev* 12: 325-48

84. Yu WL, Ko WC, Cheng KC, Lee HC, Ke DS, Lee CC, Fung CP, Chuang YC. 2006. Association between rmpA and magA genes and clinical syndromes caused by Klebsiella pneumoniae in Taiwan. *Clin Infect Dis* 42: 1351-8

85. Zheleznova EE, Crosa JH, Brennan RG. 2000. Characterization of the DNA- and metal-binding properties of Vibrio anguillarum fur reveals conservation of a structural Zn(2+) ion. *J Bacteriol* 182: 6264-7

86. Zheng M, Doan B, Schneider TD, Storz G. 1999. OxyR and SoxRS regulation of fur. *J Bacteriol* 181: 4639-43

87 賴怡琪，2002，克雷白氏肺炎桿菌 CG43 致病相關基因的搜尋，國立清華大學，生命科學所，博士論文

88 陳育聖，2006，探討大毒性質體 pLVPK 在克雷白氏肺炎桿菌致病過程中扮演的角色，國立交通大學，生物科技研究所，碩士論文

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* △ *(lac-proAB)*	Laboratory stock
S17-1λ*pir*	Tp^r Sm^r *recA, thi, pro,* $hsdR^-M^+$ [RP4-2-Tc::Mu:Km^rTn*7*] (*pir*)	De Lorenzo *et al.*, 1994
K. pneumoniae:		
CG43	Clinical isolate of K2 serotype	Laboratory stock
CG43-S3	△*rspl*, Sm^r	Laboratory stock
Z01	CG43-S3△*lacZ* Sm^r	Laboratory stock
△*fur*	CG43-S3△*fur* Sm^r	This study
△*feoB*	CG43-S3△*feoB* Sm^r	This study
Z01△*fur*	Z01△*fur* Sm^r	This study

Table 2. Plasmids used and constructed in this study

Plasmids	Relevant characteristic	Source or reference
yT&A vector	PCR cloning vector, Ap^r	Sigma
pKAS46	Suicide vector, Ap^r Km^r	Novagene
pRK415	Shuttle vector, *mob*$^+$, Tc^r	Laboratory stock
pLacZ15	A derivative of pYC016 (87), containing lacZ as a reporter, Cm^r	Laboratory stock
pfur2K46	pKAS46 carrying a △*fur* fragment	This study
pfeoB2K46	pKAS46 carrying a △*feoB* fragment	This study
pfcpRK	A 843 bp PCR product of the *fur* locus with the putative promoter cloned into pRK415	This study
pirocyyT	A 455 bp PCR product of the *iro* putative promoter region cloned into yT&A	This study
piuccyyT	A 721 bp PCR product of the *iuc* putative promoter region cloned into yT&A	This study
pfeoyT	A 564 bp PCR product of the *feo* putative promoter region cloned into yT&A	This study
pfuryT	A 426 bp PCR product of the *fur* putative promoter region cloned into yT&A	This study
pirocyZ15	A *Bam*HI/*Bgl*II fragment of pirocyyT cloned into the pLacZ15	This study
piuccyZ15	A *Bam*HI/*Bgl*II fragment of piuccyyT cloned into the pLacZ15	This study
pfeoZ15	A *Bam*HI/*Bgl*II fragment of pfeoyT cloned into the pLacZ15	This study
pfurZ15	A *Bam*HI/*Bgl*II fragment of pfuryT cloned into the pLacZ15	This study
pRmpAZ15	A 0.5 kb fragment of the *rmpA* putative promoter region cloned into the pLacZ15	Laboratory stock
pRmpA2Z15	A 0.5 kb fragment of the *rmpA2* putative promoter region cloned into the pLacZ15	Laboratory stock
pRcsBZ15	A 0.4 kb fragment of the *rcsB* putative promoter region cloned into the pLacZ15	Laboratory stock
pKvgAZ15	A 0.2 kb fragment of the *kvgA* putative promoter region cloned into the pLacZ15	Laboratory stock
porf1-2Z15	A 0.8 kb fragment of the *cps orf1-2* promoter region cloned into the pLacZ15	Laboratory stock
porf3-15Z15	A 0.9 kb fragment of the *cps orf3-15* promoter region cloned into the pLacZ15	Laboratory stock
porf16-17Z15	A 0.4 kb fragment of the *cps orf16-17* promoter region cloned into the pLacZ15	Laboratory stock

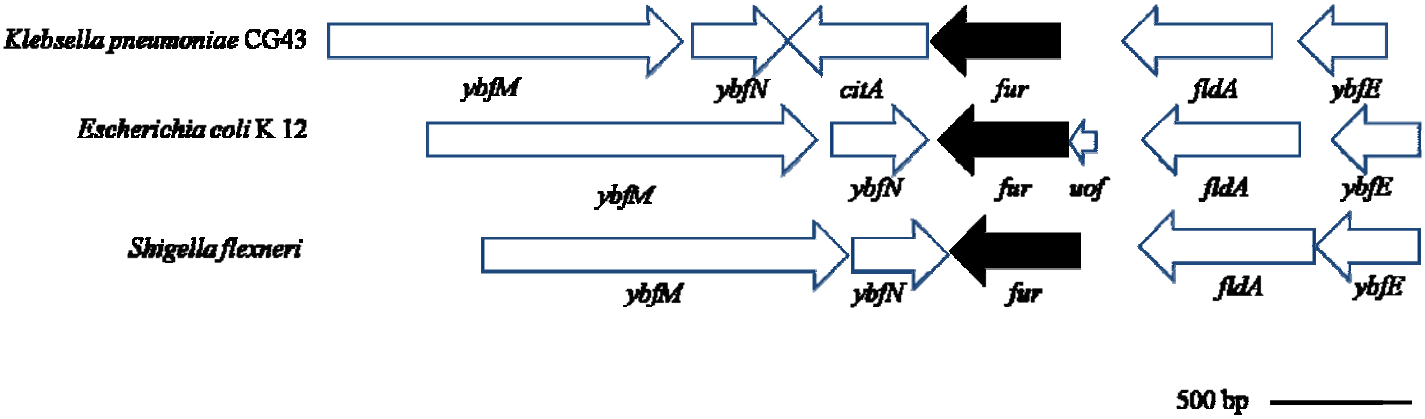
Table 3. Primers used in this study

Primer	Sequence
CY001	5'-GAATTCTGCTGATGACCCAGTTAACC-3'
CY002	5'-GGATCCGTTGTCAGTCATGCGGAATC-3'
CY003	5'-GGATCCACGCGGTGGAAACATAATTC-3'
CY004	5'-GAATTCACCTCTGGGAGAACGACAATG-3'
CY007	5'-TCTAGAGGCAGGTTGGCTCTTCAGTC-3'
CY008	5'-GGATCCATGAAGACAGCCAGCCGGA-3'
CY009	5'-AGATCTGCCCAGCCTTCTTTAATGCGG-3'
Pfeo01	5'-GAATTCTCACCAACGTCACCAACTTC-3'
Pfeo04	5'-TCTAGACAAACCATGGGCACAGAGA-3'
Pfeo05	5'-GGTACCGTGGTCTTGCTGGAGTTAGG-3'
Pfeo06	5'-GGTACCTGCCACTAAGGAGGGACTGT-3'
Pfeop01	5'-GGATCCCAACAGCGCGATGATGGAT-3'
Pfeop02	5'-AGATCTGCCAGCATGCCGAGGGAGA-3'
Piucp01	5'-GGATCCAGAGGGTGATTTGCCAGCAT-3'
Piucp02	5'-AGATCTGGAAGCACTGAGCAGCCACA-3'
Pirop01	5'-GGATCCGATTTCAGTACGGCATGGAC-3'
Pirop02	5'-AGATCTACGGGAAACGCCTGTGCCA-3'

Table 4. LD_{50} using mouse lethality assay

strain	CG43S3	*Δfur*	*ΔfeoB*
LD_{50}(cfu)	1.33×10^{4}	8.8×10^{3}	1.35×10^{4}

(A)



(B)

```
                1                                                  50
K. pneumoniae CG43  MTDNNTALKKAGLKVTLPRLKILEVLQEPDNHHVSAEDLYKRLIDMGEEIGLATVYRVLNQFDDAGIVTRHNFEG
E. coli K-12        MTDNNTALKKAGLKVTLPRLKILEVLQEPDNHHVSAEDLYKRLIDMGEEIGLATVYRVLNQFDDAGIVTRHNFEG
S. flexneri 5b      MTDNNTALKKAGLKVTLPRLKILEVLQEPDNHHVSAEDLYKRLIDMGEEIGLATVYRVLNQFDDAGIVTRHNFEG

Consensus           MTDNNTALKKAGLKVTLPRLKILEVLQEPDNHHVSAEDLYKRLIDMGEEIGLATVYRVLNQFDDAGIVTRHNFEG

                77                 100                                 140
K. pneumoniae CG43  GKSVFELTQQHHHDHLICLDCGKVIEFSDDSIELRQREIASRHGIRLTNHSLYLYGHCAEGDCREDEHAHDAVE
E. coli K-12        GKSVFELTQQHHHDHLICLDCGKVIEFSDDSIEARQREIAAKHGIRLTNHSLYLYGHCAEGDCREDEHAHEGK-
S. flexneri 5b      GKSVFELTQQHHHDHLICLDCGKVIIEFSDDSIEARQREIAAKHGIRLTNHSLYLYGHCAEGDCREDEHAHEGK-

Consensus           GKSVFELTQQHHHDHLICLDCGKVIEFSDDSIEARQREIAAKHGIRLTNHSLYLYGHCAEGDCREDEHAHEGK
```

Fig. 1. (A) The comparison of genes organization along with *fur* (B) Fur Protein sequence alignment. Sequences shown are *Klebsella pneumoniae* CG43 Fur, *Escherichia coli* K-12 Fur, and *Shigella flexneri* Fur (http://www.ncbi.nlm.nih.gov /GenBank). The putative regulatory Fe-sensing site residues (S1) are indicated with circles, and the structural Zn-binding site residues (S2) are indicated with triangles.

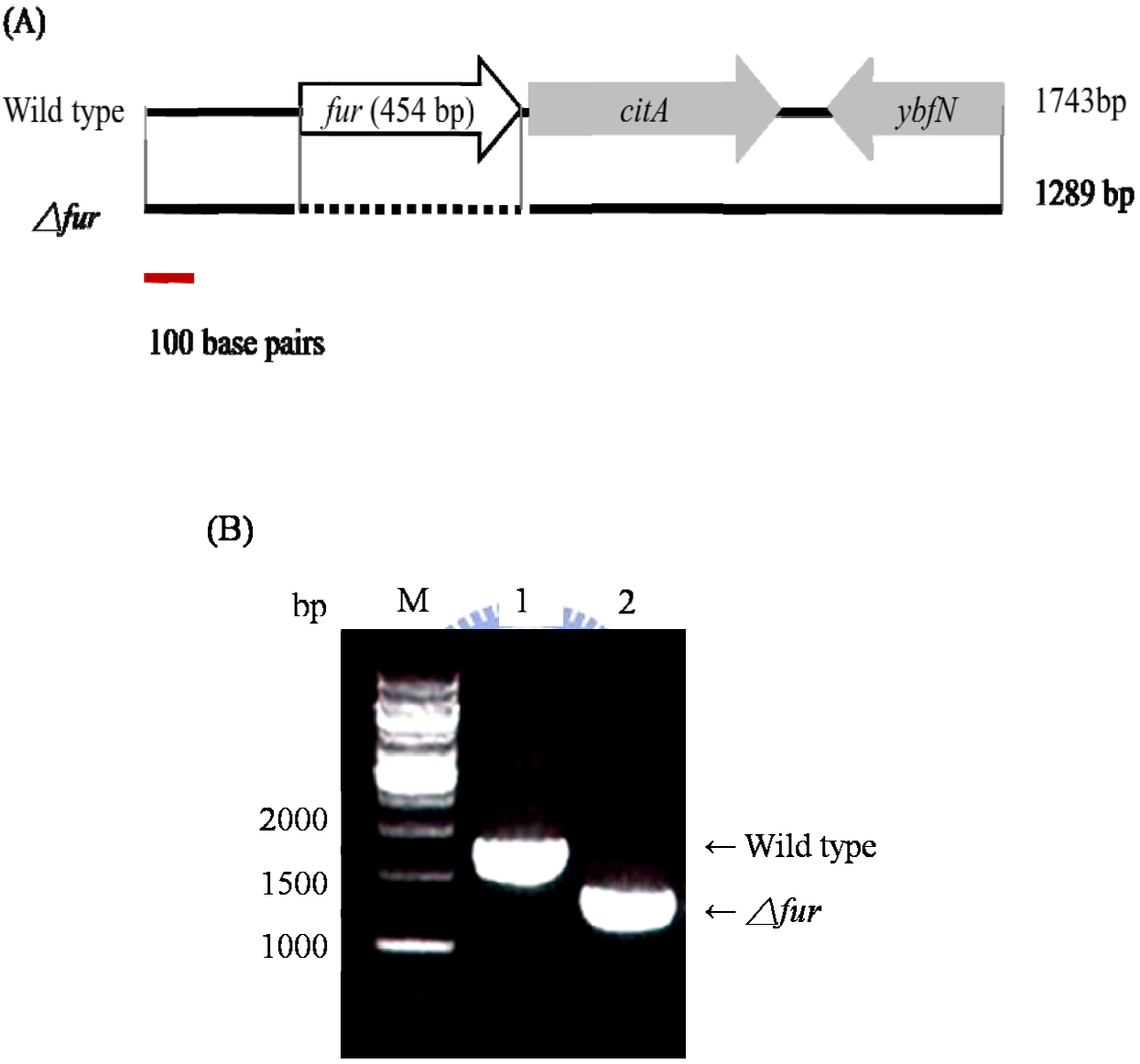


Fig. 2. Schematic representation of the *fur* deletion (A) and the deletion verified by PCR (B). M: marker. Lane 1: *K. pneumoniae*CG43S3. Lane 2: *K. pneumoniae*CG43S3△*fur.*

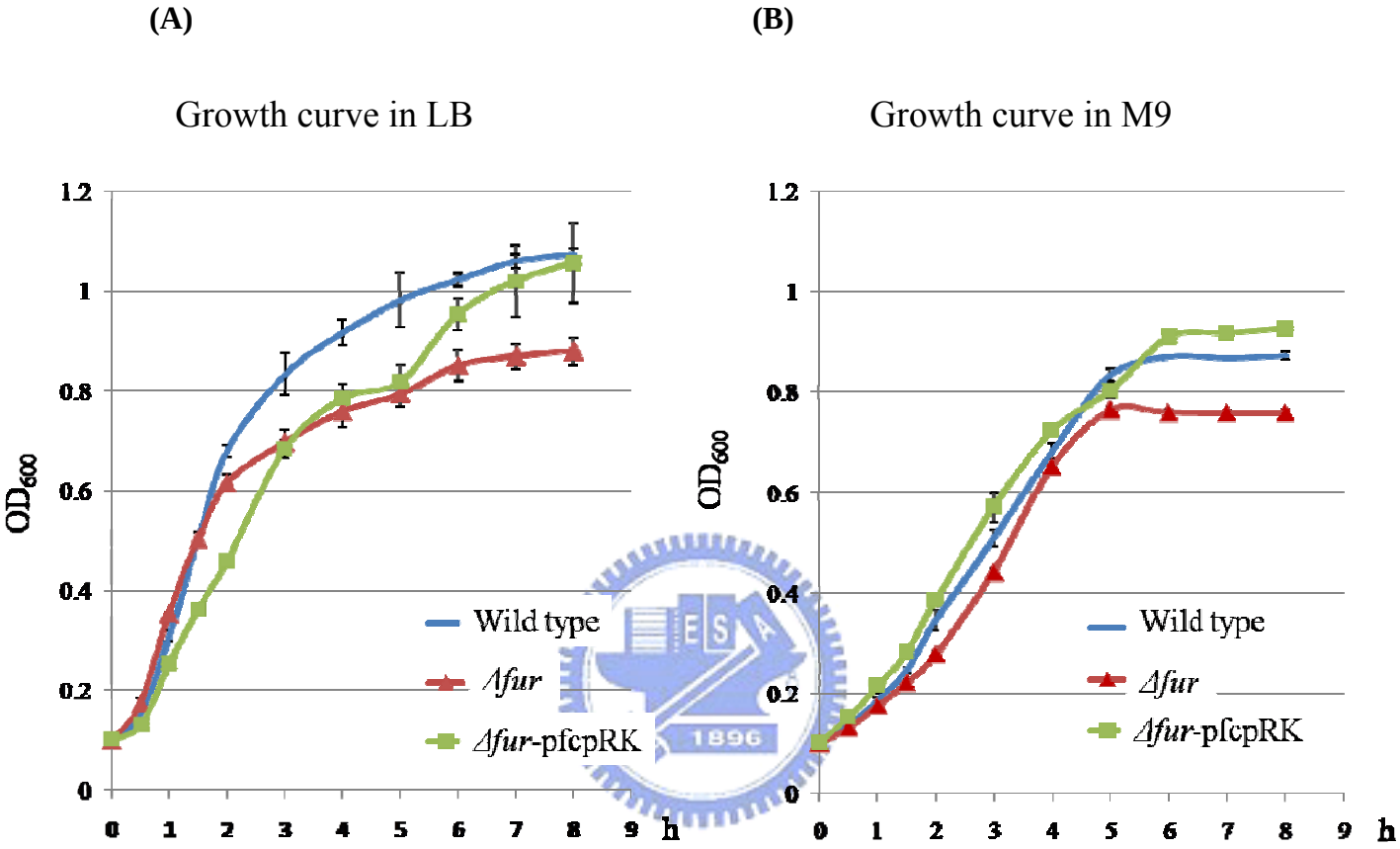


Fig. 3. Fur gene deletion effect on the growth. *K. pneumoniae* CG43S3 (blue line), *fur* deletion mutant (triangle line) and *fur* complement (square line) were growth in LB broth (A) or M9 broth (B). The concentrations of antibiotics added in mediums include streptomysin (500 μg/ml) for *K. pneumoniae* CG43S3 and *fur* deletion mutant; tetracycline (5 μg/ml) for *fur* complement.

(A)

Δfur  **Wild type**

(B)

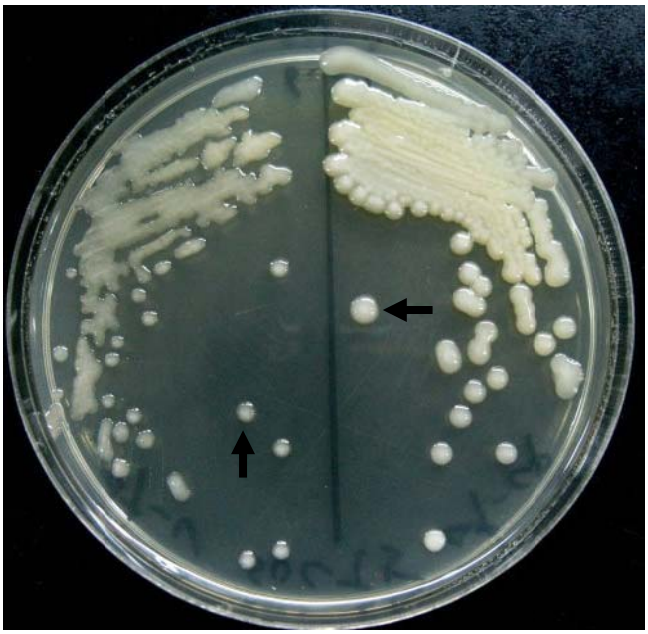
***Δfur*-pRK415**  ***Δfur*-pfcpRK**

Fig. 4. Fur gene deletion rendered a more viscous phenotype. The bacteria were streaked on SOC agar (42) at 37℃, 20 h. The concentrations of antibiotics added in mediums include streptomysin (500 μg/ml) for *K. pneumoniae* CG43S3 and *fur* deletion mutant; tetracycline (5 μg/ml) for *Δfur*-pfcpRK and *Δfur*-pRK415.

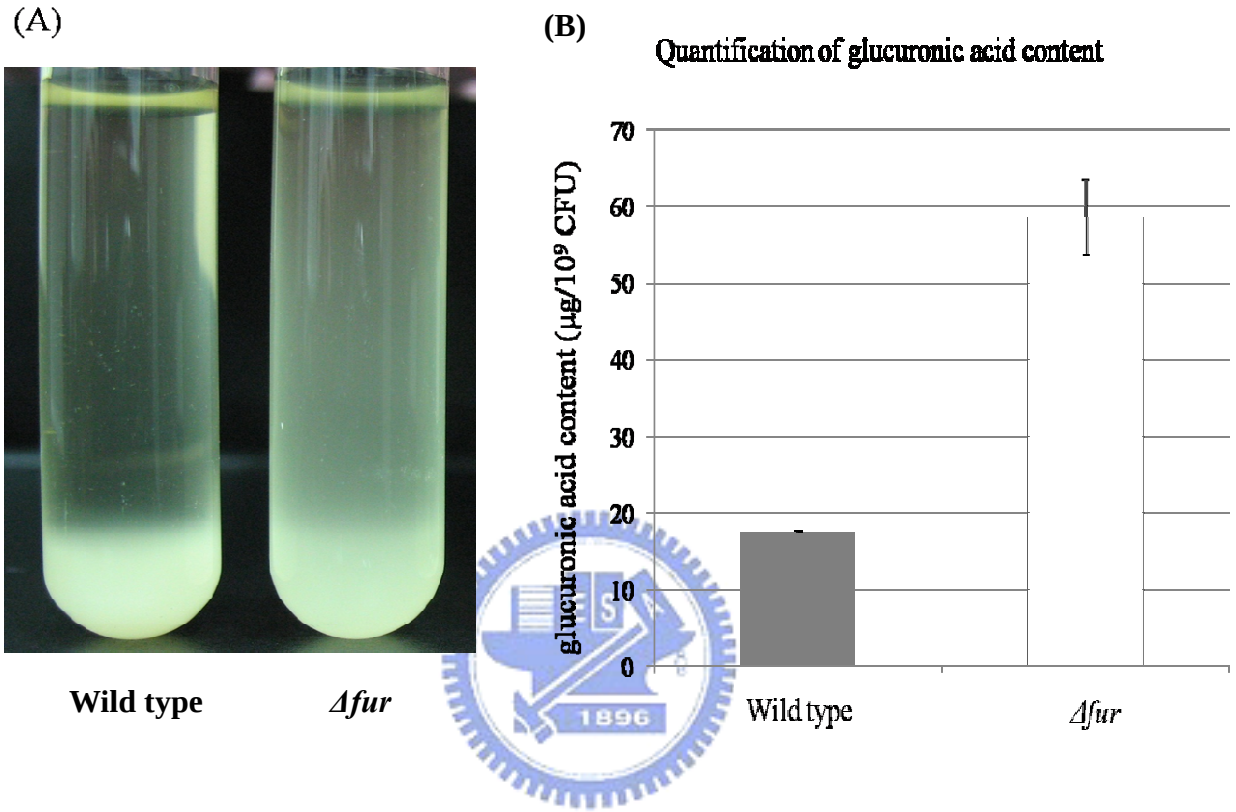


Fig. 5. CPS analysis of *K. pneumoniae* CG43S3 and *K. pneumoniae* CG43S3△*fur* using sedimentation assay (A), and glucuronic acid measurement (B). The glucuronic acid contents were determined after the bacteria were incubated in LB broth added streptomysin (500 μg/ml) for 16 h.

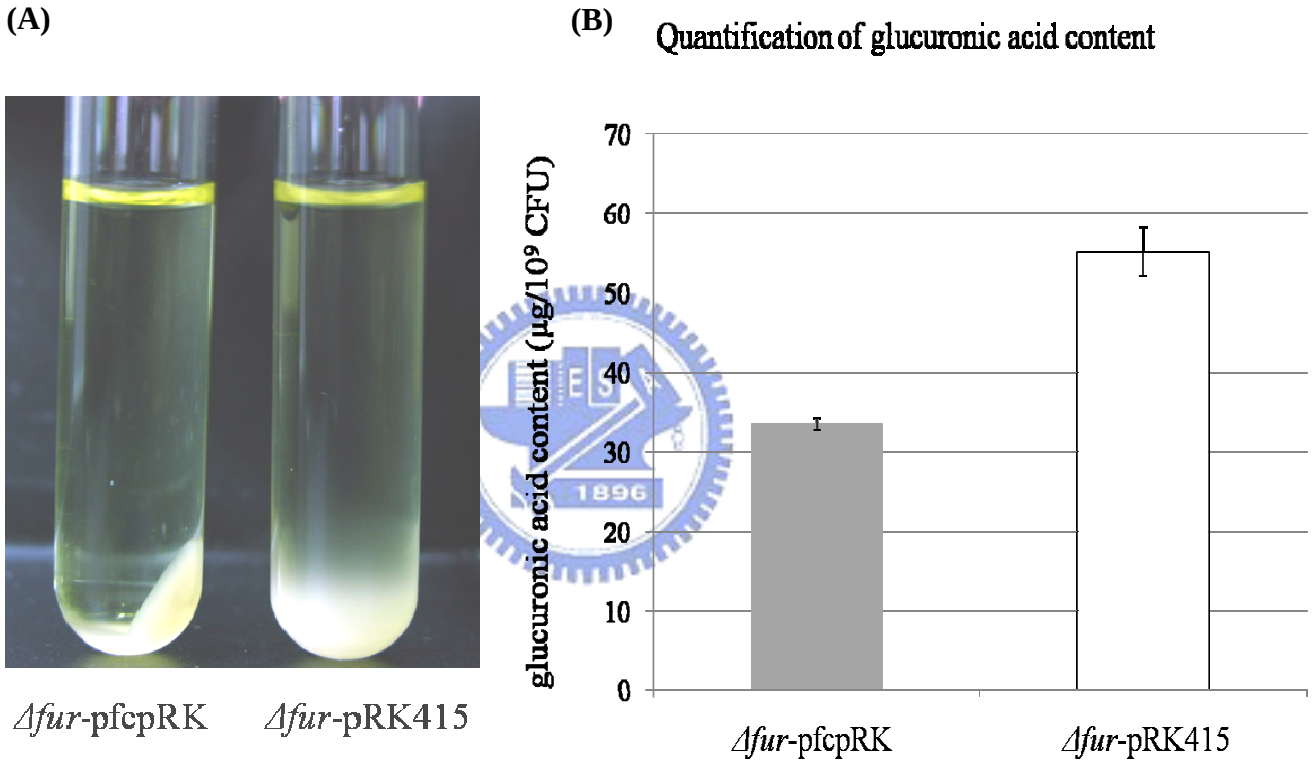


Fig. 6. CPS analysis of *K. pneumoniae* CG43S3-pRK415 and *K. pneumoniae* CG43S3△*fur*-pfcpRK using sedimentation assay (A), and glucuronic acid measurement (B). The glucuronic acid contents were determined after the bacteria were incubated in LB broth added tetracycline (5 μg/ml) for 16 h.

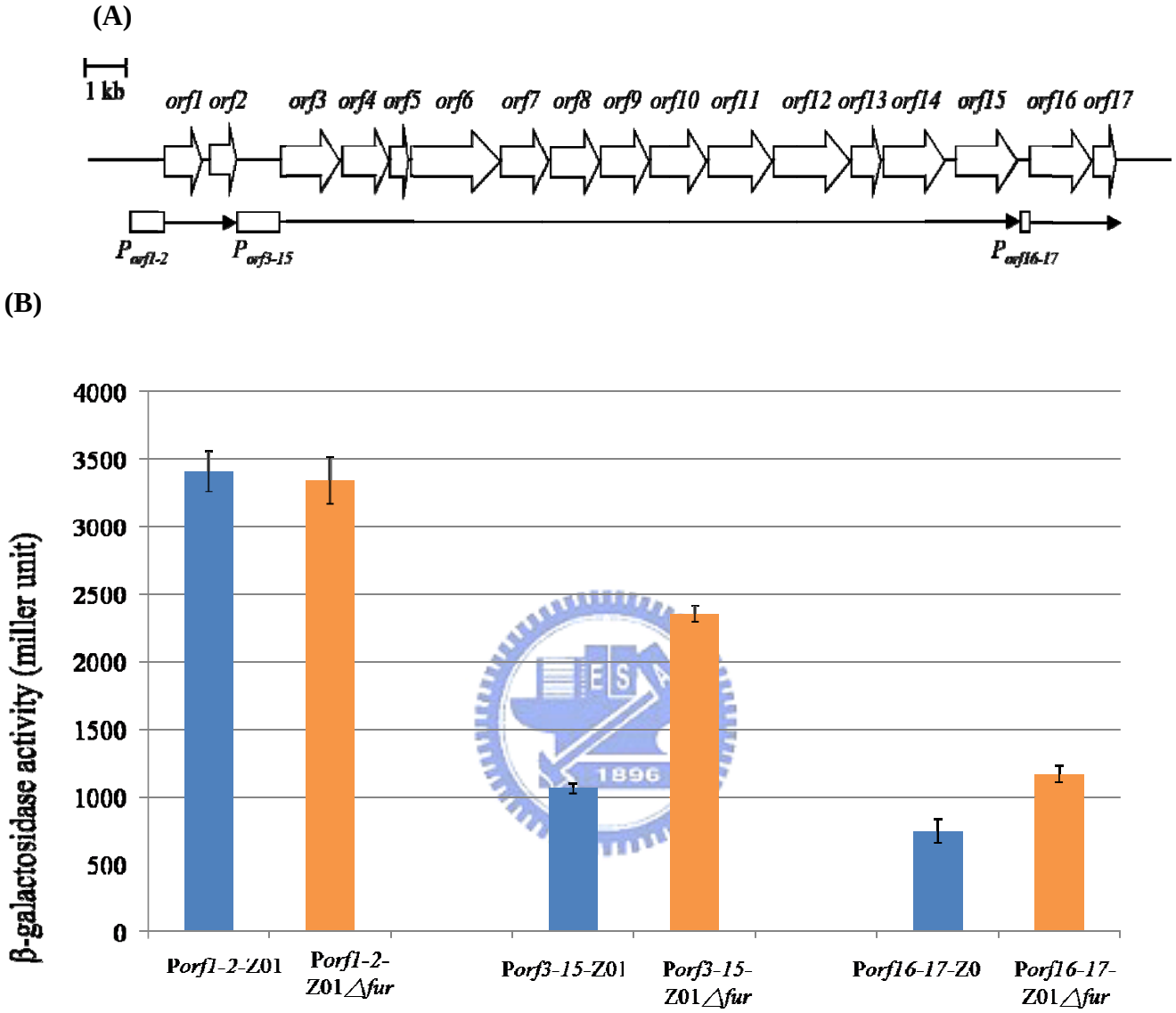


Fig. 7. Deletion effect of Fur on the expression of CPS genes. Schematic representation of the CPS genes promoters of *K. pneumoniae* CG43 (88) (A)**,** and the activity of P_{orf1-2}, $P_{orf3-15}$ and $P_{orf16-17}$ (B). The promoter activities were determined for 1/20 dilution of the overnight grown bacteria subjected to refreshed grown for 6 h.

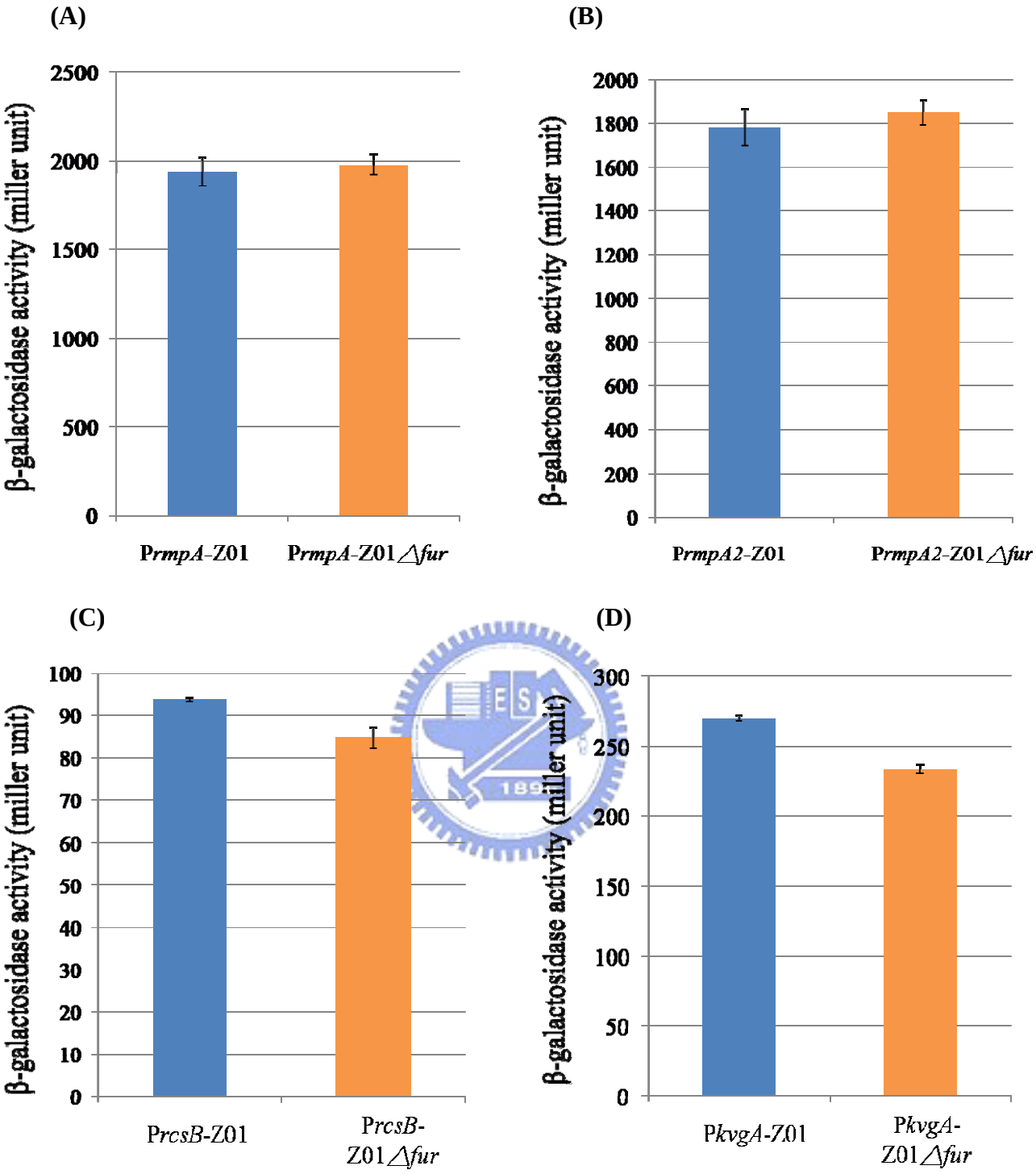


Fig. 8. Deletion effect of Fur on the expression of *rmpA, rmpA2, rcsB* and *kvgA.* The activity of P_{rmpA} (A), P_{rmpA2} (B), P_{rcsB} (C) and P_{kvgA} (D) were determined after the overnight grown bacteria in 1/20 dilution suspended in LB and refreshed grown for 6 h.

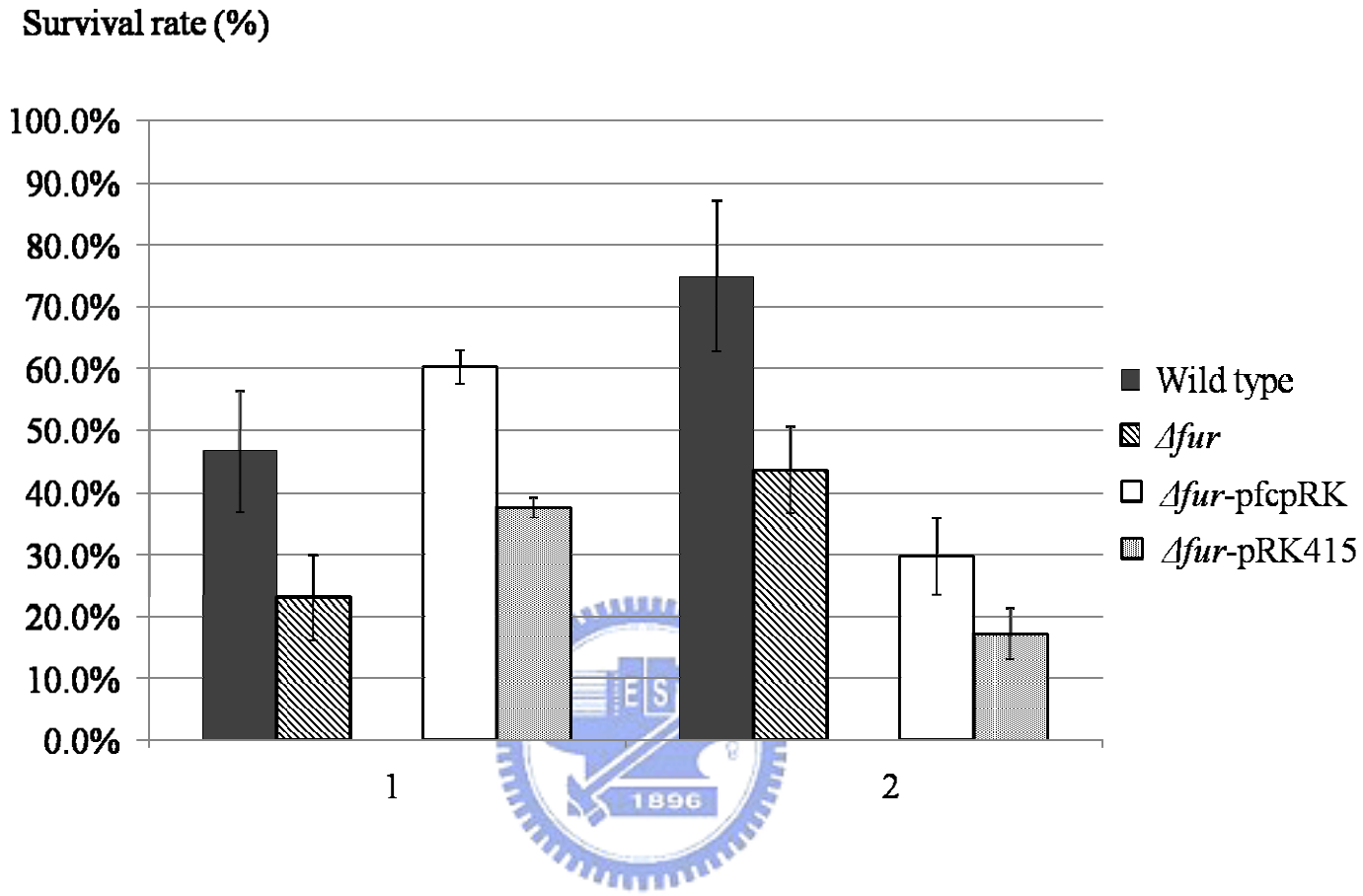


Fig. 9. Deletion effect of *fur* on the acid stress response. Wild type (black bar), Fur deletion mutant (twill bar), *fur* complement strain (white bar) and pRK415 containing strain (gray bar) were grown to mid-log phase, adapted at pH 5.8 (1) or pH 4.4 (2) for 1 h and then transferred to pH 3 for 1 h.

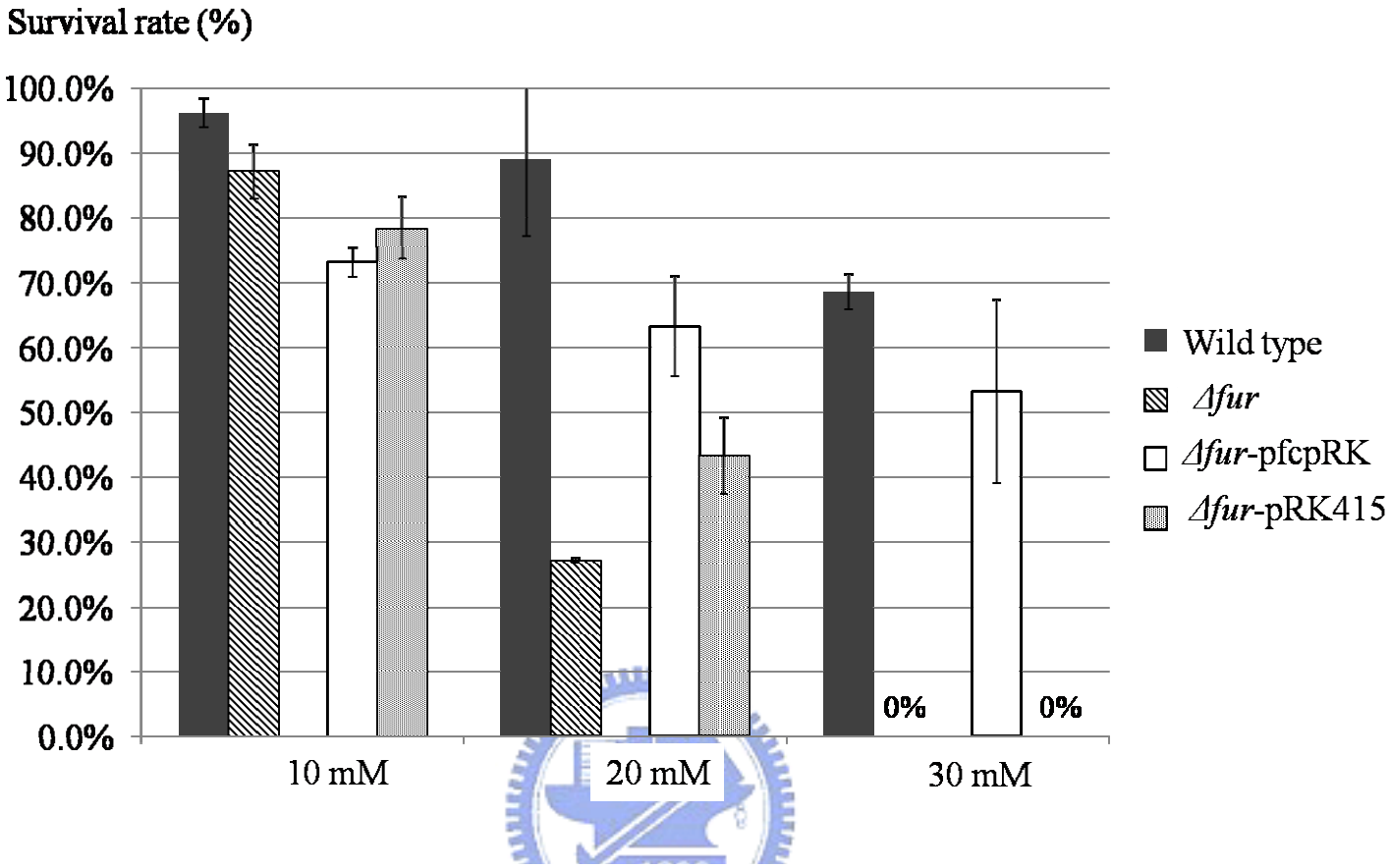


Fig. 10. Deletion effect of *fur* on the sensitivity to H_2O_2. Survivals of wild type (black bar), Fur mutant (twill bar), *fur* complement strain (white bar) and pRK415 containing strain (gray bar) were measured after 1/20 dilution of the overnight grown bacteria refreshed grown in LB for 3 h followed by 20 min of challenge with 10 mM, 20 mM and 30 mM H_2O_2, respectively.

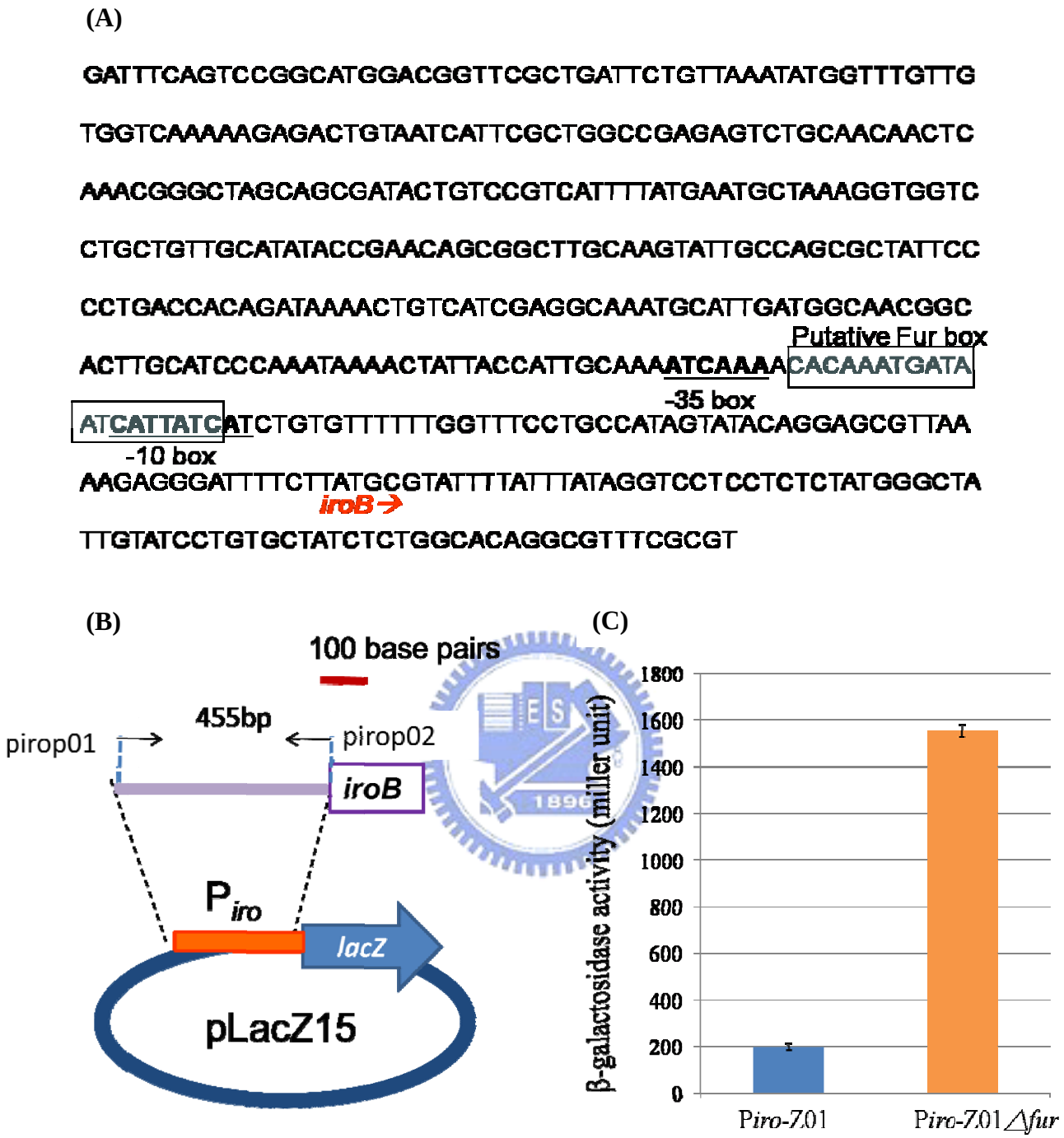


Fig. 11. Deletion effect of Fur on the expression of *iro* system. (A) The upstream non-coding sequences of *iro* and the putative Fur box are shown. (B) Schematic representation of the P_{iro}–*lacZ* fusion plasmid. (C) LacZ activity measurement. The activity of P_{iro} was determined after the overnight grown bacteria in 1/20 dilution suspended in LB and refreshed grown for 8 h.

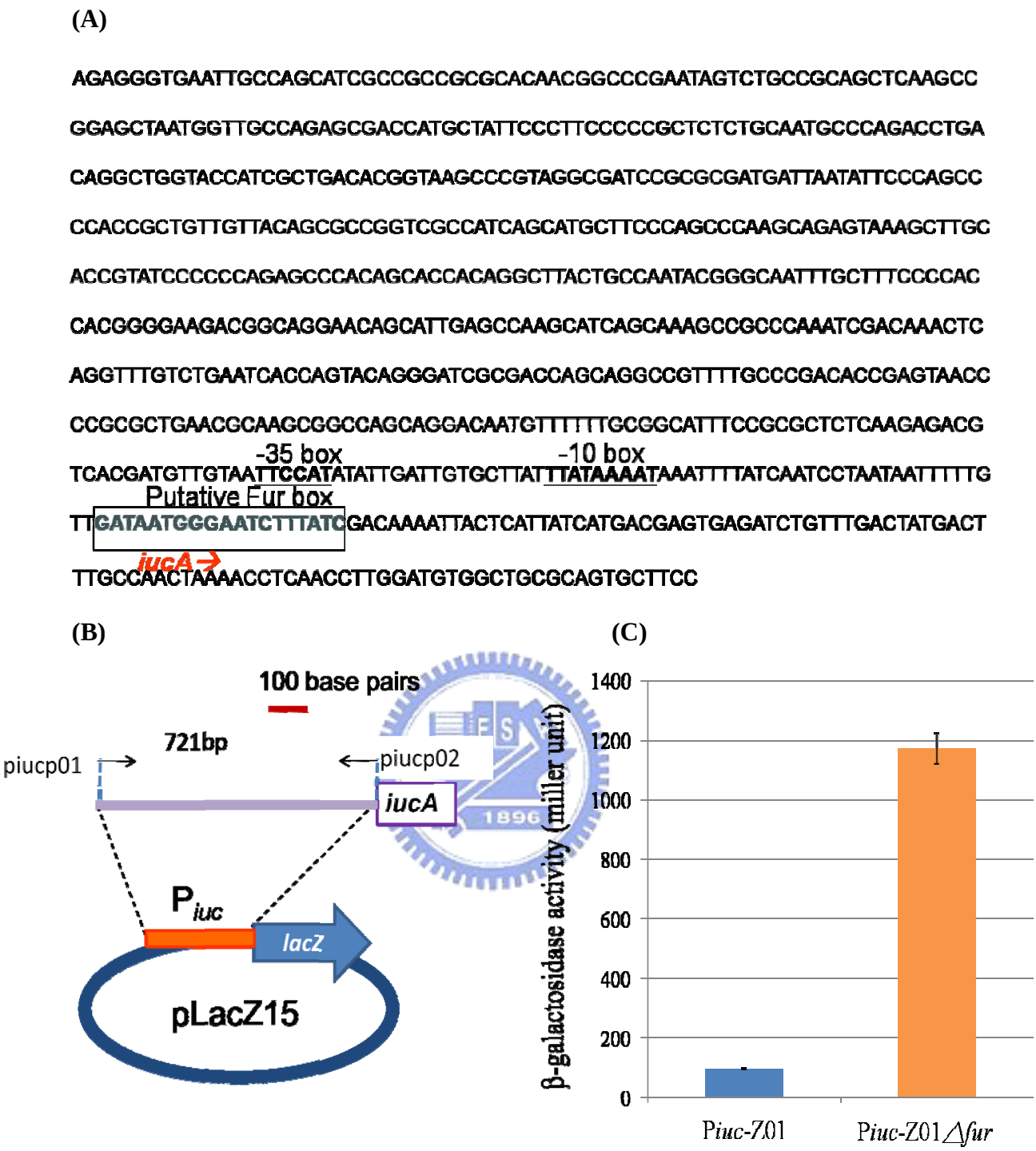


Fig. 12. Deletion effect of Fur on the expression of *iuc* system. (A) The upstream non-coding sequences of *iuc* and the putative Fur box are shown. (B) Schematic representation of the P_{iuc} –*lacZ* fusion plasmid. (C) LacZ activity measurement. The activity of P_{iuc} was determined after the overnight grown bacteria in 1/20 dilution suspended in LB and refreshed grown for 8 h.

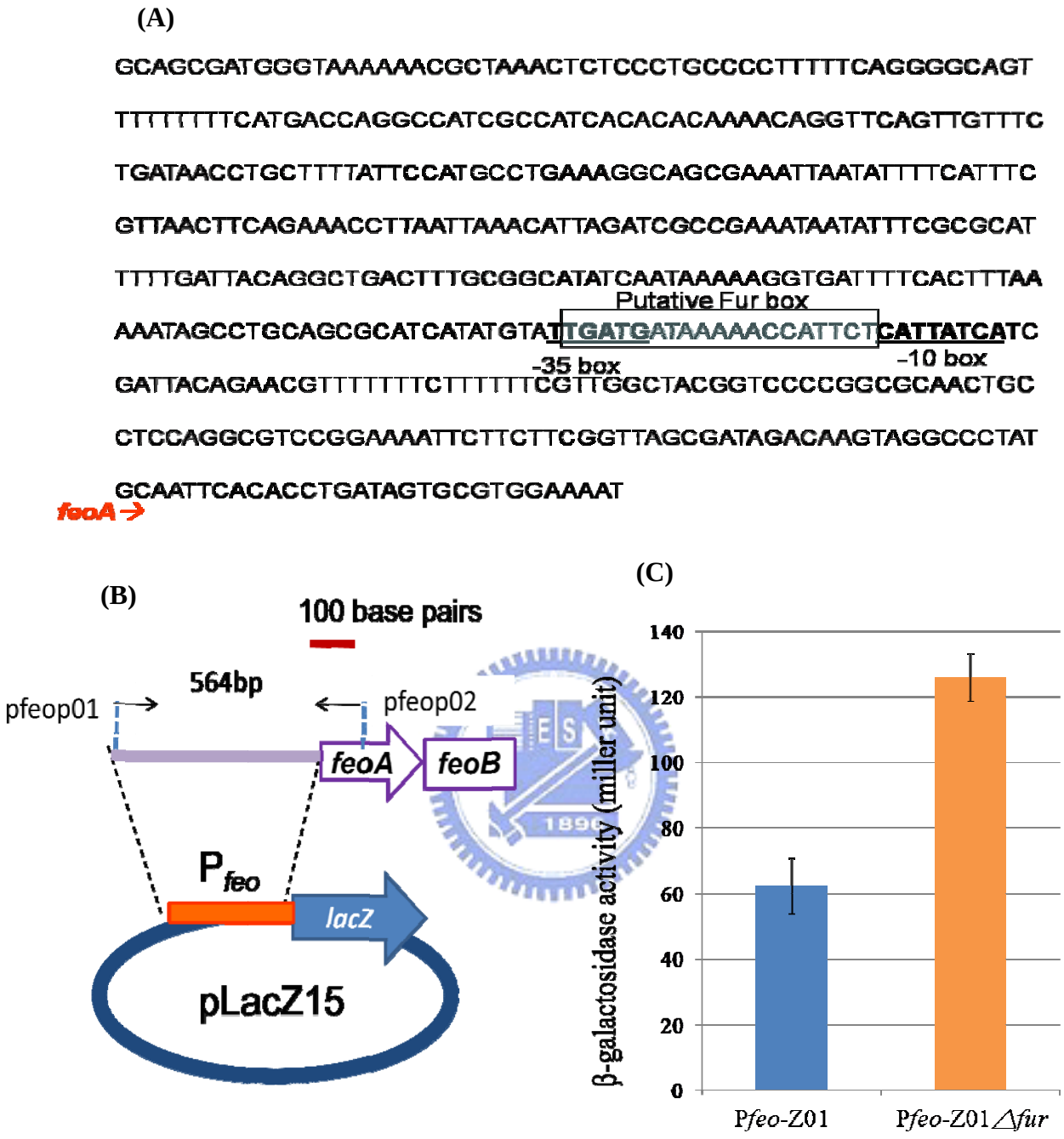


Fig. 13. Deletion effect of Fur on the expression of *feo* system. (A) The upstream non-coding sequences of *feo* and the putative Fur box are shown. (B) Schematic representation of the P_{feo} –*lacZ* fusion plasmid. (C) LacZ activity measurement. The activity of P_{feo} was determined after the overnight grown bacteria in 1/20 dilution suspended in LB and refreshed grown for 8 h.

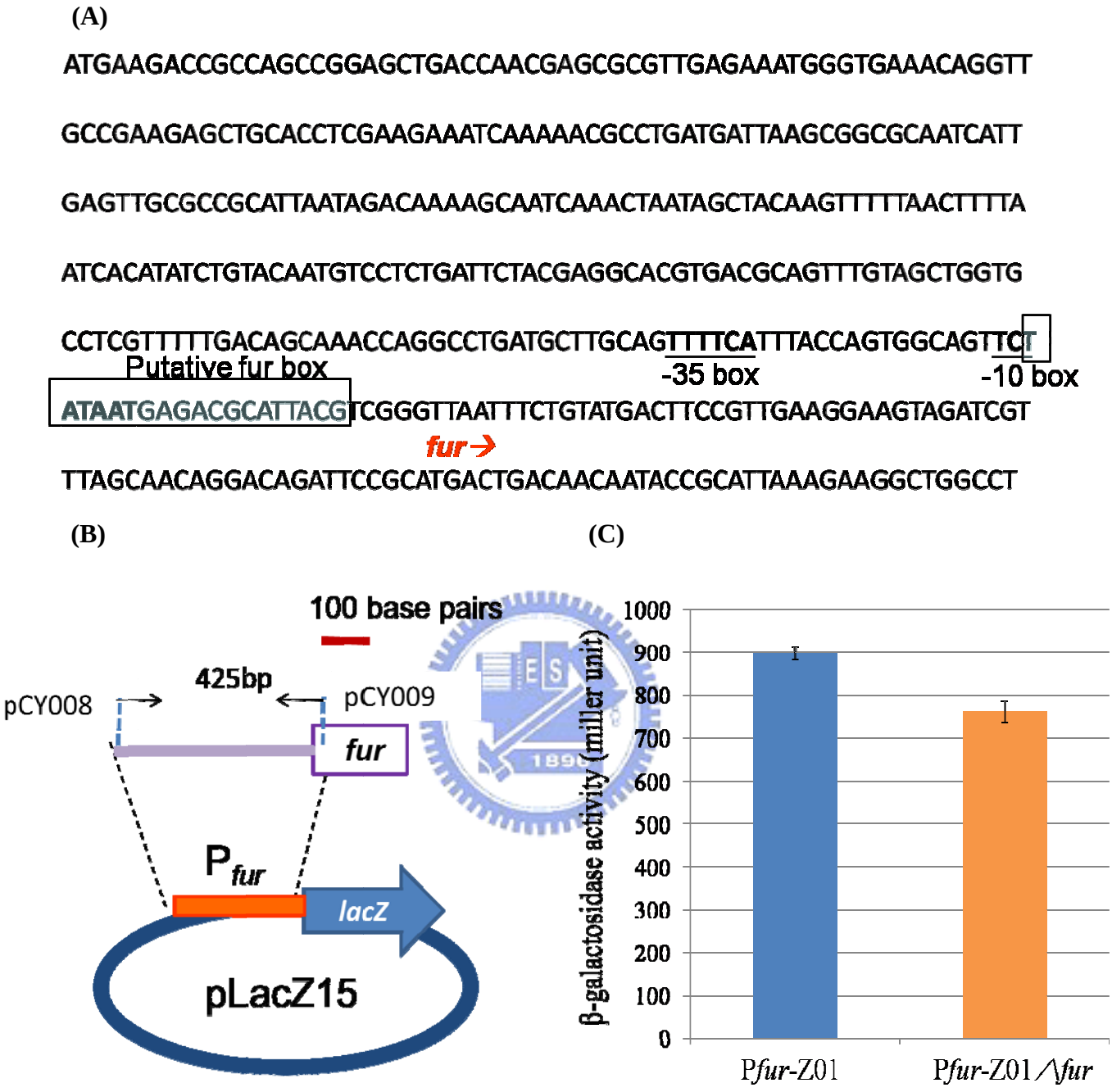


Fig. 14. Deletion effect of Fur on the autoregulation of *fur*. (A) The upstream non-coding sequences of *fur* and the putative Fur box are shown. (B) Schematic representation of the P_{fur} –*lacZ* fusion plasmid. (C) LacZ activity measurement. The activity of P_{fur} was determined after the overnight grown bacteria in 1/20 dilution suspended in LB and refreshed grown for 8 h.

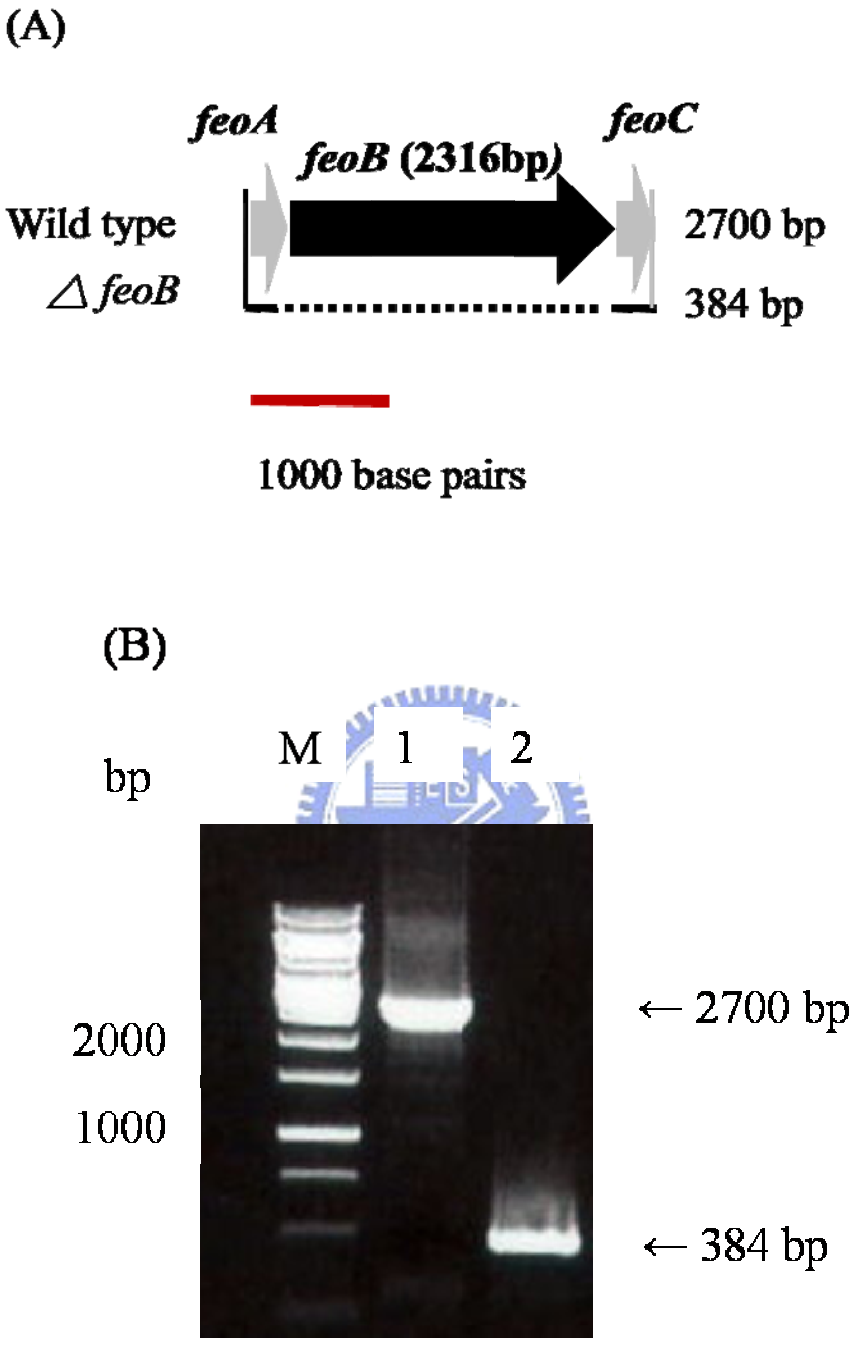


Fig. 15. Schematic representation of the *feoB* deletion (A) FeoB gene deletion mutant was verified by PCR. (B) M: marker. Lane 1: CG43S3. Lane 2: CG43S3△*feoB*.

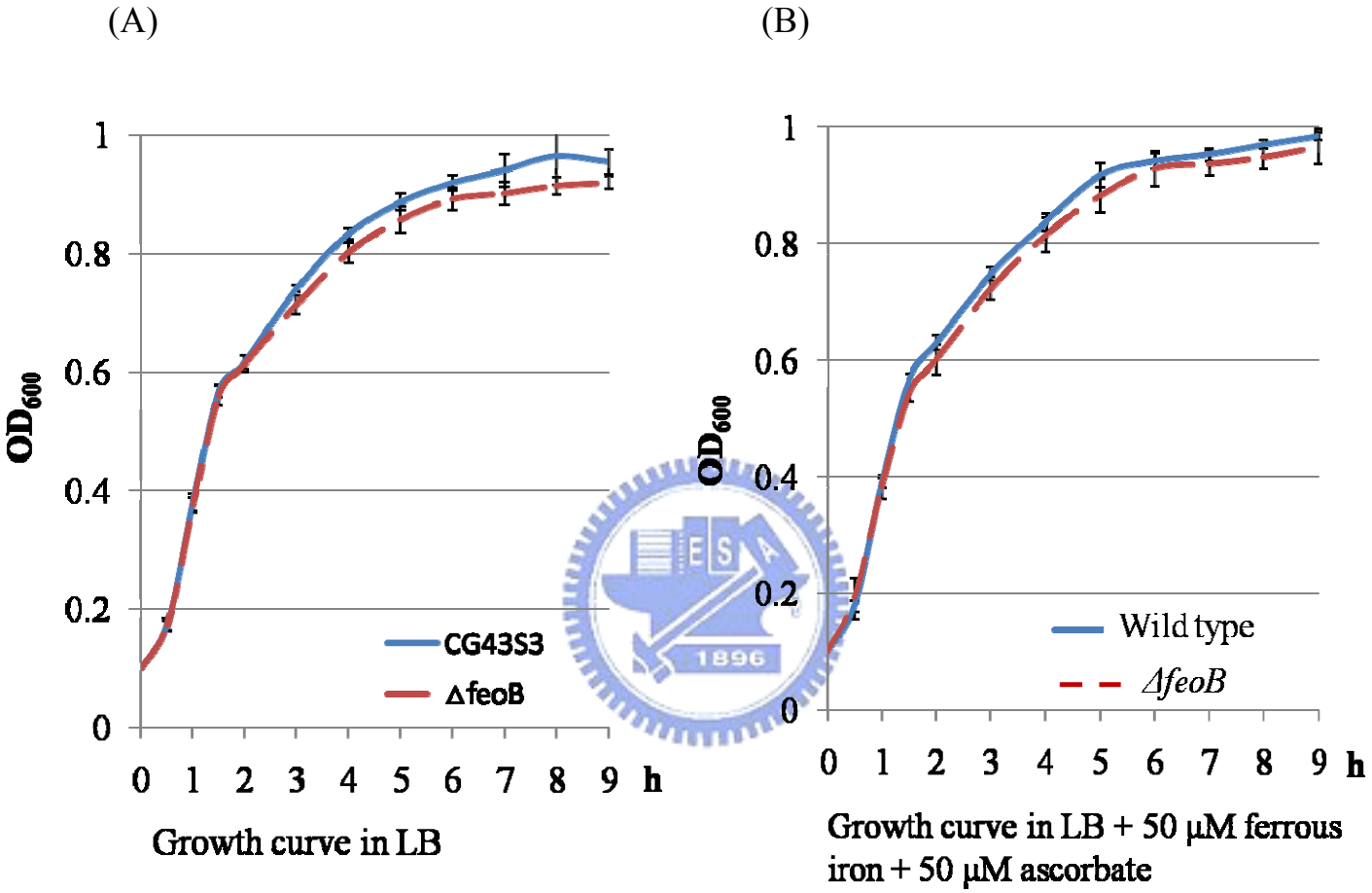


Fig. 16. FeoB gene deletion effect on the growth of *K. pneumoniae* CG43S3. Wild type (blue line), *feoB* mutant (dotted line) were growth in LB broth (A) or LB added with 50 μM ferrous iron and 50 μM ascorbate (B).

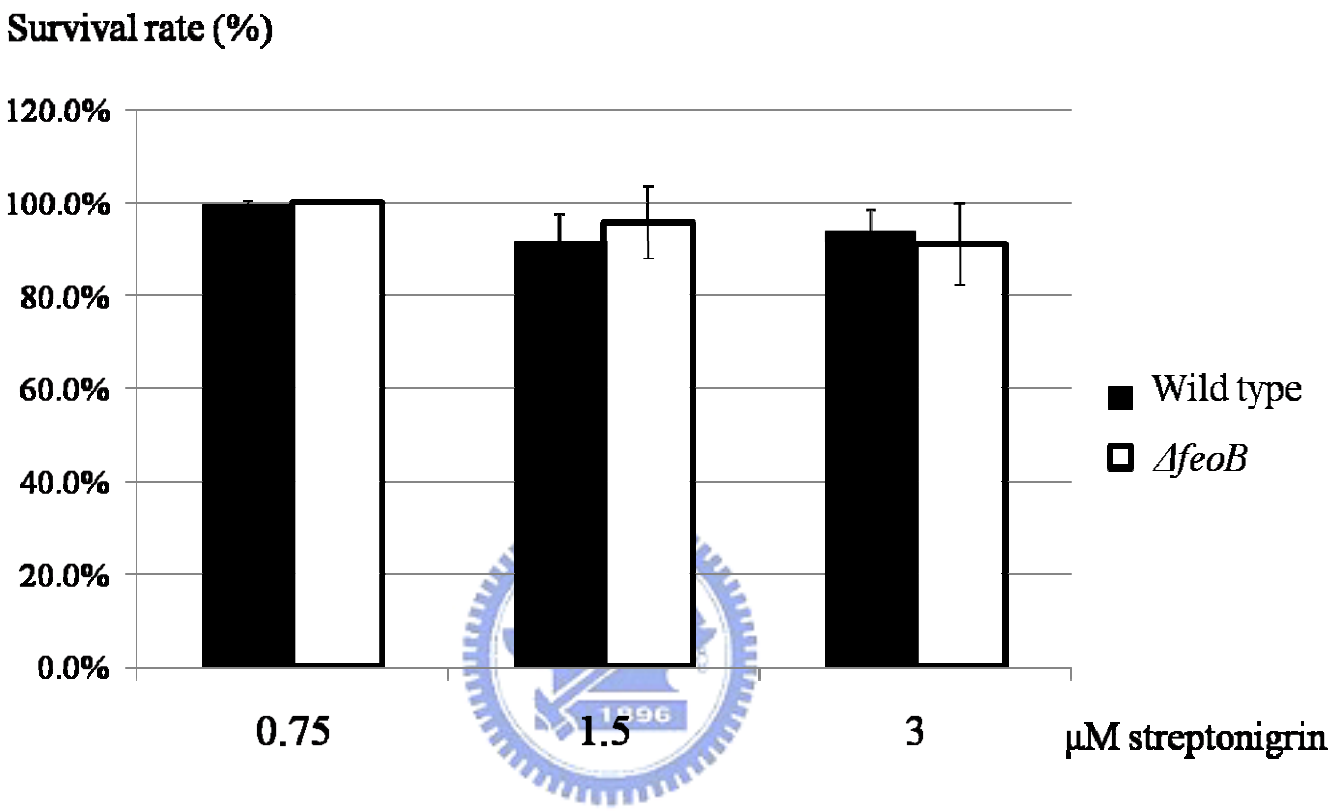


Fig. 17. FeoB gene deletion effect on the sensitivity to streptonigrin. Survivals of the wild type (black bar) and the FeoB mutant (white bar) were measured after the bacteria grown overnight in LB with the indicated concentration of streptonigrin.