國立交通大學生物科技學系

碩 士 論 文

克雷白氏肺炎桿菌 CG43 中 *yfiD* 基因表現的調控

Regulation of *yfiD* gene expression in *Klebsiella pneumoniae* CG43

研究生：蔡秉熹

Student: Bing-Shi Tsai

指導教授： 彭慧玲 博士

Advisor: Hwei-Ling Peng, Ph.D

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Contents

Abbreviation

AR	acid resistance
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
CFU	colony forming unit
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetic acid
EMSA	electrophoretic mobility shift assay
Evg	*Escherichia* virulence gene
HK	histidine kinase
IPTG	isopropyl-β-D-thiogalactopyranoside
kvg	*Klebsiella* virulence gene
kvh	*Klebsiella* virulence gene homologue
LB	Luria-Bertani medium
MES	morpholineethanesulfonic acid
NBT	nitro blue tetrazolium
PEG	polyethylene glycol
PNK	T4 polynucleotide kinase
PCR	polymerase chain reaction
RBS	ribosome binding sequence
Rcs	regulator of capsular polysaccharide
RR	response regulator
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis

中文摘要

我們利用蛋白質體分析方法探討高致病性克雷白氏肺炎桿菌 CG43 中 KvhAS 雙分子系統的調控角色時發現:在弱酸培養後的細菌 CG43S3U9451 的二維電泳膠片中，有一蛋白質點的強度較 CG43S3 U9451*kvhAS*膠片中的相對蛋白質點高出許多，經截取、純化此蛋白，再經質譜分析確認此蛋白質點為 YfiD。接著以 LacZ 為報導基因分析 *yfiD* 啟動子活性，結果顯示只有在微氧培養的狀態下，*yfiD* 啟動子在 Δ*kvhA* 突變株中的活性減少；有趣的是，Δ*rpoS* 突變株之 *yfiD* 啟動子活性也相對減少；而在截短 *yfiD* 啟動子上的一個 RpoS 結合序列後，受 RpoS 基因缺損影響的現象即消失。我也利用膠體遲滯電泳分析，證實 KvhA 直接黏附 *yfiD* 啟動子而達到正向轉錄調控的作用。進一步，我還選殖了 *yfiD* 基因並將 *yfiD* 殖入大腸桿菌表現系統來大量表現 YfiD 蛋白，並將蛋白純化後注射 Balb/c 老鼠來取得多株抗體。以西方墨點法分析的結果顯示 *kvhA* 的缺損造成 YfiD 蛋白量的下降，而轉型入 KvhA 表現質體時，YfiD 蛋白表現量即回升；另外，以帶有定點突變的 $KvhA_{D52A}$（持續去磷酸化）或 $KvhA_{D52E}$（持續磷酸化）的質體轉入 *kvhA* 的缺損株，我發現 KvhA 是否被磷酸化會影響 *yfiD* 基因的表現調控。*yfiD* 基因缺損株在有氧條件之下，對於酸處理後的感受性提高，而 *yfiD* 缺損對細菌的生長並沒有顯著的影響。最後，我

構築了 $YfiD_{G102A}$ 點突變株，證實 YfiD 的酵素活性參與其酸性逆境表現型的改變。

Abstract

A proteomic approach has been employed to unravel the regulatory role of the two-component system KvhAS in the highly virulent strain *K. pneumoniae* CG43. In comparing the two dimensional protein profiles derived from mild acid-growth bacteria, a protein spot highly expressed in the gel of *K. pneumoniae* CG43S3U9451 but not in that of CG43S3U9451*kvhAS*$^-$ mutant was isolated and later identified as YfiD by mass spectrum analysis. Promoter activity measurement using LacZ as reporter revealed a reduction of P_{yfiD} activity in KvhA$^-$ mutant only under a microaerobic environment. Interestingly, deletion of *rpoS* also decreased *yfiD* expression. The *rpoS* deletion effect was no more observed while the putative RpoS-binding element was truncated. Furthermore, EMSA demonstrated a direct binding of KvhA to P_{yfiD} DNA which supporting a positive transcriptional regulation on the expression of *yfiD*. In the meantime, *yfiD* encoding gene was cloned and overexpressed in *E. coli*, and the recombinant YfiD purified to homogeneity to inject Balb/c mice for polyclonal antibody preparation. The analysis of western blotting hybridization against the YfiD polyclonal antibody indicated that the deletion of *kvhA* reduced the expression of YfiD. The introduction of the plasmid carrying KvhA into the *kvhA* deletion mutant appeared to enhance the expression of *yfiD*. The transformation of the plasmid carrying $KvhA_{D52A}$ (constitutively dephosphorylated), or $KvhA_{D52E}$ (constitutively phosphorylated) confirmed the phosphorylation of KvhA is required for the regulation of

yfiD expression. Finally, deletion of *yfiD* was found to increase the bacterial susceptibility to acid treatment under aerobic condition while no effect on the bacterial growth. The $YfiD_{G102A}$ was generated and the involvement of its enzymatic activity in the acid-shock resistance was also demonstrated.

Introduction

Klebsiella pneumoniae is a gram-negative bacillus of the family *Enterobacteriaceae* with a world-wide distribution and is an important cause of human disease resulting in significant morbidity and mortality (54). On the basis of Voges-Proskauer (VP) reaction (38), *Enterobacteriaceae* are divided into two groups. *K. pneumoniae* excretes acetoin (3-hydroxy-2-butanone) into outer cellular environments whereas *E. coli* does not. Acetoin is an important physiological metabolic product excreted by various microorganisms when they grow in environmental niche containing glucose or other fermentable carbon sources (24, 43). This neutral product allows the bacteria to ferment large amounts of carbohydrates without self-inhibition due to the overacidification of intercellular environments (16, 70). Acetoin can also be reutilized when glucose has been depleted (21). Moreover, owing to the reversible transformation between acetoin and 2,3-butanediol coupled with the NAD/NADH conversion, the pathway has been regarded as participating in regulation of the NAD/NADH ratio in bacteria (29).

K. pneumoniae is also a commonly reported nosocomial pathogen, which often causes pneumonia, septicemia, bacteremia, suppurative lesion, wound infection, burn infection, and urinary or respiratory tract infections in chronic alcoholics and immunocompromised patients (54). In Taiwan, *K. pneumoniae* has been found to be closely associated with liver abscess leading to serious complications among diabetic patients (10,

51, 66). There are five virulence factors identified to be involved in *K. pneumoniae* infections, which include capsular polysaccharides (23), lipopolysaccharides (64), adhesins (63), iron-acquisition systems (53), and antibiotic resistance (59). In addition to these virulence factors, very little is known about the other factors that may participate in the pathogenesis. The increasing prevalence of extended spectrum β-lactamase, which leads to ESBL-producing *K pneumoniae*, has promoted the development of new drugs to overcome drug-resistance difficulties (47).

Much of the success of infections depends on the bacterial ability to survive and thrive in adverse environments. *K. pneumoniae* has evolved mechanisms to cope with diverse conditions from human guts to soil (54). These different environments present ever changing challenges such as oxygen availability, pH shift, and osmolarity change and the adaptation ability is ultimately rooted in altering patterns of gene expression. Two-component systems (2CSs) are widely distributed in gram positive and negative bacteria, which play diverse regulatory roles in bacterial physiology, such as stress resistance, virulence, energy metabolism, nutrient acquisition, and quorum sensing (9, 18, 52, 58, 62). 2CSs consist of a transmembrane sensor histidine kinase (HK) and a cytosolic response regulator (RR). Upon sensing input signals, the sensor HK catalyzes an autophosphorylation which transfers a phosphate from ATP to a conserved histidine residue on the transmitter domain. The phosphate group is subsequently transferred to a specific aspartate residue on the receiver domain of the cognate RR. The phosphorylated RR is thus

activated and begins to regulate the expression of downstream genes (4). Recently, drug development targeting 2CSs to intervene the signal transduction involved in microbial infections has been established (49). The specific aspartate residue in RcsB, the RR of RcsCDB signal transduction system, has been demonstrated by site-directed mutagenesis showing its pivotal role in the His-Asp phosphorelay for the regulation of downstream genes (6). Since signal transduction in mammals occurs by a different mechanism, inhibition of histidine kinases provides another potential target for antimicrobial agents (46).

Besides 2CSs which are responsible for the regulation to adapt to environmental changes, RpoS (σ^S), an alternative sigma subunit of RNA polymerase, has been identified to regulate bacterial general stress response (68). RpoS controls more than 80 genes (16, 30). Some of the gene expression has been found to be involved in the acid resistance mechanism (8, 67), which is important for enterobacterial infection. For gastrointestinal pathogens such as *K. pneumoniae* and *E. coli*, penetrating the barrier of gastric acid is crucial for the colonization and infection. This requires either a large number of organisms or a powerful acid resistance (AR) mechanism that allows small numbers of bacteria to survive until the stomach empties its contents into the more alkaline intestine. Four AR systems have been identified in bacteria to resist acidic environments. AR system 1 is repressed by glucose evident in stationary-phase cells, and protects cells in minimal medium. It has been shown in *E. coli* that RpoS and cyclic AMP receptor protein (CRP) play a role in control of this pathway (8, 67). The other systems are not

repressed by glucose and require the addition of glutamic acid (AR system 2), arginine (AR system 3), or lysine (AR system 4) in pH 2.5 acid challenge medium (8, 26, 42). These amino acid-dependent systems utilize matched decarboxylases and antiporters to protect the cell. The decarboxylases are glutamic acid decarboxylase isozymes GadA and GadB for system 2, the arginine decarboxylase AdiA for system 3, and presumably, the inducible lysine decarboxylase CadA for system 4 (8, 14, 22, 26). However, whether these AR systems exist in *K. pneumoniae* remains to be investigated.

We have previously identified a 2CS, KvhAS, by PCR-based cloning from *K. pneumoniae* CG43 (40). The analysis using dot-blotting hybridization revealed that *kvhAS* is presented in all strains collected in the laboratory, suggesting an important role of the 2CS in *K. pneumoniae* (40). According to the sequence analysis, *kvhAS* shows high homology to *evgAS* (*Escherichia* virulence gene). KvhA shares 66.7% protein identity with EvgA, while KvhS shares 48% protein identity with EvgS. EvgAS has been demonstrated to regulate multi-drug resistance (48), acid resistance (44) and heat-resistance (12). Therefore, a similar role of KvhAS as EvgAS was proposed. The subsequent studies showed that the overexpression of KvhA altered the susceptibility to fosfomycin, and deletion of *kvhS* reduced *kvhAS* promoter activity in the presence of 300 mM NaCl in M9 medium (41). A heat shock (44℃) activated promoter activity using GalU as reporter has been demonstrated (25). Moreover, an interacting regulation of KvhAS with KvgAS and KvhR for the expressions of *cps* gene cluster was also demonstrated (40), but the

molecular mechanism of the regulation remains unknown.

A proteomic approach has been employed to uncover if KvhAS is involved in acid resistance in *K. pneumoniae* (74). In comparison of the two dimensional protein profiles that were extracted from the bacteria incubated in mild acid (pH 5.5), a protein spot was found highly expressed in *K. pneumoniae* CG43S3U9451 but not in that of CG43S3U9451Δ*kvhAS* (Appendix I). The protein was then isolated, subjected to trypsin digestion and mass spectrum analysis and identified as YfiD.

YfiD is a homolog to the C-terminal region of pyruvate formate-lyase (PFL), the product of *pflB* gene. For a functional activity, YfiD and PFL have to be post-translationally activated in anaerobic environment by PFL activase (encoded by *pflA*) through inserting a radical to them. The radical which is crucial for the catalytic activity is susceptible to oxidative cleavage thereafter leading to inactivation of the enzyme. The inserted radical in PFL can be quenched by PFL deactivase AdhE microaerobically to prevent oxidative cleavage, however, YfiD does not respond to the deactivated AdhE (69). It has also been shown that YfiD acts as a "spare part" by providing an active glycyl radical domain to restore activity of the oxygenolytically cleaved PFL (65) for balance of the carbon flux in microaerobic environment. Upon the activation, a glycyl radical forms around a glycine residue (Glycine 102) contained in the highly conserved pentapeptide motif (RVXGY) of YfiD (Appendix III). The activated YfiD, via catalyzing the reaction to convert pyruvate into acetyl-coenzyme A, is able to reduce the accumulation of

acidic metabolic end product thereby to modulate the intracellular pH (69, 73). A phosphorylated form of YfiD has been demonstrated *in vitro* in stagnant growth of *E. coli* L form NC-7 (19), suggesting a phosphorylation regulation for the YfiD activity.

The expression of *E. coli yfiD* has been shown to be under the regulation of two well-known global regulators FNR (fumarate-nitrate reduction regulator) and ArcA (aerobic respiration control regulator) (69), and a pyruvate sensor PdhR (55) (Appendix II). In oxygen deprived condition, FNR, which contains a Fe-S cluster as a redox sensor, upregulated the *yfiD* expression at FNR element I (high affinity FNR binding element). While increased expression of FNR under low oxygen level allowed it to occupy the FNR element II (low affinity FNR binding element) to prevent from the *yfiD* expression. In contrast, ArcA competes with FNR for binding the FNR element II under the anaerobic condition to increase the expression of *yfiD*. Consequently, the *yfiD* predominantly expressed in microaerobic environment (20). While PdhR was shown to mediate repression of *yfiD* indicating a link to pyruvate/central metabolism. Previous reports have also indicated that intracellular YfiD levels are enhanced by growth in the presence of pyruvate (5).

YfiD protein has been identified in a wide range of Gram-negative and positive bacteria including *Serratia* sp., *S. liquefaciens* and *Haemophilus infuenzae*, as well as in T4 and T5 bacteriophages (69). Sequence analysis revealed that *K. pneumoniae yfiD* is located in the *srmB-ung* intergenic region, which is similar to that of *E. coli* and *H. influenzae yfiD* gene. However, the T4 *yfiD* is located next to the

thymidine kinase gene while the T5 *yfiD* is next to a tRNA gene cluster. In *Serratia*, the *yfiD* gene is associated with the *phlA* gene encoding an extracellular phospholipase A1 (69).

In this study, we investigate if KvhAS plays a regulatory role in the expression of *yfiD* and the functional role of YfiD in *K. pneumoniae* CG43. Firstly, P_{yfiD} activity measurement has been used to investigate the regulation of KvhA on *yfiD* expression at transcriptional level. In the meantime, *rpoS* deletion mutant has been utilized to inspect if RpoS is involved in *yfiD* regulation. Secondly, EMSA has been employed to demonstrate the specific binding of KvhA to *yfiD* promoter. Thirdly, anti-YfiD polyclonal antibody, YfiD expression plasmid and *yfiD* deletion mutant have been generated to demonstrate if the decreased YfiD expression in *K. pneumoniae* CG43S3Δ*kvhA* mutant could be restored by introducing a KvhA expression plasmid. The involvement of KvhAS phosphorelay in the regulation of *yfiD* expression is also investigated. Finally, the deletion effect of *yfiD* on bacterial growth in acidic environment and the acid stress response were studied and shown.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1 and 2. *K. pneumoniae* CG43S3 (36), CG43S3*kvhA*$^-$, CG43S3*rpoS*$^-$, CG43S3*yfiD*$^-$, CG43S3Z01, CG43S3Z01*kvhA*$^-$, CG43S3Z01*rpoS*$^-$, CG43S3U9451 and CG43S3U9451*kvhA*$^-$ are derivative strains of CG43 which is a highly virulent strain of K2 serotype with an LD50 of 10 cfu in laboratory mouse (11). Tryptone, yeast extract, and agar were purchased from Difco Laboratories (Detroit, Mi). Unless otherwise indicated, bacterial cultures were grown in Luria-Bertani broth (LB) supplemented with appropriate antibiotics at 37°C. Microaerobic growth was carried out essentially as described by Croinin (13).

Recombinant DNA technique

The recombinant DNA experiment was carried out by standard procedures (30). Primers used in PCR are listed in Table 3. Plasmid DNA was prepared by VIOGENE Miniprep kit. Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolab (Beverly, MA), MBI (Fermentas, Hanover, MD), or Roche Molecular Biochemicals (Mannheim, Germany), and were used according to the recommendations of the suppliers.

Promoter activity assessment

The putative promoter of *yfiD* and the derived truncation regions were PCR amplified from *K. pneumoniae* CG43S3 by the designed primers yfiD01, yfiD02, yfiD05, yfiD07, yfiD08, and yfiD09 (Table 2) and subcloned into placZ15 (40) to fuse them with the promoterless *lacZ* reporter gene. One-hundredth overnight culture of the bacteria carrying each of the plasmids were subcultured and freshly grown in LB medium in microaerobic or aerobic condition for 4 h. β-galactosidase activity assay was carried out essentially as described by Miller (47). The data presented were derived from a single experiment which was representative of at least three independent experiments. Every sample was assayed in triplicate, and the average activity and standard deviation were presented.

Construction of *yfiD* mutant

Two 1.1 kb DNA fragments flanking the *yfiD* gene were PCR amplified from *K. pneumoniae* CG43S3 using yfiDm06/yfiDm07 and yfiDm08/yfiDm09. The generated DNA fragments were ligated and subcloned into a suicide vector pKAS46 (61). The resulting plasmid pYm2KAS, containing a 308-bp deletion in *yfiD*, was transformed into *E. coli* S17-1 *λ-pir* by electroporation. The *E. coli* S17-1*λ-pir* and *K. pneumoniae* CG43S3 were cultured to mid-logarithmical phase and mixed at a ratio of 1:2, and pelleted by centrifugation. The mating mixture was then suspended in 100 μl of saline and spread onto a

sterilized nitrocellulose membrane on a prewarmed LB plate. After overnight incubation at 37℃, the cells were washed off from the membrane with 5 ml of LB. The transconjugants were selected by plating with a 10^5-fold dilution of the culture on M9 minimal medium containing 30 μg/ml kanamycin and 100 μg/ml ampicillin for the integration of the suicide vector. Subsequently, several of the transconjugants were cultured in LB without selection to late logarithmic phase followed by selection on LB plates containing 500 μg/ml streptomycin for the loss of vector plasmid. The deletion was further demonstrated by PCR analysis.

Expression and purification of the histidine-tagged YfiD

The recombinant plasmid pY34B containing the His_6-tagged YfiD was constructed and the plasmid was transformed into *E. coli* BL21(DE3). The transformants carrying pY34B were cultured in LB to log phase, and expression of the His_6-YfiD recombinant protein was induced with 0.1 mM IPTG for 1 h. The IPTG-induced bacterial cells were harvested by centrifugation at 4℃, 3300 *Xg* for 15 min, resuspended in 1 X binding buffer (5 mM imidazole, 0.5 M NaCl and 20 mM Tris-HCl pH 7.9), and then disrupted by sonication. Subsequently, the cell lysate was centrifuged at 20,400 *Xg* for 25 min at 4℃, and the supernatant was applied to a column containing His_6-Bind Resin (Novagen, Madison, WI). After washing the column with 1 X binding buffer and then 1 X wash buffer (60 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl pH7.9), the

His_6-YfiD protein was eluted with 1 X elution buffer (1 M imidazloe, 0.5 M NaCl, and 20 mM Tris-HCl pH 7.9). The eluted His_6-YfiD protein was subjected to dialysis with 1 X phosphate-buffered saline (PBS) by CelluSep Dialysis Membranes purchased from MFPI (Seguin, TX). Finally, the dialyzed His_6-YfiD protein was concentrated by covering with polyethylene glycol 20,000 on dialysis membranes.

Preparation of YfiD antisera

To obtain anti-YfiD antibody, 5 μg of the purified YfiD was injected intraperitoneally to five-week old female BALB/c mice which were purchased from the animal center at National Taiwan University. Ten days later, the mice were immunized again with 5 μg of the protein and the antisera obtained by intra-cardiac puncture.

Western blotting analysis

Total cellular lysates from *K. pneumoniae* CG43S3 grown overnight in LB medium were resolved by SDS-PAGE. The proteins were then electrophoretically transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). After incubation with 5% skim milk at room temperature overnight, the membrane was washed 3 times with 1 X phosphate-buffered saline (PBS). Subsequently, the membrane was incubated at room temperature for 2 h with diluted anti-YfiD serum. After washes three times with 1 X PBS, a 3000-fold diluted alkaline

phosphatase-conjugated anti-mouse immunoglobulin G was added and the incubation continued for one more hour. The blot was again washed and the bound antibodies were detected by using the chromogenic reagent BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (Nitro blue tetrazolium).

Determination of protein intensity

The protein intensity was determined by analyzing the Western blot image using Gel-Pro Analyzer™ (Media Cybernetics, Inc.) according to the instruction.

Generation of a *kvhA* expression plasmid

A DNA fragment containing the *kvhA* coding region and a RBS sequence was PCR amplified from *K. pneumoniae* CG43S3 using its primers kvhAc01/ kvhAc02 and cloned into pCPP45. The resulting plasmid was designated as pKvhAcPP.

Generation of *yfiD* expression plasmids

A DNA fragment containing the *yfiD* coding region and a RBS sequence was PCR amplified from *K. pneumoniae* CG43S3 using primers yfiDc01/yfiDc02 and cloned into pRK415 (32) and pCPP45. The resulting plasmids were designated as pYcRK and pYcPP respectively.

Construction of $KvhA_{D52A}$, $KvhA_{D52E}$ and $YfiD_{G102A}$ expression plasmids by site-directed mutagenesis

Site-directed mutagenesis in KvhA and YfiD was carried out essentially as described by QuikChange® Site-Directed Mutagenesis Kit (Stratagene, USA). Asp52 in KvhA is responsible for receiving an orthophosphate, which was changed into alanine and glutamic acid residues in pKvhAcPP by primer sets hAd52a-F3/hAd52a-R3 and hAd52e-F2/hAd52e-R2, and the resulting plasmids were designated as pHAcAPP and pHAcEPP respectively. Meanwhile, gly102 in the YfiD active site was changed into an alanine in pYcPP by primer set yfiDg102a-F3/ yfiDg102a-R3, and the resulting plasmid was named pYc102PP.

Electrophoretic mobility shift assay (EMSA)

The probes used in the electrophoretic mobility shift assay were the restriction fragments of the putative *yfiD* promoter-containing region including $P_{yfiD\text{-}2}$ and $P_{yfiD\text{-}5}$, putative *kvhA* promoter-containing region and *kvhA* coding sequence. The restriction fragments were then separated on a 2% agarose gel and recovered. They were 5' end-labelled with [γ-^{32}P]-ATP (25 mCi/ml) using T4 polynucleotide kinase. The His_6-KvhA was phosphorylated by incubating in 1 X gel shift binding buffer [20 mM Tris-HCl, pH 8.0, 0.4 mM $MgCl_2$, 50 mM KCl, 1 mM $CaCl_2$, 1 mM DTT, and 10% glycerol] (Karimova *et al.* 1996) in the presence of 50 mM

acetylphosphate and 1μg of acetylated bovine serum albumin for 30 min at 25℃. And then the radioactively-labeled DNA fragments was added and incubated at 37℃ for 30 min. The samples were applied to 5% non-denaturing polyacrylamide (acrylamide: bis-acrylamide, 19: 1) gels that were pre-electrophoresed at a constant voltage of 100 V for 30 min at 4℃ before the samples were loaded. Electrophoresis was performed at 25 mA for 40 to 80 min at 4℃. The gels were read by InstantImager™ (Packard Instrument Company) or visualized by autoradiography.

Bacterial growth analysis

One-hundredth overnight grown bacteria were inoculated to LB or pH 5.5 LBMES medium (1, 56) and incubated at 37°C in aerobic or microaerobic condition. The optical density at the wavelength of 600 nm (OD_{600}) was measured every hour, which was representative of at least three independent experiments.

Acid survival assessment

One-hundredth overnight cultured bacteria were inoculated to pH 5.5 LBMES medium and grown at 37°C aerobically to OD_{600} of about 0.7. Aliquots of the bacteria were then collected by centrifugation and resuspended in pH 3 LB medium, and subjected to 37°C incubation aerobically for 35 min. CFU of the bacteria before and after the stress treatment were counted, and the survival rate was determined by the ratio

of the CFU after to before the acid stress treatment. The representative data of at least three independent experiments were presented. Every sample was assayed in triplicate, and the average activity and standard deviation were presented.

Results

Sequence analysis of the *yfiD* putative promoter revealed conserved binding sequences of FNR, ArcA, RpoS and EvgA

DNA sequence upstream to the *yfiD* coding region was analyzed by BPROM of Softberry (http://www.softberry.com/) and -10 and -35 boxes were identified. As shown in Fig. 1, FNR and ArcA binding elements (69), a putative EvgA binding box (31), and two putative RpoS elements (34, 37) were also found.

Deletion of *kvhA* or *rpoS* reduced P_{yfiD} activity

As shown in Fig. 2A, the respective LacZ reporter plasmids containing the putative promoters P_{yfiD-1}, P_{yfiD-2}, P_{yfiD-3} of *yfiD* were constructed and transformed into *K. pneumoniae* CG43Z01 and derived bacteria. The bacteria were cultivated in aerobic or microaerobic conditions in LB at 37 ℃ for 4 hours and then subjected to activity assessment. In aerobic condition, the activity of P_{yfiD-1}, P_{yfiD-2}, P_{yfiD-3} in Z01, Z01Δ*kvhA* or Z01Δ*rpoS* showed no apparent difference (Fig. 2C).

Under microaerobic condition, P_{yfiD-1} activity was slightly reduced in Z01Δ*kvhA*. The *kvhA* deletion appeared to have more profound effect on the activity of P_{yfiD-2}, suggesting the presence of KvhA binding site after the truncation from P_{yfiD-1} to P_{yfiD-2}, and the negative effects of *rpoS* deletion apparently reduced the activity of P_{yfiD-1} and P_{yfiD-2}. As shown in

Fig. 2B, the negative effect of *rpoS* deleltion was alleviated in the expression of P_{yfiD-3} that contains no RpoS binding element. This implied the RpoS element I as shown in Fig. 2A is the major regulatory sequence for the RpoS dependent expression.

Preparation of His_6-KvhA and His_6-KvhAΔHTH proteins

His_6-KvhA expression plasmid pHP4005 (40) and His_6-KvhAΔHTH expression plasmid pHArdB were introduced into *E. coli* BL21-CodonPlus(DE3)-RIL and BL21(ED3), respectively. One-tenth of the overnight bacterial cultures were freshly grown in LB at 37℃ for 2 h, then 0.1 mM IPTG was added for another 5 h incubation followed by SDS-PAGE resolution of the total proteins. Overexpression of the proteins with expected molecular mass of KvhA (Fig. 3A) and the truncation form of KvhA (Fig. 3B) could be observed. After disruption by sonication, large amounts of His_6-KvhA and His_6-KvhAΔHTH proteins were found in the supernatant fraction (Figure 3A, B). After purification by His_6-Bind Resin column, the collected fractions (Figure 4A, B) were subjected to dialysis and concentration. The purified His_6-KvhA and His_6-KvhAΔHTH proteins were then confirm by SDS-PAGE (Figure 4C, D).

The phosphorylated His_6-KvhA directly bound to P_{yfiD-2} and P_{yfiD-5} DNA fragments

The response regulator, KvhA, like many other response regulator of the two-component system, possesses a helix-turn-helix motif at the C-terminus to bind to specific DNA for its regulatory function. In order to demonstrate direct binding of KvhA to $P_{yfiD\text{-}2}$ (containing a putative EvgA binding box shown in Fig. 1 and $P_{yfiD\text{-}5}$ (Fig. 1), EMSA was carried out. As shown in Fig.5, the recombinant protein His_6-KvhA directly bound to $P_{yfiD\text{-}2}$ (Fig.5A) and $P_{yfiD\text{-}5}$ (Fig.5B) DNA fragments. While the presence of excess BSA, the retarded DNA was no more detected, indicating the DNA shift was not resulted from the BSA addition.

Construction of *yfiD* deletion mutant

To construct an *yfiD* deletion mutant, the DNA fragments flanking the deleted region were amplified by PCR and cloned into the suicide vector, pKAS46 (61). The resulting plasmid pYm2KAS was introduced into *K. pneumoniae* CG43S3 by conjugation. PCR using the primer set ung01/yfiD04 was employed to confirm the deletion of the resulting mutant (Figure 6).

Expression and purification of His_6-YfiD protein

The plasmid pY34B, a derivative of pET-30b containing the *yfiD* coding sequence, was transformed into *E. coli* BL21(DE3) for protein expression. As shown in Fig. 7A, overexpression of YfiD could be observed after 5 h induction with IPTG. After disruption by sonication, a large amount of

His_6-YfiD protein was found in the supernatant fraction (Figure 7A). After purification by His_6-Bind Resin column, the collected fractions (Figure 7B) were subjected to dialysis and concentration. The purified His_6-YfiD was then confirmed by SDS-PAGE (Figure 7C).

YfiD protein content was reduced in Δ*kvhA* mutant strain

One-hundred dilution of the overnight grown bacteria including *K. pneumoniae* CG43S3, CG43S3Δ*kvhA*, CG43S3Δ*yfiD*, CG43S3Δ*kvhA* [pHAm] and CG43S3Δ*yfiD* [pYcRK], were refreshly and aerobically cultured in LBMES (pH 5.5) or M9MES (pH 5.5) at 37℃ for 6 h. Total protein was then harvested and the concentration was determined as described (7). Five micrograms of each sample was incubated at 100℃ for 10 min before loaded onto SDS-polyacrylamide gel. Western-blot analysis using anti-YfiD polyclonal antibody was then performed. As shown in Fig. 8A, in comparing with that of the wild type bacteria, a reduced YfiD protein content was observed for the total protein extracts from the *kvhA* deletion mutant grown in LBMES (Fig. 8A) or M9MES (Fig. 8B).

KvhAS phosphorelay is required for the regulation of YfiD

Expression

Upon sensing extracellular signals, a sensor protein is

auto-phosphorylated, the orthophosphate then transferred to the cognate response regulator (RR), and finally the activated RR regulates the expression of downstream genes (4). To answer whether the phosphorylation of KvhA is essential for *yfiD* regulation, two mutant forms $KvhA_{D52A}$ (constitutively dephosphorylated) and $KvhA_{D52E}$ (constitutively phosphorylated) were generated by site-directed mutagenesis. As shown in Fig. 9, the reduced expression of YfiD in Δ*kvhA* strain was slightly restored by introducing a KvhA expression plasmid pKvhAcPP, while introducing pHAcAPP (expressing $KvhA_{D52A}$) did not appear to increase YfiD content. The YfiD protein content was apparently increased by introducing with pHAcEPP (expressing $KvhA_{D52E}$), suggesting the KvhAS phosphorelay is required for the regulation of YfiD expression.

Deletion of *yfiD* had no apparent effect on growth

Overnight cultures of *K. pneumoniae* CG43S3 or CG43S3Δ*yfiD* were refreshly grown in LB (pH 7.0) or LBMES (pH 5.5) at 37℃ under aerobic and microaerobic conditions, and the OD_{600} was measured each hour. As shown in Fig. 10, no obvious effect of *yfiD* deletion on the bacterial growth was observed.

YfiD affected the acid-shock resistance

As shown in Fig. 11A, the deletion of *yfiD* apparently reduced the

survival of *K. pneumoniae* CG43S3 while the bacteria in LBMES (pH 5.5) changed to pH 3.0 medium. The deletion effect could be complemented by introducing into the mutant with the YfiD expression plasmid pYcRK. As shown in Fig. 11B, the survival analysis of CG43S3Δ*yfiD* [pYcRK] showing no full restoration may be resulted from the loss of pYcRK plasmid. Furthermore, the introduction of pRK415 into CG43S3 led to apparent decrease of the survival. An *yfiD* expression plasmid pYcPP contain *yfiD* coding gene and its RBS was thus cloned into pCPP45 (Dr. David Bauer at Cornell University), carrying the partition region from RK2 plasmid and hence leading to high stability, to resolve the problem. As shown in Fig. 11(C), the survival rate still remained low when introducing pCPP45 into CG43S3.

Although the vast majority of the studies on YfiD focuses on the microaerobic regulation and activation, YfiD was found to be phosphorylated *in vitro* in stagnant growth of *E. coli* L form NC-7 (19), suggesting an functional role of YfiD under aerobic environment. Sequence analysis has shown that glycine 102 (g102) is contained in the active site to execute the YfiD enzymatic function (57, 69), pYc102PP containing an $YfiD_{G102A}$ coding region and an RBS sequence has thus been generated to determine if YfiD enzyme activity is involved in the regulation of the acid stress response. As shown in Fig. 11(C), the survival rate of CG43S3Δ*yfiD* [pYc102PP] apparently decreased comparing to that of CG43S3Δ*yfiD* [pYcPP], suggesting an involvement of YfiD enzyme activity in phenotypic alteration.

Discussion

Two-component signal transduction systems, which are widely distributed in gram positive and negative bacteria to sense extracellular signals, render bacteria to respond environmental changes and thus to survive and thrive in the environments or hosts. KvhAS has previously been identified and found to regulate the synthesis of CPS (40) , which is a crucial virulence factor for *K. pneumoniae* infection (23). We have also shown that the overexpression of KvhA resulted in an alteration of bacterial susceptibility to a small number of antibiotics; the deletion of *kvhS* reduced *kvhAS* promoter activity in the presence of 300 mM NaCl (41), and 44℃-heat shock treatment increased *kvhAS* promoter activity (25).

Sequence analysis revealed that KvhAS has high sequence homology to EvgAS that has been demonstrated to regulate multi-drug resistance (48), heat-resistance (12) and acid resistance (44) in *E. coli*. A similar role was hence proposed. Herein we show a positive role of KvhAS on the expression of YfiD by 2D-PAGE comparative analysis of the proteome patterns of *K. pneumoniae* CG43U9435 and CG43U9435Δ*kvhA*. Upon analyzing the regulation in aerobic culture, the *kvhA* deletion showed no apparent effect on *yfiD* expression in mild acid (pH 5.5) condition (74). The activity measurement was thus carried out under microaerobic condition which has been shown to be optimal for *yfiD* expression *E. coli* (69). Under the microaerobic condition, both KvhA and RpoS were found to play positive roles in the control of the

expression of *yfiD*. Activity analysis of P_{yfiD-1}, P_{yfiD-2} and P_{yfiD-3} containing different sizes of the *yfiD* promoter suggesting RpoS element I is for RpoS binding to regulate *yfiD* expression. Unknown repressive element may be located near RpoS element II since an apparent increase of P_{yfiD-2} activity. While the reduced activity of P_{yfiD-3} suggested unknown up-regulatory element exists around RpoS element I. Nevertheless, further work have to be carried out for an insight view of the regulation of RpoS on *yfiD* expression.

Interestingly, the previous study has shown that RpoS negatively regulates *kvhAS* expression (41). Herein a model of RpoS-KvhAS-YfiD regulatory pathway is proposed. As shown in Fig. 12, we speculate that the RpoS-dependent regulation is much stronger than KvhAS for the expression of *yfiD*. Acetate stress simultaneously induced the expression of YfiD and RpoS growth phase regulon in *E. coli* W3110 (33), also supporting a role of RpoS in *yfiD* regulation. Nevertheless, a regulatory role of KvhA in *yfiD* expression at transcriptional level could be supported by EMSA. The putative EvgA box in P_{yfiD-2} was not required for the binding by the recombinant KvhA, suggesting a different regulatory pattern of KvhAS from that of EvgAS. The non-identical residues in the highly homologous (63.8% sequence identity) DNA binding domain possibly contribute to the different DNA binding specificity.

By comparing YfiD content of mild acid-grown bacteria, it was shown that Δ*kvhA* mutant strain had a reduced YfiD protein expression. However, *kvhA* complement plasmid using pHAm (41) carrying a *kvhA*

coding region did not increase the expression of YfiD protein, possibly due to low expression of KvhA. Introducing the plasmid pKvhAcPP with *kvhA* coding gene and RBS into Δ*kvhA* mutant showed a small increase of YfiD expression. The small increase may be due to the interference of some non-phosphorylated KvhA protein and thus no fully-complementing effect was observed. The possibility was further confirmed by transformation of pHAcAPP or pHAcEPP to the *kvhA* mutant. The substitution of the receiver residue by glutamate $KvhA_{D52E}$, which was able to mimic a continuously phosphorylated status of KvhA, led to apparent increase of YfiD expression.

Acid-resistant role was proposed since YfiD was demonstrated to reduce acidic-metabolite accumulation in cytosol under microaerobic environment (69). In addition, expression of *yfiD* has been found to be activated by acid either in aerobic and anaerobic environments (5). Using acid-shock resistance assay, we have also shown an involvement of *yfiD* at aerobic condition and the glycine residue 102 determines the catalytic activity in acid-shock resistance. Although YfiD was demonstrated to be highly expressed and activated in microaerobic condition (69), the expression level remains high under aerobic environment suggesting a role in aerobic environment.

The complementation assay using pRK415 and pCPP45 with or without the addition of the antibiotics tetracycline resulted in a steep drop of the survival rate, an involvement of tetracycline-resistant gene was hence proposed. TetA is a membrane-spanning H^+-$[tc\text{-}Mg]^+$ antiporter embedded in the cytoplasmic membrane (71, 72). Overexpression of TetA

has been shown to be lethal for *E. coli* (3), which has been attributed to membrane potential collapse (17). Moreover, expression of TetA in the absence of tetracycline was also harmful to bacterial cells (2). It is hence other selection markers containing plasmids should be used to resolve the problem.

E. coli yfiD mutant also revealed growth defect upon acid treatment (50). However, no apparent growth retardation of the Δ*yfiD* strain was observed in the growth curve comparison under pH 7.0, pH 5.5, aerobic and microaerobic environments. The *yfiD* null mutant of *E. coli* W3110 showed an apparent increase of acid-metabolite accumulation including pyruvate, formate, succinate and lactate (69). No growth defect was observed for *K. pneumoniae* CG43S3Δ*yfiD* in microaerobic growth could be attributed to the reutilization of the neutral metabolite acetoin to prevent for the accumulation of acidic metabolic end products in the environment (70). Analysis of *K. pneumoniae* CG43 genome sequence (http://140.113.173.58) revealed two proteins TdcE and YbiW which may compensate YfiD activity since a conserved radical-enzyme domain was identified. However, whether the radical-enzyme domain in these proteins is crucial for bacterial growth remains to be investigated.

No study has indicated a regulatory role of EvgAS on the expression of *yfiD* in *E. coli*. The acid-shock resistance assay indicated a reduced survival of Δ*yfiD* mutant comparing to the parental strain implying a role of YfiD in the acid shock resistance in *K. pneumoniae* CG43. EvgAS has been demonstrated to regulate the expression of *gadA and gadBC* (45) which have been identified to be the pivotal components of AR system 2

(42). The AR 2 assay condition (28) could also be employed to investigate if the phenotypic alteration of *yfiD* deletion is related to the acid resistance pathway.

The cDNA subtractive hybridization analysis (39) revealed a KvhA-activated gene *dcuB*, encoding an anaerobic C4-dicarboxylate transporter (60). KvhAS to sense extracellular status or to cross-react with other regulator to execute the YfiD- and DcuB- mediated metabolic pathway is hence proposed. As shown in Fig. 13, the energy-source materials are ingested and flow through glycolysis and TCA cycle to derive energy, and finally go through an electronic transport chain using O_2 as a terminal e^- acceptor. While under microaerobic environment where fumarate respiration dominates (27), pyruvate, the end product of glycolysis, is introduced into further oxidation pathway by YfiD with fumarate as a terminal e^- acceptor. It is then DcuB transports fumarate into the bacterium to replenish the fumarate pool, and pull succinate, a reduced form of fumarate, out to the environment (60).

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74. 林佳融，大專生參與專題研究計畫，核定計畫編號 94-2815-C-009-045-B，探討 KvhAS 雙分子系統在克雷白氏肺炎桿菌抗酸能力上所扮演的調控角色，中華民國九十五年六月。

Tables

Table 1. Bacterial strains used in this study

Strain	Genotype or relevant property	Reference or source
E. coli:		
BL21(DE3)	F$^-$ *ompT hsdS$_B$(r$_B^-$ m$_B^-$) gal dcm met* (DE3)	Laboratory stock
JM109	*RecA1 supE44 endA1 hsdR17 gyrA96 rolA1 thi* Δ*(lac-proAB)*	Laboratory stock
BL21-CodonPlus(DE3)-RIL	F$^-$ *ompT hsdS$_B$(r$_B^-$m$_B^-$)gal dcm*(DE3)	Laboratory stock
S17-1λ*pir*	Tp^r Sm^r *recA, thi, pro, hsdR$^-$M$^+$* [RP4-2-Tc::Mu:Km^r Tn*7*](*pir*)	(15)
K. pneumoniae:		
CG43	K2 serotype	(51)
CG43S3	*rspl* mutant, Strep^r	(51)
CG43S3-Z01	*lacZ* mutant in CG43 *rspl*$^-$	(40)
CG43S3- Z01Δ*kvhA*	*kvhA* mutant in CG43 *rspl*$^-$ *lacZ*$^-$	(41)
CG43S3- Z01Δ*rpoS*	*rpoS* mutant in CG43 *rspl*$^-$ *lacZ*$^-$	(41)
CG43S3-U9451	*galU* deletion mutant	(11)
CG43S3Δ*kvhAS*	*kvhAS* mutant in CG43 *rspl*$^-$	(40)
CG43S3-U9451Δ*kvhAS*	*kvhAS* mutant in CG43 *rspl*$^-$ *galU*$^-$	(39)
CG43-S3Δ*kvhS*	*kvhS* mutant in CG43 *rspl*$^-$	(40)
CG43-S3Δ*yfiD*	*yfiD* mutant in CG43 *rspl*$^-$	This study

Table 2. Plasmids used in this study

Plasmid	Relevant characteristic	Source or reference
yT&A	PCR cloning vector, Ap^r	Yeastern Biotech Co.
pET-30b	His_6-tagging protein expression vector, Km^r	Novagene
pKAS46	Suicide vector, rpsL, Km^r	(61)
pHP4001	putative *kvhA* promoter region cloned into pUC-T	(35)
pHP4005	pET-30c carrying *kvhA* gene	(40)
pHAm	*Bam*HI digested fragment of pHP4005 cloned into *Bam*HI site of pETm-c	(41)
pRK415	broad-host-range IncP cloning vector, Tc^r	(32)
pLacZ15	A derivative of pYC016 (36), containing a promoterless *lacZ* from *K. pneumoniae* CG43S3 as the reporter, Cm^r	(40)
pCPP45	broad-host-range cloning vector with the partition region from RK2 plasmid, Tc^r	Dr. David Bauer at Cornell University
pYm67TA	1081-bp PCR product of the region upstream of *yfiD* cloned into yT&A	This study
pYm89TA	1148-bp PCR product of the region downstream of *yfiD* cloned into yT&A	This study
pYm2KTA	*Bam*HI digested fragment of pYm89TA cloned into pYm67TA	This study
pYm2KAS	*Eco*RI/XbaI digested fragment of pYm2KTA cloned into pKAS46	This study
pKvhAcTA	698-bp PCR product carrying *kvhA* cloned into yT&A	This study
pKvhAcPP	*Bam*HI/*Eco*RI digested fragment of pKvhAcTA cloned into pCPP45	This study
pHAcATA	698-bp PCR product expressing $KvhA_{D52A}$ cloned into yT&A	This study
pHAcAPP	*Bam*HI/*Eco*RI digested fragment of pHAcATA cloned into pCPP45	This study
pHAcETA	698-bp PCR product expressing $KvhA_{D52E}$ cloned into yT&A	This study
pHAcEPP	*Bam*HI/*Eco*RI digested fragment of pHAcETA cloned into pCPP45	This study

Plasmid	Relevant characteristic	Source or reference
pYcTA	502-bp PCR product carrying *yfiD* cloned into yT&A	This study
pYcPP	*Bam*HI/*Eco*RI digested fragment of pYcTA cloned into pCPP45	This study
pYcRK	*Bam*HI/*Eco*RI digested fragment of pYcTA cloned into pRK415	This study
pYc102TA	502-bp PCR product expressing $YfiD_{G102A}$ cloned into yT&A	This study
pYc102PP	*Bam*HI/*Eco*RI digested fragment of pYc102TA cloned into pCPP45	This study
pYc102RK	*Bam*HI/*Eco*RI digested fragment of pYc102TA cloned into pRK415	This study
pY34TA	429-bp PCR product carrying *yfiD* cloned into yT&A	This study
pY34B	441-bp *Bam*HI/*Hind*III digested fragment of pY34TA cloned into pET-30b	This study
pHArdTA	337-bp PCR product	This study
pHArdB	*Bam*HI/*Eco*RI digested fragment of pHArdTA cloned into pET-30b	This study
pYfiD01	302-bp PCR product carrying P_{yfiD-2} cloned into yT&A	Laboratory Stock
pYfiD02	*Bam*HI/*Bgl*II digested fragment of pYfiD01 cloned into the *Bam*HI site of pLacZ15	Laboratory Stock
pY25TA	417-bp PCR product carrying P_{yfiD-1} cloned into yT&A	This study
pY25Z	*Bam*HI/*Bgl*II digested fragment of pY25TA cloned into the *Bam*HI site of pLacZ15	This study
pY27TA	274-bp PCR product carrying P_{yfiD-3} cloned into yT&A	This study
pY27Z	*Bam*HI/*Bgl*II digested fragment of pY27TA cloned into the *Bam*HI site of pLacZ15	This study
pY28TA	204-bp PCR product carrying P_{yfiD-4} cloned into yT&A	This study
pY28Z	*Bam*HI/*Bgl*II digested fragment of pY28TA cloned into the *Bam*HI site of pLacZ15	This study
pY29TA	130-bp PCR product carrying P_{yfiD-5} cloned into yT&A	This study
pY29Z	*BamH*I/*Bgl*II digested fragment of pY29TA cloned into the *Bam*HI site of pLacZ15	This study
pUNY	1010-bp PCR product carrying *yfiD* and P_{yfiD} cloned into yT&A	This study

Table 3. Primers used in this study

Primer	Sequence
yfiDm06	5’- GAATGGTGGCGTCAGAACAAGGCG -3’
yfiDm07	5’- GGATCCCAGGCAGTGTTTATTCGA -3’
yfiDm08	5’- GGATCCGCGGTACGCTTCAACTCT -3’
yfiDm09	5’- TCTAGACATCGCCACCATTACCGG -3’
kvhAc01	5’- CGCGGATCCTGCACCAGGC -3’
kvhAc02	5’- GATAGAATTCGACGAACCGCCATG -3’
hAd52a-F3	5’- CCTGCTCATCGTCGCTGTCGATATTCCG -3’
hAd52a-R3	5’- CGGAATATCGACAGCGACGATGAGCAGG -3’
hAd52e-F2	5’- CCTGCTCATCGTCGAAGTCGATATTCCGGA -3’
hAd52e-R2	5’- TCCGGAATATCGACTTCGACGATGAGCAGG -3’
yfiDc01	5’- GGATCCGGCTGATATATATACAC -3’
yfiDc02	5’- GAATTCACATCTGGCGTTAGCCT -3’
yfiDg102a-F3	5’- CAGCTGACCATTCGTGTTTCCGCATACGCG -3’
yfiDg102a-R3	5’- CGCGTATGCGGAAACACGAATGGTCAGCTG -3’
kvhA-rd-F	5’- TCTGCAGCCCATGAATGCGATAAT -3’
kvhA-rd-R	5’- GATTCAATTCATTCCCTCTTTTTT -3’
yfiD01	5’- CATCGCTAGATCTTAACGGC -3’
yfiD02	5’- CCAGTAATCATTGGATCCCTC -3’
yfiD03	5’- CGAATTCACACTGCCTGGGA -3’
yfiD04	5’- CACATCTGGCGTTAGCCTTA -3’
yfiD05	5’- GGAGATCTTCGGCGACGGTC -3’
yfiD07	5’- ATAGATCTAAATCGGCACGG -3’
yfiD08	5’- AGATCTCACAATGGCGTAAC -3’
yfiD09	5’- AGATCTATATCAAGGAATGCCG -3’

Figures

Figure 1. Sequences of the putative *yfiD* promoter. A putative -10 box and -35 box are highlighted. FNR element I and II, ArcA element, RpoS element I and II, and a putative EvgA binding box (31, 34, 37, 69) are also indicated. The promoter containing regions used in the study include $P_{yfiD\text{-}1}$ (pY25Z), $P_{yfiD\text{-}2}$ (pYfiD02), $P_{yfiD\text{-}3}$ (pY27Z), $P_{yfiD\text{-}4}$ (pY28Z) and $P_{yfiD\text{-}5}$ (pY29Z).

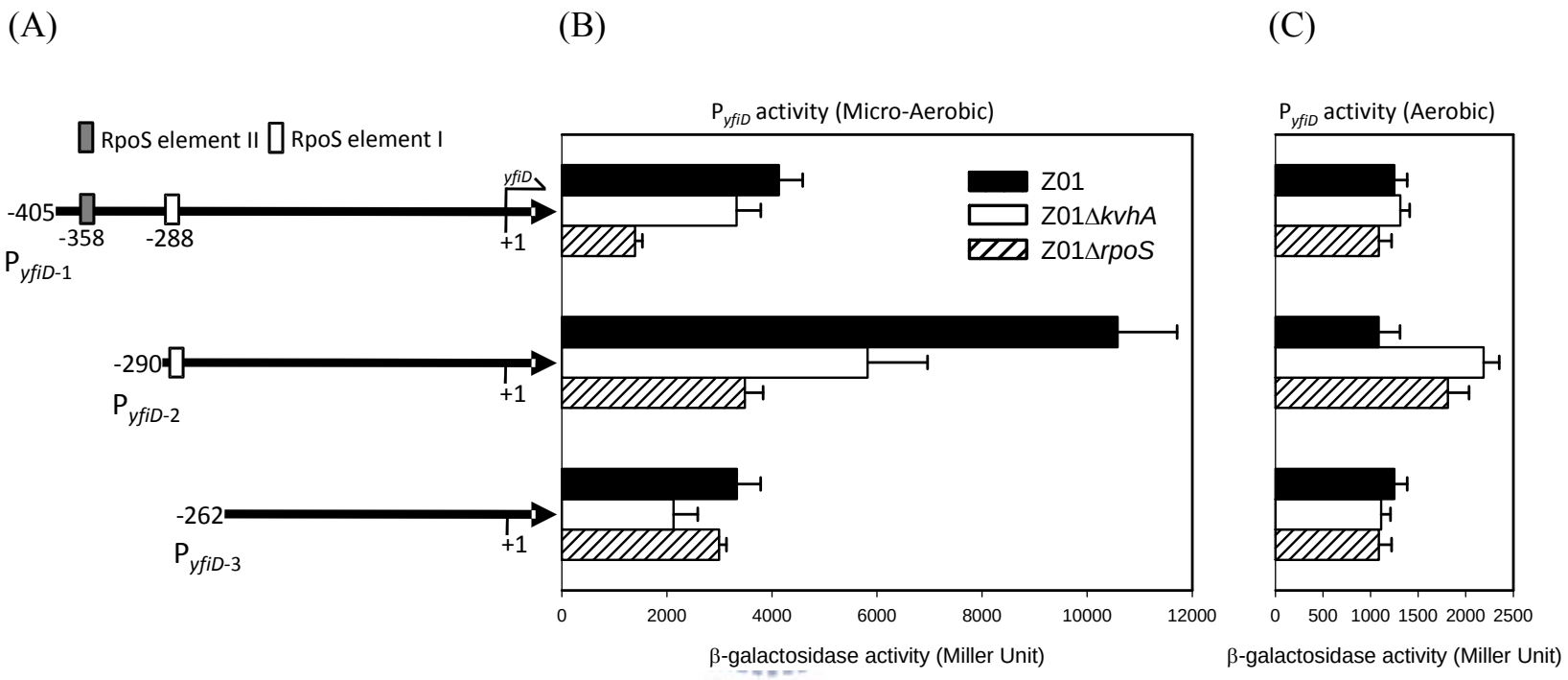


Figure 2. The deletion of *kvhA* or *rpoS* reduced the expression of *yfiD* in microaerobic environment. (A) Two RpoS binding elements (I & II) and the putative promoters are shown. The promoters P_{yfiD-1}, P_{yfiD-2}, and P_{yfiD-3} respectively containing 405 bp, 290 bp, and 262 bp noncoding sequence upstream of the *yfiD* start codon were isolated and inserted in front of the promoterless *lacZ* gene of pLacZ15. (B) The bacteria were cultured for 4 h in 15 ml tightly sealed culture tube filled with LB and the activity of P_{yfiD-1}, P_{yfiD-2} and P_{yfiD-3} in either of *K. pneumonae*CG43S3-Z01, CG43S3-Z01Δ*kvhA,* or CG43S3-Z01Δ*rpoS* was measured.

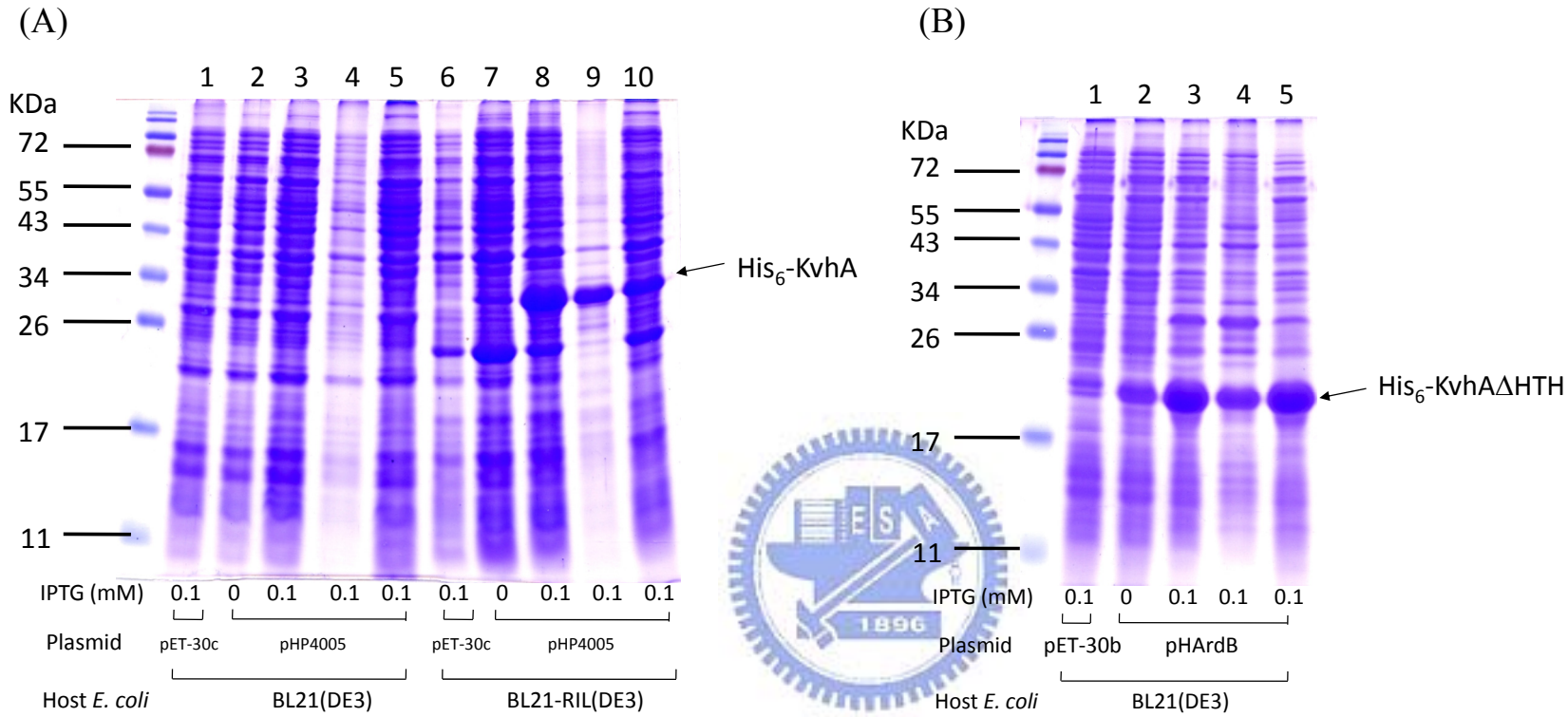


Figure 3. Overexpression of His_6-KvhA and His_6-KvhAΔHTH proteins. (A) pHP4005 carrying *kvhA* coding sequence was transformed into *E. coli* BL21(DE3) and BL21-RIL respectively. After 5 h IPTG induction (lanes 1 to 3, 6 to 8), the induced bacteria (lanes 3, 8) were disrupted and centrifugated into pellet (Lanes 4, 9) and supernatant (Lanes 5, 10) forms. The supernatant containing soluble His_6-KvhA (lane 10) was then subjected to purification. (B) pHArdB carrying the *kvhA* coding sequence deletion of HTH domain was transformed into *E. coli* BL21(DE3). After 5 h IPTG induction (lanes 1 to 3), the induced bacterium (lane 3) was disrupted and centrifugated into pellet (lanes 4) and supernatant (lanes 5) forms. The supernatant containing soluble His_6-KvhA ΔHTH (lane 5) was also subjected to purification.

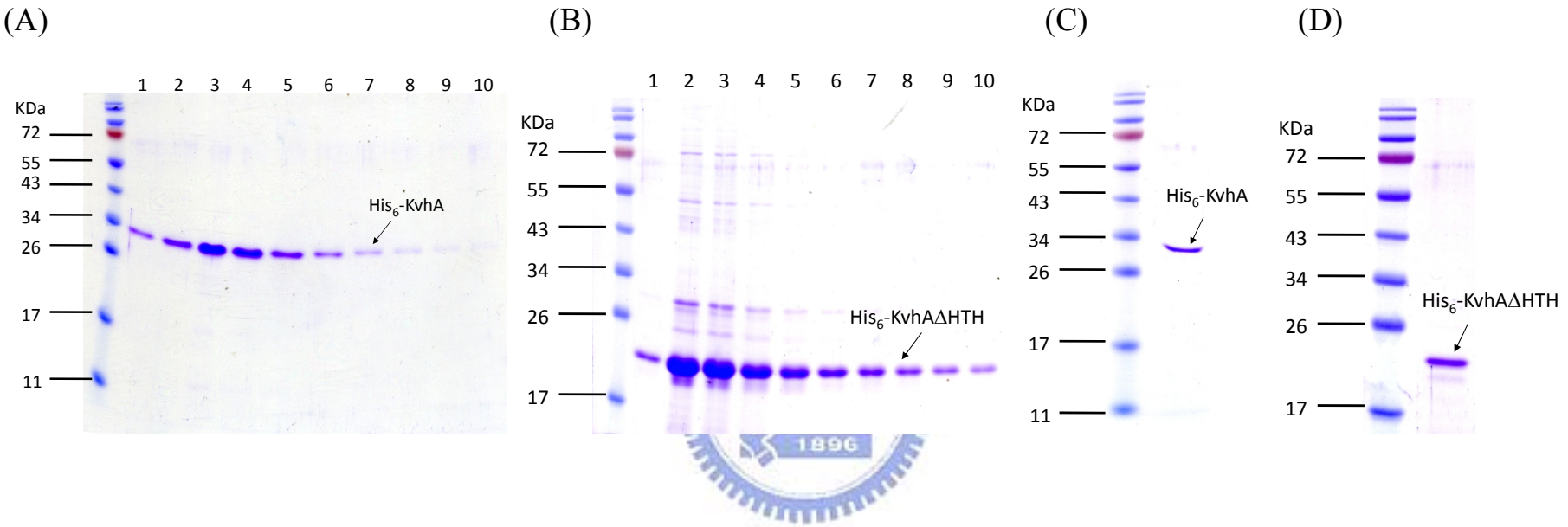
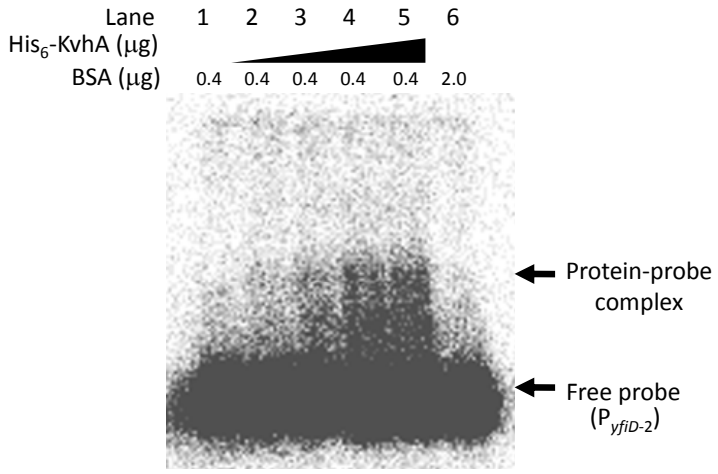


Figure 4. Purification of His_6-KvhA and His_6-KvhAΔHTH proteins. Soluble fractions of the total cell lysates respectively containing His_6-KvhA (A) and His_6-KvhAΔHTH (B) were subjected to His_6-Bind Resin column for purification. The protein purity of each eluted fractions containing His_6-KvhA (lanes 1 to 10 of (A)) and His_6-KvhAΔHTH (lanes 1 to 10 of (B)) were then analyzed by SDS-PAGE. The collected fractions were subjected to dialysis, concentration and the purity of His_6-KvhA (C) and His_6-KvhAΔHTH (D) confirmed by SDS-PAGE.

(A)



(B)

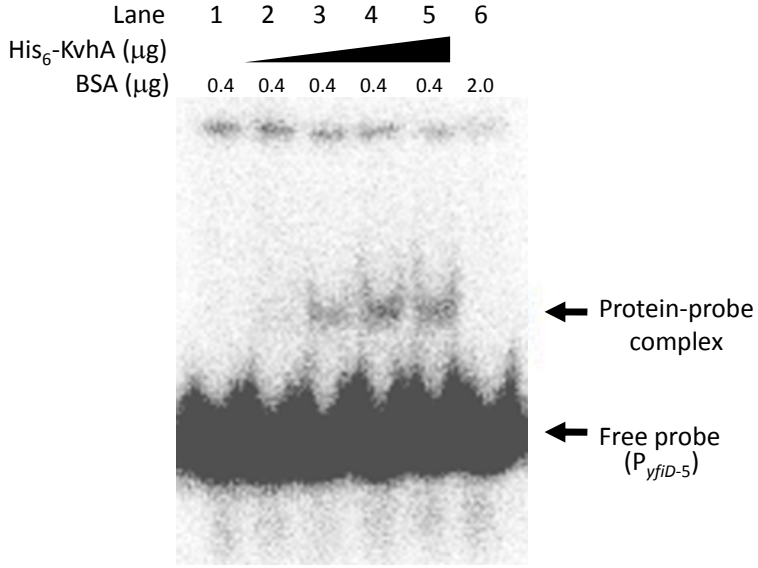
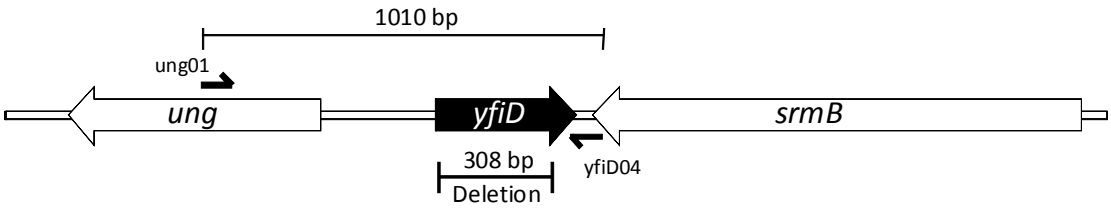


Figure 5. Direct binding of the phosphorylated His_6-KvhA to P_{yfiD}. The direct binding activity of His_6-KvhA was assayed by electrophoretic mobility shift assay with 5 % (w/v) non-denature polyacrylamide gel. Different amount of the purified His_6-KvhA were phosphorylated in 1 X binding buffer in the presence of 50 mM acetylphosphate and 1μg of acetylated bovine serum, and then the ^{32}P-labelled P_{yfiD-2} (A) or P_{yfiD-5} (B) DNA fragments was added. The His_6-KvhA added in lanes 1 to 6 respectively was 0 μg, 0.18 μg, 0.54 μg, 1.08 μg, 1.44 μg and 0 μg.

(A)



(B)

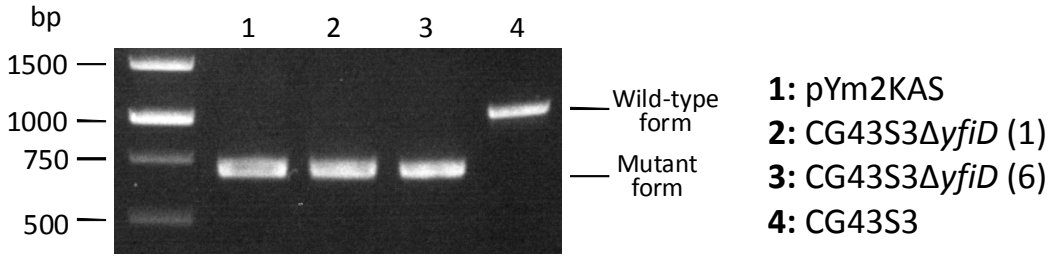


Figure 6. Construction of the *yfiD* deletion mutant. (A) Schematic representation of *yfiD* and the flanking genes. Primer set ung01/yfiD04 was designed to confirm *yfiD* mutant. (B) PCR analysis using ung01/yfiD04 primer to confirmed the deletion of *yfiD*. The plasmid pYm2KAS contains the flanking DNA of the *yfiD* for homologous recombination to generate the *yfiD* deletion mutant.

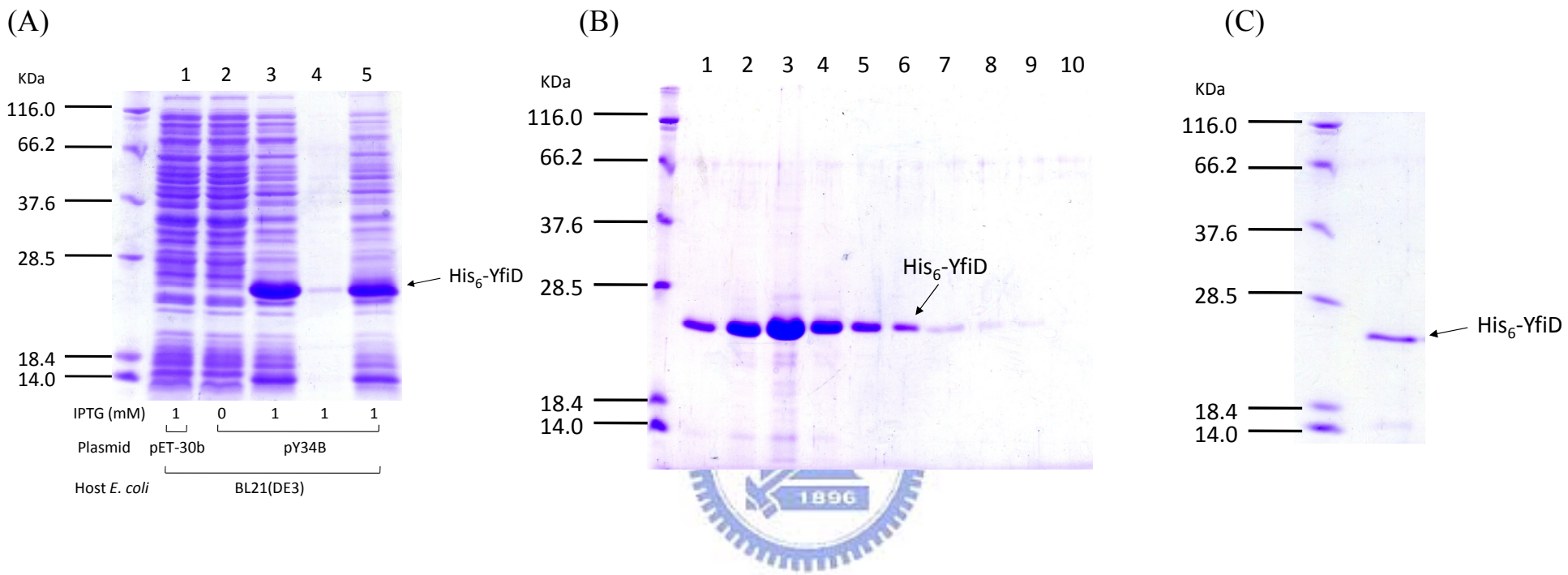


Figure 7. Expression and purification of His_6-YfiD protein. (A) The coding sequence of *yfiD* gene was cloned into pET-30b and the resulting plasmid pY34B transformed into *E. coli* BL21(DE3). After 5 h IPTG induction (lanes 1 to 3), the induced bacterium (lane 3) was disrupted and centrifugated into pellet (lane 4) and supernatant (lane 5) forms. The supernatant containing soluble His_6-YfiD (lane 5) was then subjected to His_6-Bind Resin column for purification. (B) The protein purity of each eluted fractions containing His_6-YfiD (lanes 1 to 10) were then analyzed by SDS-PAGE. The collected fractions were subjected to dialysis, concentration and the purity of His_6-YfiD (C) confirmed by SDS-PAGE.

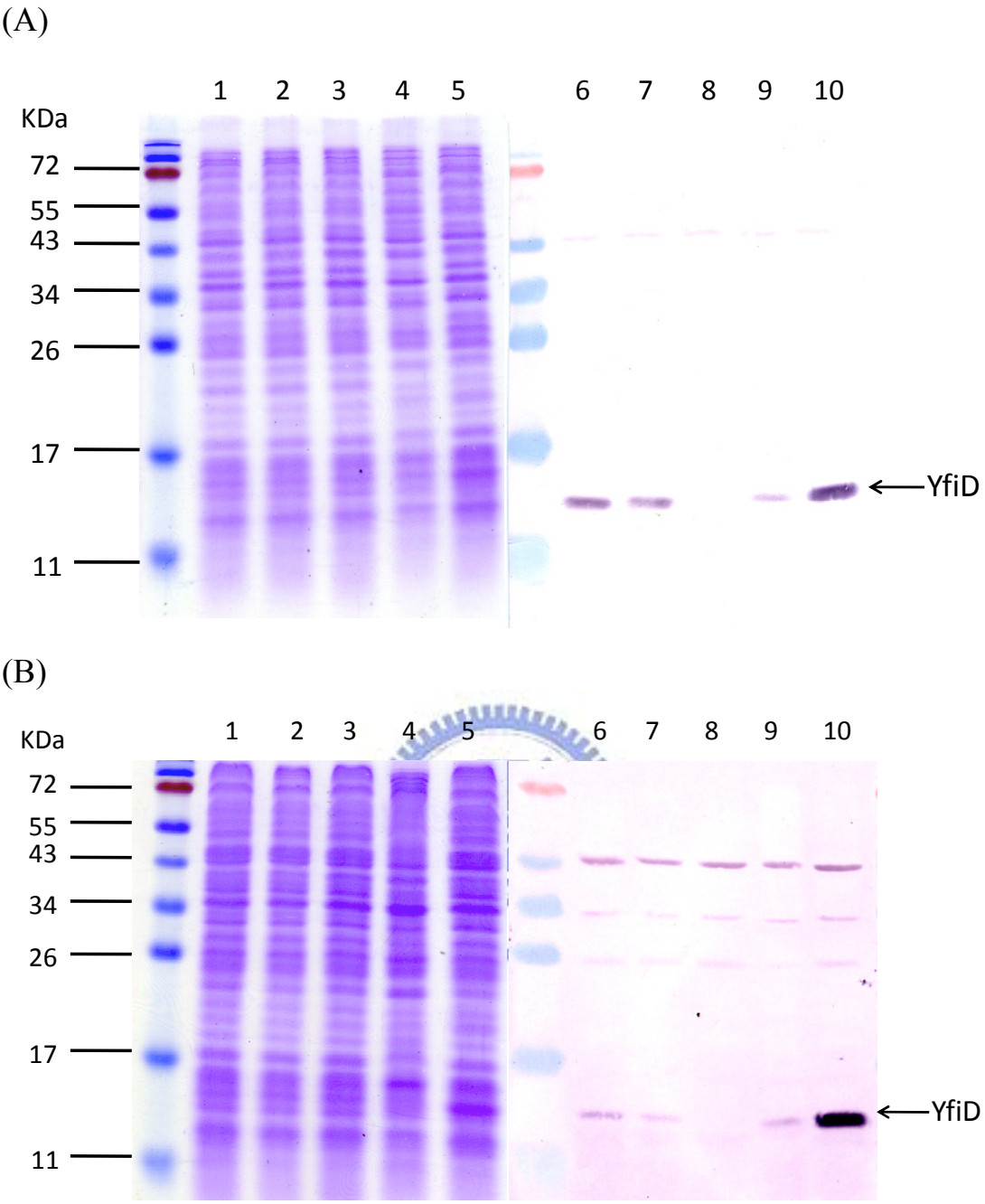


Figure 8. Western blotting analysis of the YfiD protein. Overnight bacteria were refreshed grown in (A) LBMES (pH 5.5) and (B) M9MES (pH 5.5) medium at 37℃ aerobically for 6 h and then subjected to SDS-PAGE and western blot hybridization using the YfiD antiserum. Lanes 1 and 6 are the cell lysates from *K. pneumoniae* CG43S3; 2 and 7 from CG43S3Δ*kvhA*; 3 and 8 from CG43S3Δ*yfiD*; 4 and 9 from CG43S3 [pHAm]; 5 and 10 from CG43S3Δ*yfiD* [pYcRK].

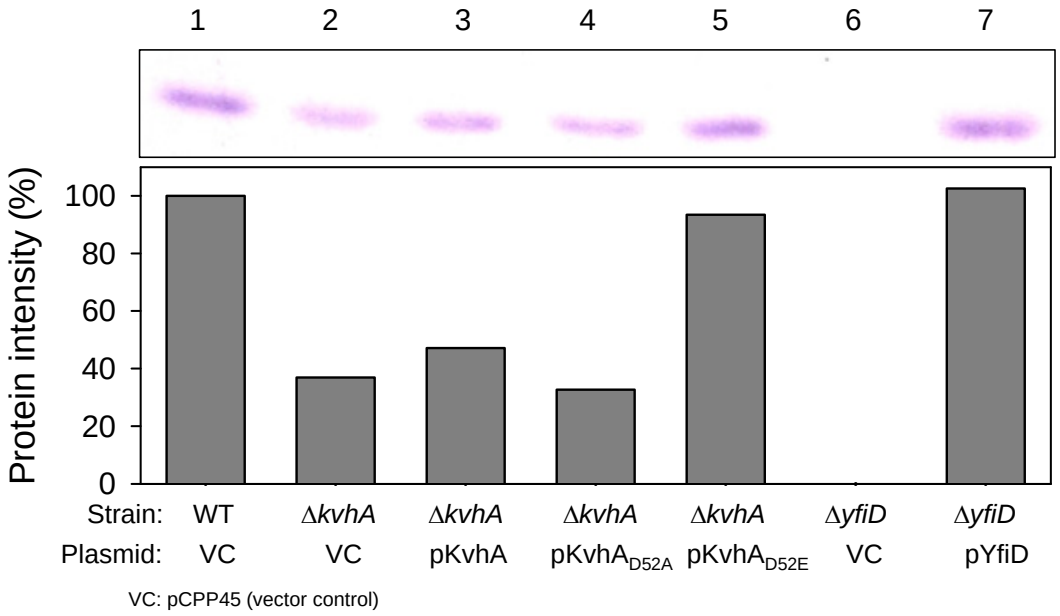


Figure 9. KvhAS phosphorelay is required for the regulation of YfiD expression. Overnight bacteria were refreshed grown in LBMES (pH 5.5) and medium at 37℃ aerobically for 6 h and then subjected to SDS-PAGE and western blot hybridization using the YfiD antiserum. Protein density was determined by Gel-Pro Analyzer™. Lanes 1 to 7 are respectively *K. pneumoniae*CG43S3[pCPP45], CG43S3Δ*kvhA* [pCPP45], CG43S3Δ*kvhA* [pKvhAcPP], CG43S3Δ*kvhA* [pHAcAPP], CG43S3Δ*kvhA*[pHAcEPP], CG43S3Δ*yfiD*[pCPP45] and CG43S3Δ*yfiD* [pYcPP].

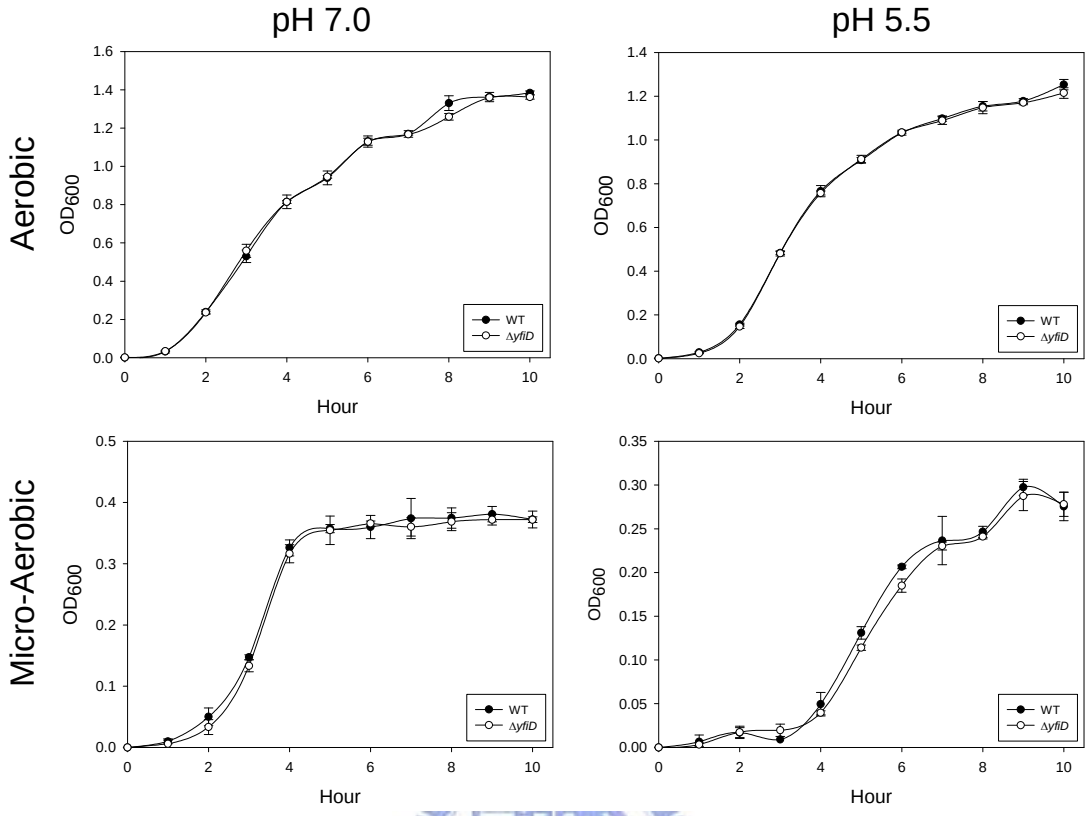
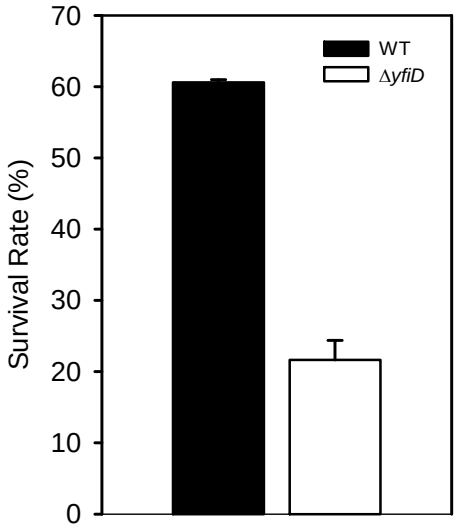
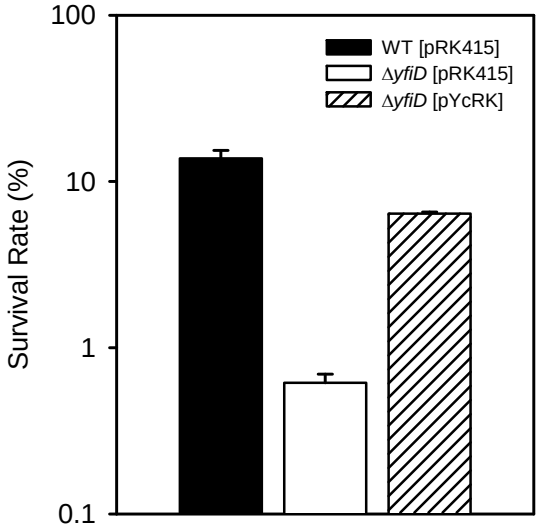


Figure 10. Growth analysis of *K. pneumoniae* CG43S3 and CG43S3Δ*yfiD*. A hundred fold dilution of the overnight grown bacteria was cultured in LB (pH 7.0) or LBMES (pH 5.5) at 37℃ in aerobic or microaerobic condition. OD_{600} was measured each hour and the charted diagrams were resulted from triplicate analysis.

(A)


70
60
50
40
30
20
10
0
Survival Rate (%)
WT
ΔyfiD

(B)


100
10
1
0.1
Survival Rate (%)
WT [pRK415]
ΔyfiD [pRK415]
ΔyfiD [pYcRK]

(C)

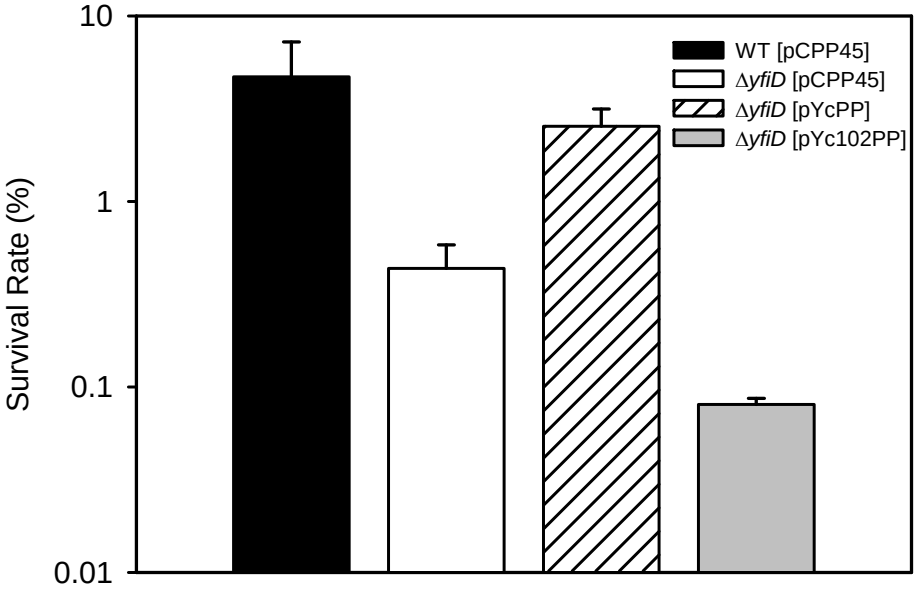

10
1
0.1
0.01
Survival Rate (%)
WT [pCPP45]
ΔyfiD [pCPP45]
ΔyfiD [pYcPP]
ΔyfiD [pYc102PP]

Figure 11. Acid-shock resistance. A hundred fold dilution of the overnight grown bacteria was cultured in (A) (C) pH 5.5 LBMES medium or (B) pH 5.5 LBMES medium with 5 μg/ml tetracycline addition at 37℃ till the OD_{600} reached 0.7. The bacteria were then refreshed grown in LB medium (pH 3.0) for another 35-minute. Finally, the cultures were plated onto LB plates for colony formation. The survival rate was determined as percentage of the viable counts of the cultures at pH 3 to that of pH 5.5. The plasmids pYcRK and pYcPP contain an *yfiD* gene with an RBS sequence in pRK415 and pCPP45 respectively. The plasmid pYc102PP contains an $YfiD_{G102A}$ coding sequence and an RBS sequence.

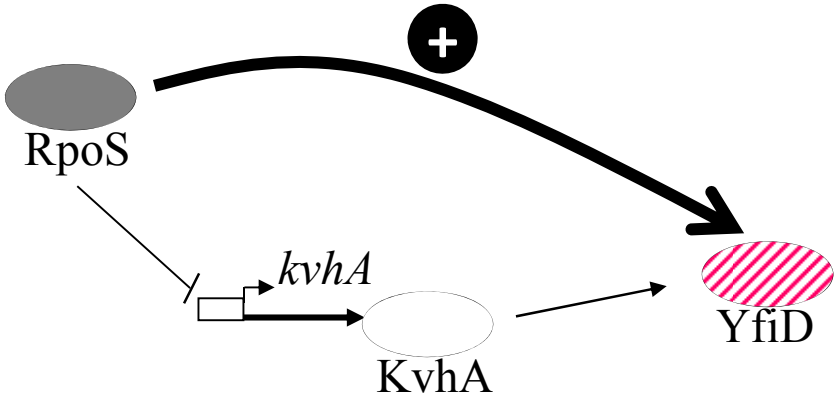


Figure 12. Schematic description of the RpoS-KvhA-YfiD regulatory model. RpoS plays a major role for the expression of *yfiD*.

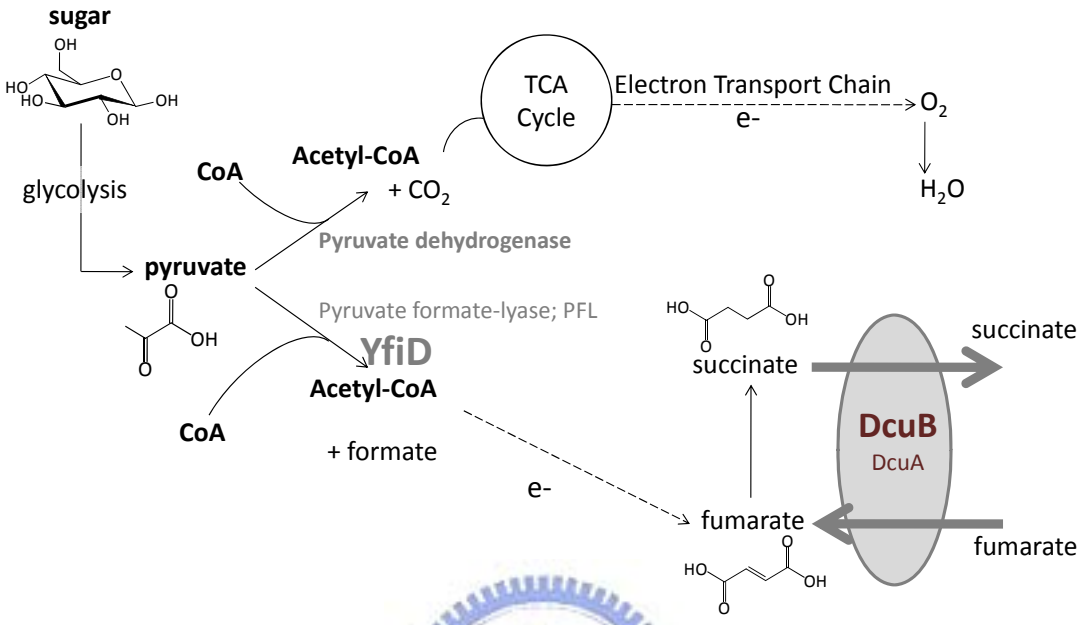
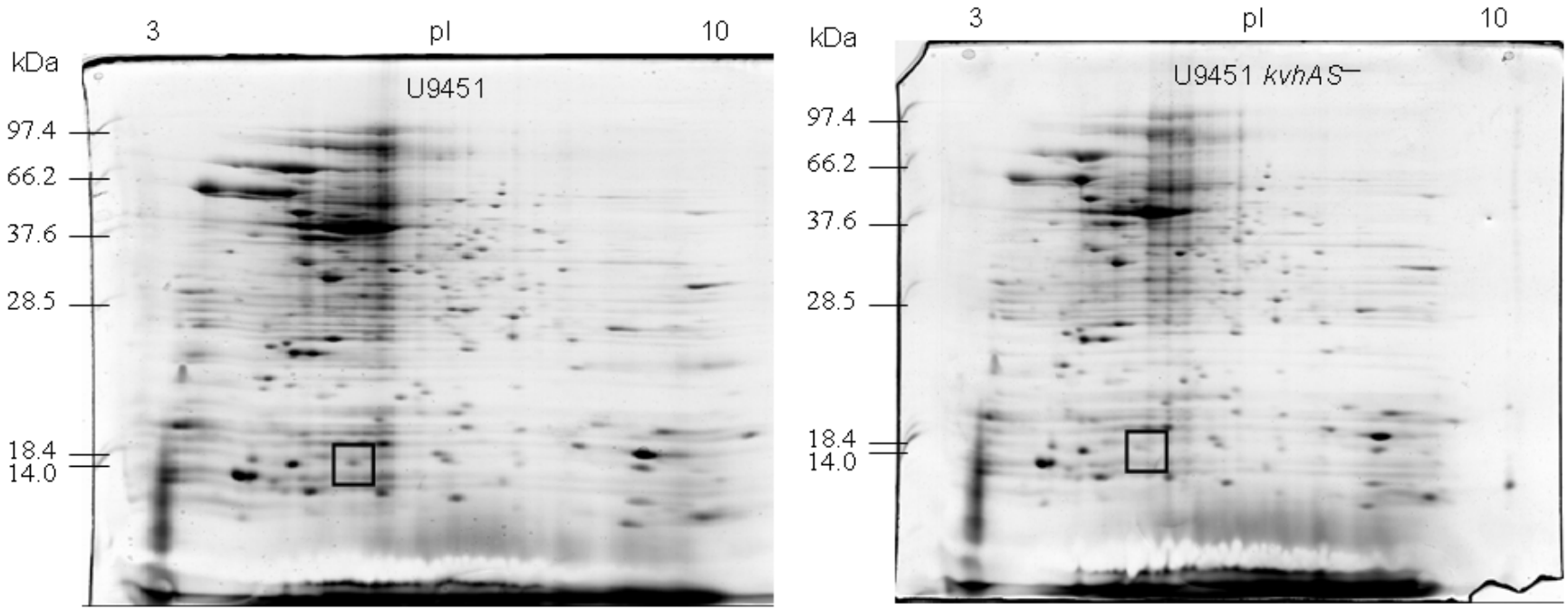


Figure 13. Schematic description of the postulated function of YfiD and DcuB in *K. pneumoniae* CG43. KvhAS is proposed to activate the expression of *yfiD* and *dcuB* for the regulation of the metabolic pathway under microaerobic environment.

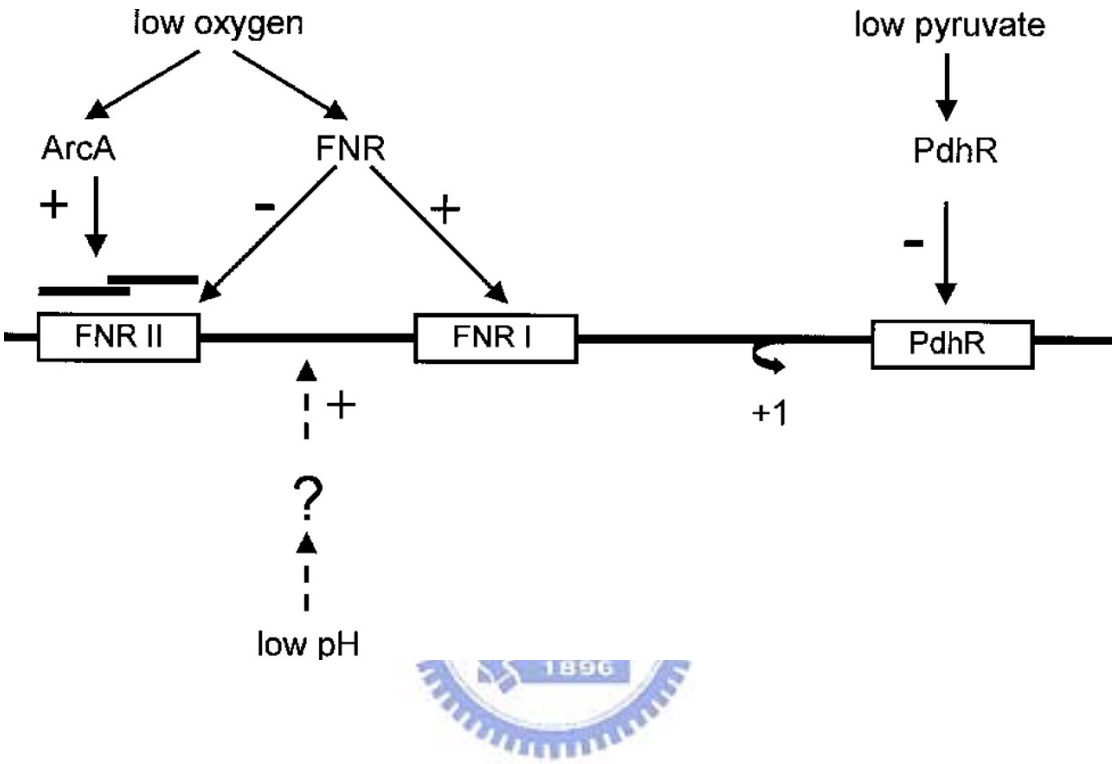
Appendix I

Two dimensional gel electrophoresis of the total proteins extracted from (A) *K. pneumoniae* U9451 and (B) U9451*kvhAS*⁻ at late-log phase culture in LB medium and the gel stained with Coomassie blue (74).



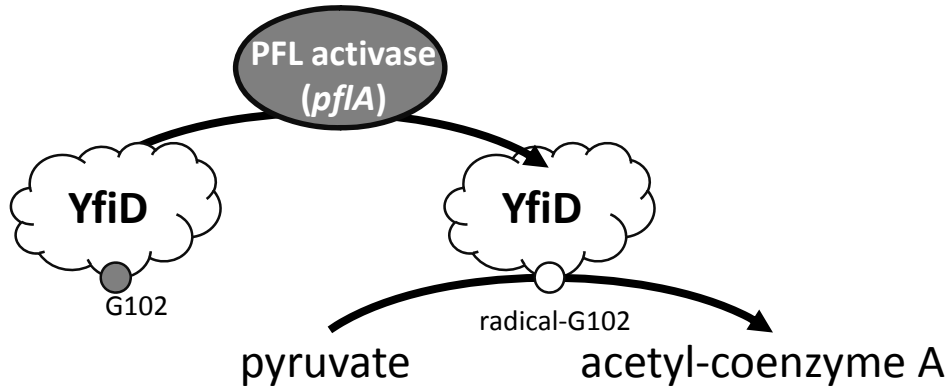
Appendix II

Regulation of the *yfiD* gene of *E. coli* showing the effects of global regulators on the activity of the *yfiD* promoter (69).



Appendix III

Schematic description of the activation steps of YfiD enzyme



In microaerobic condition, glycine 102 (G102) of YfiD protein is activated into radical-G102 which functions to convert pyruvate into acetyl-coenzyme A (69).