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Role of the *in vivo* expression protein YjcC in the oxidative stress responses in *Klebsiella pneumoniae* CG43

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I. Abstract

Ia. Abstract in Chinese

關鍵詞：克雷白氏肺炎菌 CG43，活體表現蛋白 YjcC，EAL domain 功能，抗氧化酵素 SOD 和過氧化氫去氫酶，過氧化逆境反應。

感染過程中，如何抵抗過氧化的逆境壓力，是決定細菌毒性的重要因子。我們曾報導克雷白氏肺炎桿菌 CG43 在老鼠活體表現的 YjcC 蛋白可被 10 μM 巴拉刮所誘導，同時，基因結構分析顯示過氧化逆境的兩個主要調控基因 *soxS* 和 *soxR* 座落於 *yjcC* 基因旁，我們的先導實驗更證明 *yjcC* 基因缺損會使細菌抗氧化逆境的能力大為降低，這些結果顯示 YjcC 蛋白在克雷白氏肺炎桿菌 CG43 抗氧化逆境反應中扮演重要的角色。為了更深入探討 *yjcC* 基因在其生物上的意義，近三年完成的工作成果如下:

1. 建立 *yjcC* 啟動子報導系統證明此基因確實受巴拉刮所誘導而活化，此外再將克雷白氏肺炎桿菌經 500 μM 巴拉刮或 10 mM 過氧化氫處理過後，以定量聚合酶反應分析，結果發現 *yjcC* 與已知抗氧化壓力調控基因（*soxR*、*soxS*、*oxyR*、*rpoS*、*fur* 及 *fnr*）的表現，經過巴拉刮與過氧化氫處理之後，皆會增加其表現量。

2. YjcC 蛋白在其 C 端具有 EAL domain，並且有 Phosphodiesterase 酵素的活性。在 YjcC 蛋白之 N 端部分，則被預測具有穿膜蛋白區域，而 N 端的功能在未來將被確認。

3. 將 *yjcC* gene 剔除後，經過巴拉刮和過氧化氫的作用，其生存率明顯下降相較與野生株，而此反應可被互補質體 pJR1(pRK415-*yjcC*)的表現所恢復，但不被 pJR2(pRK415-*yjcC*-E303A)所恢復，顯示 YjcC 蛋白的 EAL 之 E(麩胺酸)為 phosphodiesterase 的活性所必需，同時也暗示二次訊息分子 c-di-GMP 可能參與氧化逆境的調控。

4. 當 *yjcC* gene 剔除後細胞內的 ROS 及蛋白質過氧化 carbonyl groups 的過氧化，暗示著 YjcC 蛋白具有調控清除自由基酵素的功能。而經測定在 *yjcC* gene 剔除株中超氧歧化酶(SODs)與過氧化氫酶(catalase)之活性，結果顯示 Mn-SOD、Fe-SOD 和 KatG 活性增加而 KatE 的活性減少，進一步支持 YjcC 蛋白參與調控抗氧化酵素以利清除自由基的假設。

5. 有趣的是，*yjcC* 基因剔除不僅影響了細菌的毒性也影響生物膜的生成和第三型纖毛主要蛋白 MrkA 的表現。然而，YjcC 是否直接參與克雷白氏菌第三型纖毛的表現調控仍待研究探討。

Abstract

Ib. Abstract in English

Key words: *Klebsiella pneumoniae* CG43; IVE *yjcC*; functional EAL domain, SOD and catalase, oxidative stress responses

During infection, defenses against oxidative stress play an important role in determining the bacterial virulence. The expression of *yjcC*, previously identified by *in vivo* expression technology (IVET) in *Klebsiella pneumoniae* CG43, was found to be inducible by 10 μM paraquat. Analysis of the gene organization revealed two oxidative stress regulators SoxR and SoxS encoding genes were next to *yjcC* gene. The deletion of *yjcC* was found to decrease the bacterial survival under the treatment of paraquat further suggesting an involvement of YjcC in the oxidative stress defense. The accomplished works in the past three years for the assessment of the biological role of *yjcC* are as followings.

(1) YjcC mRNA as well as several stress response regulators is increased by the treatment of H_2O_2 and paraquat. In addition, the promoter activity is inducible upon treatment with H_2O_2 or paraquat;

(2) YjcC is a PDE and the C-terminal EAL domain is essential for the enzymatic activity. However, functional role of the N-terminal transmembrane domain remains to be determined;

(3) Deletion of *yjcC* increased the susceptibility to paraquat and hydrogen peroxide, and the deficiency could be complemented by transforming into the mutant with pJR1 (pRK415-*yjcC*), the YjcC expression plasmid, but not pJR2 (pRK415-*yjcC*-E303A), the YjcC-EAL domain critical residue E was substituted by A, indicating that the involvement of YjcC in the oxidative stress response is PDE activity dependent;

(4) The intracellular ROS and protein carbonyl groups were increased by the deletion of *yjcC* implying a role of YjcC in modulating the activity of the radical scavenging enzymes. Increasing activity of Mn-SOD, Fe-SOD and KatG but decreased KatE activity were observed for the Δ*yjcC* mutant further demonstrating a regulation of YjcC on the activity of some radical scavenging enzymes;

(5) Interestingly, deletion of *yjcC* not only affected the bacterial virulence but also influenced the biofilm formation activity and the expression of MrkA, the major pilin of type 3 fimbriae. However, if YjcC directly affects the expression of type 3 fimbriae remained to be investigated.

II. Background and Significance

Klebsiella pneumoniae is responsible for 6 to 8.6 % of all community-acquired pneumonia, and alcoholics were found to be most susceptible (23, 34). The fatality rate may reach 90 % in untreated cases and 30 % in the patients with antibiotic therapy. In Taiwan, the close association between *K. pneumoniae* liver abscess and diabetes has especially drawn a lot of concerns (13, 21, 23, 87).The exact mechanism of the tight association between the bacteria and the disease remains unclear, however. An increasing number of outbreaks in pediatric wards concerning *K. pneumoniae* meningitis have also been reported lately (45). Moreover, many clinical isolates of *K. pneumoniae* are highly resistant to antibiotics and the increasing number of the ESBLKp further limits the effectiveness of current therapy (66, 89). A search for the pathogenic mechanism is warranted for an antibacterial modification.

The identified virulence factors of *K. pneumoniae* include capsular polysaccharides (CPS), lipopolysaccharides, siderophores, serum resistance factors, and adhesions (38). Clinically isolated *K. pneumoniae* usually produces large amounts of CPS, forming sticky glistering colony on LB plate, to protect the bacterium from phagocytosis by polymorphonuclear granulocytes and to prevent from killing of the bacteria by bactericidal serum factors (8, 35, 50, and 67). The hypermucoviscosity phenotype resulted from heavy capsules in *K. pneumoniae* isolates has also been associated with the development of the invasive syndrome (37, 43). *K. pneumoniae* CG43, with LD_{50} as low as 10 CFU assessed using mouse peritonitis model, is a highly capsulated clinical strain of K2 serotype isolate (9). We have previously reported in *K. pneumoniae* CG43 that UDP glucose pyrophosphorylase (9), large virulence plasmid pLVPK (12) mucoid factor RmpA2 (41), and the two component system (2CS) KvgAS (39, 47) each played a role as virulence determinants. Besides, *in vivo* expression technology (IVET) has been used to isolate the genes specifically induced in mouse from *K. pneumoniae* CG43 (40) since it is well recognized that the expression of genes that participate in pathogenesis could be specifically induced within the host. Two out of the 20 *ivi* genes were found to be turned on expression under iron deprivation, while the expression of another five was activated in the presence of 10 μM paraquat, a superoxide generator, indicating their likely roles in oxidative stress defense.

Oxidative stress responses During infection, pathogens have equipped to protect themselves from the oxidative burst of phagocytic cells and the challenging oxidative environments within cellular and extracellular compartments. The defense mechanisms, which play an important role in determining the bacterial virulence, include sensing, avoiding, and removing the oxidants. The most commonly discussed oxidants that cause damage to DNA, proteins, and cell membranes and often results in cell death are the reactive oxygen species (ROS) including superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\cdot}$), and the reactive nitrogen species (RNS), which include nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^-$) (75). In *Escherichia coli*, superoxide is removed by SODs (SodA, SodB,

SodC), generating hydrogen peroxide which is then removed by catalases (KatE, KatG) and peroxidases (AhpC). The transcriptome analysis of *Pseudomonas aeruginosa* or *Staphylococcus aureus* response to H_2O_2 revealed many more genes including the virulence genes, the genes encoding products involved in DNA repair and anaerobic metabolism were induced (10,11).The genome-wide analysis in *E. coli* also reported the expression of more than 100 genes which were induced in responding to either H_2O_2 or paraquat (83). Taken together, these indicated the complexity of the antioxidant strategies and many more genes remained to be identified.

Regulation of oxidative stress defenses Many of the stress defenses are controlled by regulators that respond to iron (e.g., Fur), oxygen tension (e.g., FNR and ArcAB), superoxide (e.g., SoxRS,), and hydrogen peroxide (e.g., OxyR). In *E. coli*, OxyR is known to regulate more than nine genes including *kat*G (hydroperoxidase I), *ahpC* (alkylhydroperoxide reductase), *gorA* (gluthione reductase), *oxyS* (regulatory RNA), and *fur* that are involved directly or indirectly in the oxidative stress response (27).The antioxidant genes *katE*, *katG*, and *sodC* have been reported to be components of RpoS regulon (88). While the expression of superoxide dismutase SodA and SodB are respectively controlled by at least 3 global regulators including SoxRS, ArcAB, and Fur (83).In *K. pneumoniae* CG43, the 2CS response regulator encoding gene *kvgA* deletion was found to reduce the expression of *katG* and *sodC* (49). The expression of *kvgAS* was shown to be inducible upon treatment with 0.2 mM paraquat (49), further supporting an involvement of the 2CS in oxidative stress defense in the bacteria.

Fur, which is complexed with iron as an iron responsive regulator, binds to multiple sites (a 19-bp Fur box) with differential affinities to repress the transcription of genes required for iron acquisition, acid and oxidative stress responses (74). However, Fur has also been demonstrated to function as a transcriptional activator for several genes including *fur* itself (*Hp*and *Vv*), and virulence associated genes (15, 27, and 56). RpoS is a sigma factor which regulates expression of a variety of genes in *E. coli*, *Salmonella* spp, and *V. vulnificus* that allows the bacteria to adapt, resist, and survive under stress condition (25, 59, and 88). The deletion of *rpoS* in *V. vulnificus* was found to down-regulate the expression of *fur* (44), indicating a cascade regulation between the two global regulators. Moreover, we have reported in *K. pneumoniae* CG43 that *rpoS* deletion reduced the *kvgAS* expression which also showing a cascade regulation on the expression of 2CS (49). Besides, the involvement of interaction between 2CS(s) and global regulator(s) in virulence regulation has been increasingly recognized (6, 63). We anticipate that complex signaling networks with inter-connection regulatory circuits are required for multiple stress signal integration.

***Klebsiella pneumoniae yjcC*, an IVE (*in vivo* expression) gene** As previously reported, the five paraquat-inducible *ive* genes include *lysA*, *yfjB*, *yjcC* and two unknown function sequences. LysA encodes the last enzyme in lysine biosynthetic pathway which has been considered as potential targets for antibacterial drugs and herbicides (46). *Mycobacterium tuberculosis lysA*

deletion mutant has been demonstrated to be attenuated and safe as vaccine candidate in mice (72). YfjB is an NAD kinase which has been regarded as the key enzyme for the regulation of the NADP level and NADP-dependent pathways in cells (36). While analysis of the yet known function YjcC revealed similar gene organization and significant sequence homology with that of the *E. coli* and *S. yclase* Typhimurium homologs. Gene organization analysis revealed that the global oxidative stress regulators SoxR and SoxS encoding genes are upstream located suggesting a role of YjcC in the response to oxidative stress and hence *yjcC* was chosen for further study.

Cyclic-di-GMP, second messenger Increasing evidences have shown that cyclic diguanylate (c-di-GMP), a bacterial second messenger, acts to modulate the expression of virulence genes which are required for the infection of bacterial pathogen during the transition from environmental reservoirs to the human host (69, 71). The genome-wide transcriptional profile of *E. coli* in response to high level of c-di-GMP showed an important number of genes encoding cell surface and membrane-bound proteins are altered in their transcription activity (55). The genes encoding several transcription regulators such as Fur, SoxS, and RcsA were up-regulated implying a repression of iron uptake system, increase expression of oxidative stress response and synthesis of CPS.

Bacterial genome sequencing revealed a surprising abundance of GGDEF and EAL domains, indicating that c-di-GMP-dependent regulation is more widespread than was first expected. The level of c-di-GMP has been demonstrated to be controlled inversely by GGDEF- and EAL-domain containing proteins, which respectively function as diguanylate yclase (DGC) and phosphodiesterase (PDE) (73). Many of the sensory proteins contain N-terminal cytoplasmic sensor domains that affect the enzymatic activities of the downstream domains (28). However, the ligands of most sensory domains remain obscure. The comparative analysis of the N-terminal sequences revealed 41% identity between Kp-YjcC and Ec-YjcC, and 50% identity between Kp-YjcC and St-YjcC. The Blast search for the sensory domain at the N-terminus of YjcC revealed no classified functional motif. However, a couple of conserved blocks including aa 40~100 and 130~188 could be identified. In contrast, the C-terminal sequences containing EAL domain appeared to be highly conserved in three bacteria.

The pilot study revealed that under the treatment of 50 μM paraquat, a dramatic decrease of the bacterial resistance to paraquat was observed and the introduction of the *yjcC*-expression plasmid enhanced the bacterial resistance to higher level of paraquat (200 and 400 μM), further supporting a role of YjcC in the bacterial resistance to oxidative stress.

Specific aims The proposed studies are (1) To investigate the functional role of YjcC in the oxidative stress defense; (2) To identify the regulatory system(s) for optimal expression of *yjcC*; (3) To show how and why YjcC is expressed *in vivo* to encounter the oxidative stress.

Materials and methods

Paraquat and H_2O_2 survival assessment One-hundredth overnight grown bacteria were inoculated to LB and incubated at 37°C to OD600 of 0.6~0.7. Aliquot of the bacteria were then collected by centrifugation and resuspended in 200 μM of Paraquat and 10 mM H2O2 respectively, then subjected to 37°C incubation for 35 min. CFU of the bacteria after the stress treatment were counted, and the survival rate was determined by the ratio of the CFU. The representative data at least three independent experiments were presented. Every sample was assayed in triplicate, and the average activity and standard deviation were presented.

Promoter activity assessment β-galactosidase activity was determined essentially as described by Miller. 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.

Mouse lethality assay Female BALB/c mice with an average weight of 25 g will be obtained. The tested bacteria suspended in 10-fold steps graded dose in 0.2 ml saline will be injected intraperitoneally. The LD50 will be determined by the method of Reed and Muench (58)and expressed as CFU.

Real-Time PCR analysis Three micrograms of the Dnase I-treated total RNA extracted from *K. pneumoniae* CG43 or the derived strains treated with or without paraquat was reverse transcribed to generate cDNA using AMV Reverse Transcriptase and random primer pDN(5) according to the manufacturer's instructions (Roche). Real-time PCR (RT-PCR) analyzing the transcription of *rpoS*, *oxyR*, *fur*, *yjcC*, *soxR*, *soxS* and 23S rRNA using each cDNA as a template was performed using SYBR Green PCR Master Mix and SDS 2.1 software on a PRISM 7000 HT platform (Applied Biosystems). Relative quantification of *rpoS*, *oxyR*, *fur*, *yjcC*, *soxR* and *soxS* transcription in various conditions and various strains was analyzed by the comparative Ct (threshold cycle) method. The internal control used here was 23S rRNA.

***In trans* complementation analysis for the assessment of phosphodiesterase activity**

Swimming activity assay

Swimming motility was tested in the LB medium solidified with 0.3% Bacto Agar, 0.5% NaCl, and 1% tryptone. Five microliters of the bacterial suspension was spot on the plate and then incubated for 16 h at 30°C.

Site-directed mutagenesis

Site directed mutagenesis was performed on the plasmids pJR1 to substitute critical residue in the EAL domain of YjcC according to the QuickChange site-directed mutagenesis kit

following the manufacturer's protocol (Stratagene). The generated PCR product contained one point mutation corresponding to E303 to A303 change in the active EAL site. The resulting PCR product was digested with *Bam*HI and *Hin*dIII and ligated into *Bam*HI/*Hin*dIII-digested plasmid pRK415-pJR2.

Determination of SOD and catalase enzyme activity

(a) Cell-free extracts preparation. To measure specific activities of SOD and catalase, cell-free extracts were harvested (15000 rpm, 4°C, 20 min) from the exponential phased bacteria (OD_{600}= 0.7~0.8) and suspended in ice-cold potassium phosphate buffer (50 mM, pH 7). After cells disrupted by ultra-sonication, cell debris was removed by 12000 rpm centrifugation for 10 min at 4°C, and the supernatant collected. The extraction of total proteins was carried out on ice and the concentrations were estimated according to Bradford (1976) method using bovine serum albumin as standard.

(b) Native-PAGE and gel activity staining. Approximately same quantity of the extracted proteins was individually loaded onto 10% native polyacrylamide gels and the proteins separated at a constant voltage of 150 V for 2 h. The gels were then removed and stained for SOD and CAT activity by the methods of Beauchamp and Fridovich (1971), and Woodbury *et al*. (1971), respectively. Essentially, SOD was localized by soaking gels in 2.45 mM nitroblue tetrazolium for 20 min, followed by immersion in a saluting of 50 mM phosphate buffer, pH 7.0, 0.028 mM riboflavin and 0.028 M TEMED. The gels were then removed from the solution and exposed to light for about 20 min. SOD activity results in achromatic zones in the otherwise purple gel. The expression of CAT activity was identified by soaking gels in 10 mM hydrogen peroxide for 30 min with gentle shaking and then the gels transferred to a solution of 1% ferric chloride and 1% potassium ferricyanide for 10 min. The localized-CAT gave rise to colorless bands on a dark green background.

(c) SOD specific activity determination. SOD activity was determined spectrophotometrically at 25°C by the xanthine oxidase–cytochrome C method (McCord & Fridovich, 1969). The assay mixture in 0.7 ml contained 50 mM potassium phosphate, pH 7.8, 0.1 mM EDTA, 50 mM xanthine, 1.7 mU xanthine oxidase, and 10 mM cytochrome C. The reduction of cytochrome C was measured at A550. One unit (U) of SOD activity was defined as the amount of enzyme required to inhibit the reduction rate of cytochrome C by 50% (McCord & Fridovich, 1969).

(d) Catalase specific activity determination. Catalase activity was also determined spectrophotometrically at 25°C (1 cm cuvette, JascoV-550 spectrophotometer) by monitoring the decrease in A240 of 10 mM H_2O_2 in 50 mM Tris/HCl buffer, pH 8.0 (Beers & Sizer, 1952; Nelson & Kiesow, 1972). One unit (U) of activity was defined as the amount of enzyme that catalyses the oxidation of 1 mmol H_2O_2 min^{-1} under the assay conditions.

Evaluation of antioxidant activity with DPPH assay

DPPH radical scavenging activity was estimated using the method of Liyana-Pathirana and Adedapo (2, 51). A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract in methanol containing 0.02-0.1 mg of the extract. The reaction mixture was thoroughly vortexed and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid and BHT were used as references. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = [(Abs control – Abs sample)]/(Abs control)] × 100 where Abs control is the absorbance of DPPH radical + methanol; Abs sample is the absorbance of DPPH radical + sample extract/standard.

Determination of intracellular ROS concentration

Bacterial cultures were grown exponentially (OD600 = 0.6~ 0.7) and exposed to 10 mM of Hydrogen peroxide or 500 μM of paraquat for 40 min. Cells were centrifuged at 13,000 g, washed with 10 mM potassium phosphate (pH 7.0) buffer (buffer A) and suspended in 500 μl of the same buffer that contained 10 μM 2',7' –dihydrodichlorofluorescein diacetate (H2DCFDA). After shaking in the darkness for defined time intervals at room temperature, cells were centrifuged as above and washed twice with 500 μl of buffer A. Cells were suspended in 500 μl of buffer A and disrupted by sonication. After centrifugation at 13,000 g, aliquots of 100 μl supernatants were used to determine fluorescence intensity (excitation 490 nm, emission 519 nm) as described (60, 61).

Oxidation of cytoplasmic proteins

K. pneumoniae CG43S3 and its derived mutants were grown to an OD600 0.6~ 0.7 in the presence of 10 mM hydrogen peroxide. After incubating for 30 min at 37°C, crude extracts were prepared and suspended in 500 μl of buffer A then disrupted by sonication. After centrifugation at 13,000 g, four volumes of 10 mM dinitrophenylhydrazine (DNPH) were added to the supernatant and the mixture was incubated at room temperature for 1 h with occasional stirring. Proteins were precipitated by adding one volume of 20% trichloroacetic acid (TCA) and centrifuged at 13,000 g for 5 min. The precipitate was washed three times with a mixture of ethanol: ethyl acetate (1:1). Finally, the sediment was dissolved in 450 μl of 6 M guanidine hydrochloride/dithiothreitol and carbonyl concentration was determined spectrophotometrically at 370 nm (e = 22,000 M-1 cm-1) (1, 60, 61).

Western blot analysis

K. pneumoniae CG43S3 and its derived mutants were grown in LB broth with agitation at 37°C. The bacterial cultures were collected using centrifugation and quantified with Bradford protein assays (Biorad). The bacterial total protein, approximately 5 µg per lane, was then subjected to western blot analysis using MrkA antiserum. MrkA antiserum preparation and western blot analysis were performed as described elsewhere (30).

Biofilm formation assay

The ability of bacteria to form biofilm was analysed as described elsewhere with a minor modification (48, 91). Bacteria diluted 1/100 in LB broth supplemented with appropriate antibiotics were inoculated into each well of a 96-well micro titre dish (Orange Scientific) and statically incubated at 37°C for 48 h. Planktonic cells were removed, and the wells were washed once with distilled water to remove unattached cells. Crystal violet (0.1% w/v; Sigma) was used for 30 min to stain the attached cells. Unattached dye was rinsed by washing 3 times with distilled water, and stained biomass was solubilised in 1% (w/v) SDS. Absorbance at 595 nm was determined, and relative bacterial biofilm-forming activities were observed.

IV. Results

YjcC is induced under paraquat or H_2O_2 treatment

The previous study has shown that *yjcC* which was selected by *in vivo* expression technology (IVET) is paraquat-inducible. Since paraquat is a superoxide anion generator, a role of YjcC in oxidative stress response was suggested. To further confirm the YjcC involvement in the oxidative stress defenses, q-PCR analysis of the expression of *yjcC* together with other well characterized stress response regulators including SoxR, SoxS, OxyR, RpoS, Fur and FNR, under the treatment with paraquat or H_2O_2 was carried out. As shown in Fig. 1, the *yjcC* mRNA level increased 0.75 fold and 1.75 fold after addition with 10 mM H_2O_2 and 500 μM paraquat, respectively. Compared to the transcript levels of the major oxidative stress response regulators OxyR and SoxS, the induction fold of the *yjcC* mRNA was lower but comparable with the levels of *rpoS*, *fur*, or *fnr*.

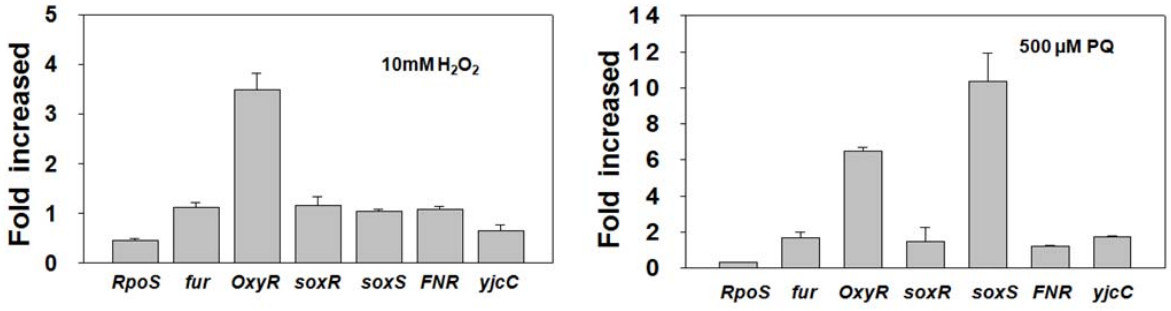
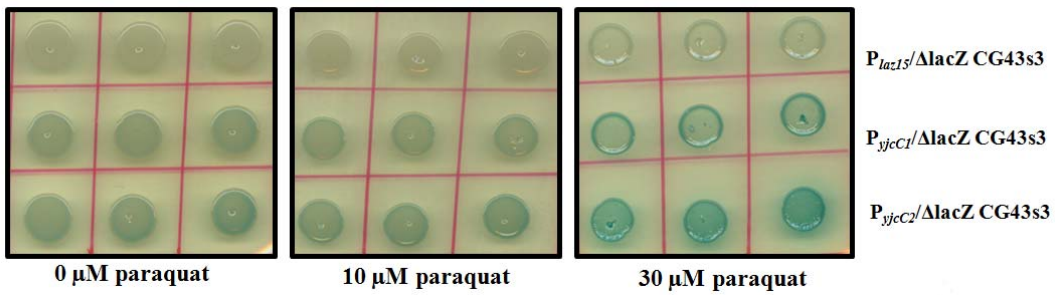


Fig 1. q-PCR analysis of the oxidative stress responses. Total RNA was isolated after the bacteria grown in 10 mM H_2O_2 or 500 μM of paraquat. Specific primer pairs were used to detect the expression of *yjcC*, *rpoS*, *fur*, *oxyR*, *soxR*, *soxS*, and *fnr*.

The YjcC promoter is paraquat-inducible

The *yjcC* expression using GalU as reporter was found to be induced by paraquat (1). The putative promoters P_{yjcC1} and P_{yjcC2}, which respectively containing 525-bp and 415-bp of the non-coding sequences upstream to the start codon of *yjcC*, were isolated and cloned in front of the promoterless *lacZ* gene of the plasmid pLacZ15 (47,57). The resulting P_{yjcC}-*lacZ* fusion plasmids p525 and p415 were then individually transformed *K. pneumoniae* CG43Z01and the transformants analyzed for LacZ activity. As shown in Fig. 2(A), the supply of 30 μM paraquat apparently induced the expression of both P_{yjcC1} and P_{yjcC2} and the activity of P_{yjcC1} revealed a higher level activity than P_{yjcC2}. The β-galactosidase activity measurement as shown in Fig. 2(B) also demonstrated that P_{yjcC1} has a higher level activity than $P_{yjcC2,}$ however, only under the induction with 30 μM paraquat.

(A)



(B)

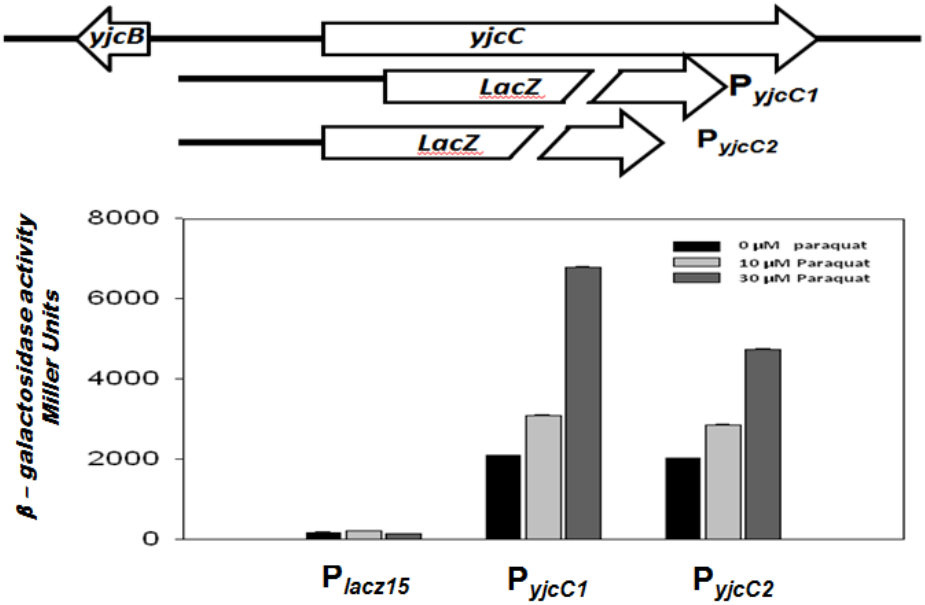


Fig 2. **Measurement of the *yjcC* promoter activity**. The putative promoters which respectively contain 525 bp (P_{yjcC1}) and 415 bp (P_{yjcC2}) of the non-coding region upstream of *yjcC* were isolated and cloned into the promoterless *lacZ* reporter plasmid placZ15. The recombinant plasmids were then transformed to *K. pneumoniae* CG43Z01 and the β-galactosidase activity of the transformants measured. The β-galactosidase activity were measured qualitatively (A) and quantitatively (B).

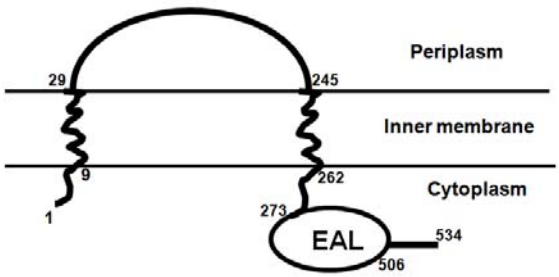
The yjcC encodes transmembrane domains and EAL motif

Sequence analysis of YjcC using P-fam prediction revealed an inner membrane protein domain containing a CSS motif and two transmembrane motifs at the N-terminal region, and an

EAL domain at the C-terminal region (Fig. 3A). The domain structure analysis of YjcC on membrane topology and protein localization using TMHMM server, TMpred or CELLO v.2.5 produced similar topology. As shown in Fig. 3(B), the topology was from TMHMM. As shown in 3(C), alignment of the EAL domain of YjcC with proteins whose activities have been tested in vitro, suggesting YjcC encodes a c-di-GMP phosphodiesterase.

(A)

(B)



(C)

Fig 3. Sequence analysis of YjcC. Two transmembrane regions and CSS motif in N-terminal of YjcC are represented by green line below the sequence and dotted line in red color. EAL domain is represented by blue boldface. (B) Domain structure of YjcC is predicted by TMHMM, TMpred and Cello on line prediction. (C) Sequence alignment of the C-terminus of YjcC with the EAL proteins grouped into active phosphodiesterase activity base on the EAL signature, was generating by Clustal W. As shown in Fig3.

The YjcC protein exerts a phosphodiesterase activity

Site directed mutagenesis on the *yjcC* expression plasmid pJR1 (pRK415-*yjcC*) to substitute the active site residue of E to A was subsequently carried out which resulting the plasmid pJR2. The plasmid pJR1 and pJR2 were individually transformed into *E. coli* MG1655 for motility analysis as an *in vivo* assessment of the c-di-GMP phosphodiesterase (PDE) activity. As shown in Fig. 4(A), the bacteria MG1655[pJR1] swims slightly better than MG1655[pRK415] implying the cellular level of c-di-GMP was reduced by the PDE activity of YjcC. By contrast, the swimming activity of MG1655[pJR2] was lower than MG1655[pRK415] or MG1655[pJR1]. This implied that YjcC encodes a functional phoshodiesterase activity and the E residue of the EAL domain is critical for the enzymatic activity. To demonstrate the phosphodiesterase activity, the EAL domain of YjcC was isolated and cloned into the expression plasmid pET30. As shown in Fig. 4(B), the recombinant EAL domain protein could be overexpressed in *E. coli* and the purified protein exerted a phosphodiesterase activity on the substrate bis (*p*Npp). Interestingly, the recombinant protein with the critical E substituted by A residue still showed a low level of phosphodiesterase activity.

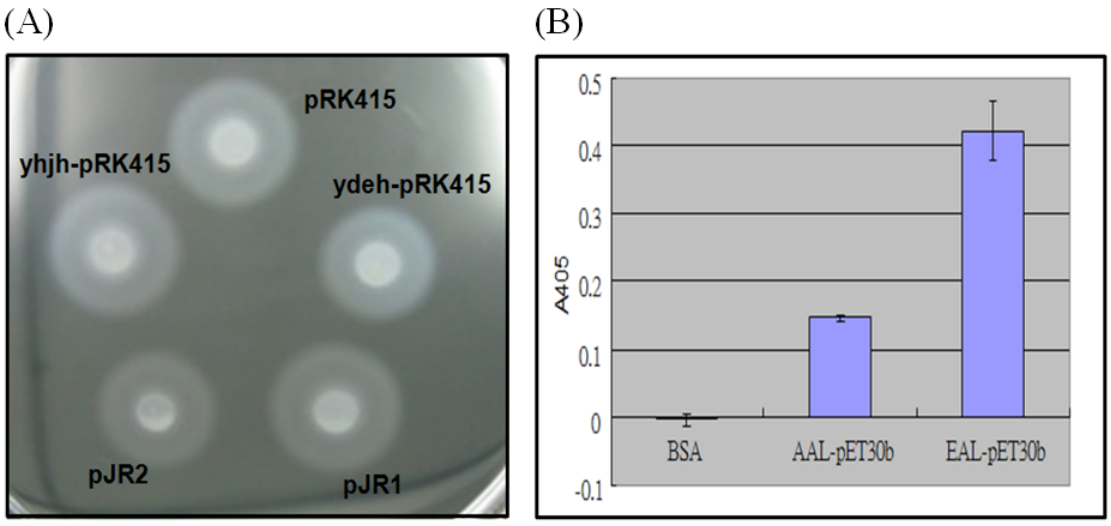
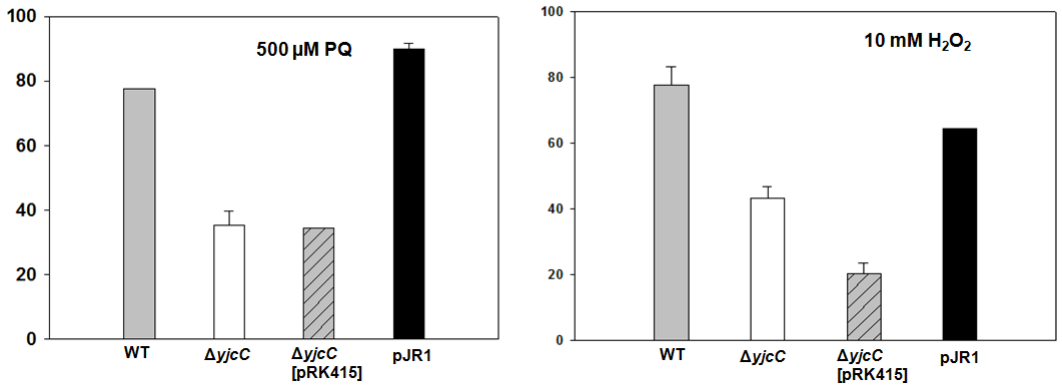


Fig. 4. (A) Trans-complementation assessment of the phoshodiesterase activity. *E. coli* MG1655 carrying the plasmid yhjH-pRK415 has been demonstrated exhibiting a phosphodiesterase activity, while the bacteria carrying ydeh-pRK415 exerted an active diguanylate cyclase activity (10, 11). (B) Overexpression of the recombinant YjcC-EAL domain protein and YjcC-AAL in *E. coli*. A phosphodiesterase activity using bis(pNpp) as substrate was demonstrated by release of p-nitrophenol determined at 410 nm.

The yjcC deletion reduced the survival under oxidative stress

To study the yjcC role in the oxidative stress response, gene deletion mutant was generated via an allelic exchange strategy. Compared to the wild type bacteria *K. pneumoniae* CG43S3, the *yjcC* deletion mutant appeared to be more sensitive to hydrogen peroxide and paraquat stresses. As shown in Fig. 5A, the deletion effect could be complemented by transforming the mutant with the *yjcC* expression plasmid pJR1. The plasmid pJR2 which carrying a YjcC with a substituted residue appeared to be unable to complement the *yjcC* deleting effect (Fig. 5B). These indicated that the E residue of the EAL domain is essential for the YjcC activity in responding to the oxidative stress.

(A)



(B)

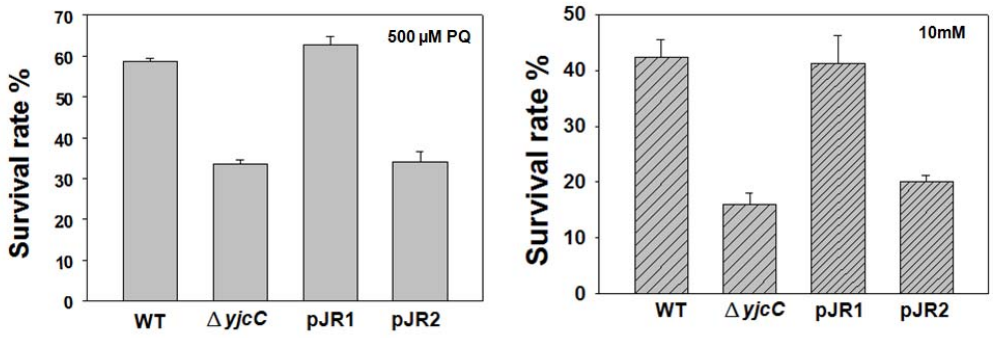
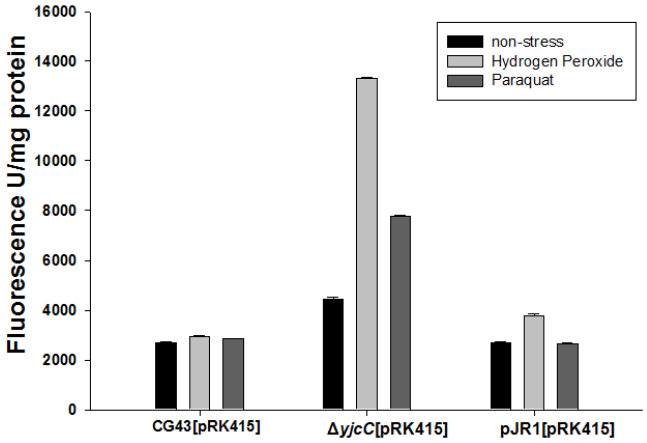


Fig 5. Paraquat and H_2O_2 sensitivity assays. Overnight cultures were collected and refreshed grown in LB until OD600 reach 0.6~0.7. Aliquots of 500 μM paraquat or 10 mM H_2O_2 were respectively added and the cultures at 37°C continued for 50 min. Finally, the cultures were plated onto LB plates for colony formation.

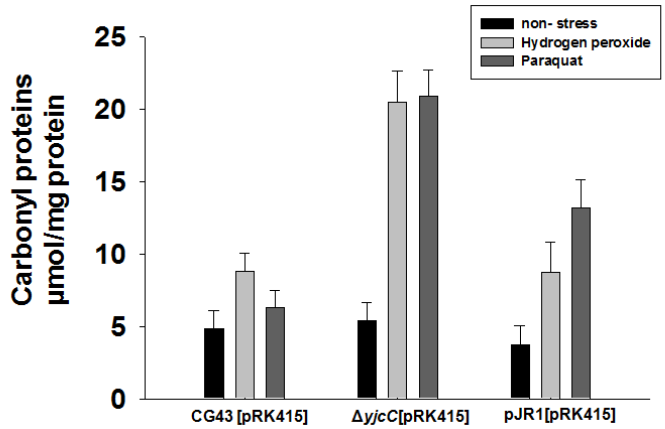
The yjcC deletion increased accumulation of the intracellular ROS and carbonyl group content but decreased the oxidant-scavenging activity.

Increase of the cellular reactive oxygen species and the content of protein carbonyl groups upon exposure to paraquat or hydrogen peroxide has been reported (1, 60, 61). The fluorescent probe H2DCFDA (2', 7'- dichlorodihydrofluorescein diacetate) was used to monitor formation of the intracellular ROS while 2, 4 dinitrophenyl hydrazine (DNPH) was measured to determine the generation of carbonyl groups in proteins. As shown in Fig. 6(A) and (B), increased amounts of the intracellular ROS and the protein carbonyl groups were found in the crude extracts of PQ and H_2O_2 -treated *yjcC* deletion mutant when compared with those of the wild type strain. To determine if the *yjcC* deletion decreased the antioxidant ability, the DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay was used as an indicator of the oxidant-scavenging activity. As shown in Fig. 6(C), the level of anti-oxidant activity in *yjcC* mutant was decreased while compared to that of the parental strain implying that YjcC may affect the activity of the anti-oxidant enzyme such as super oxide dismutase (SOD) or catalase.

(A)



(B)



(C)

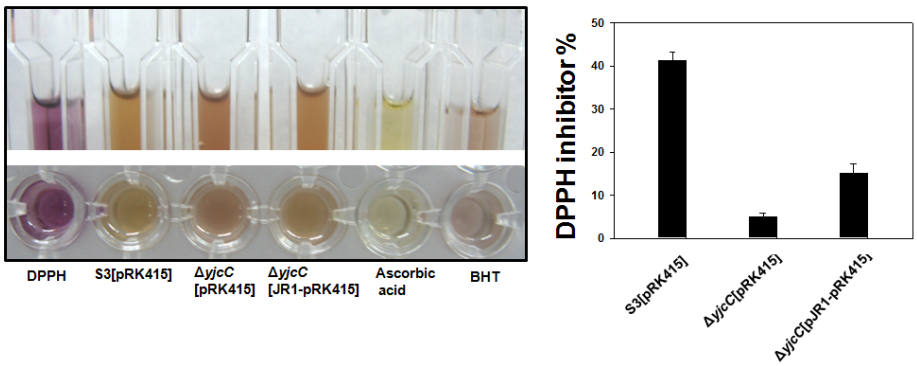
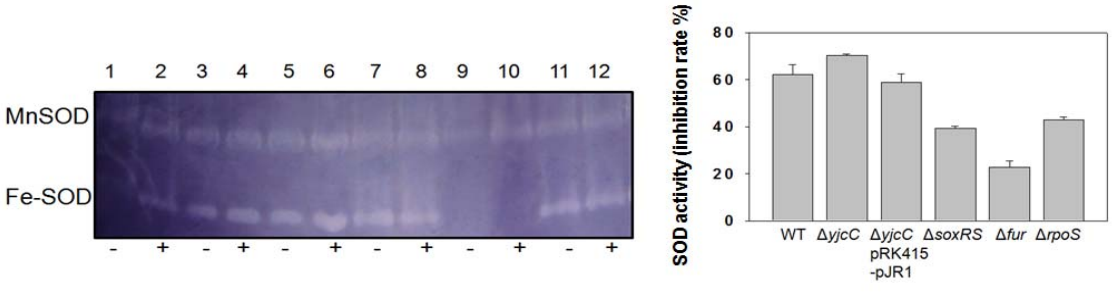


Fig 6. (A) Intracellular ROS contents after the bacteria exposed to hydrogen peroxide or paraquat. (B) Oxidation of the cytoplasmic proteins determined by a chemical protein carbonyl assay. (C) DPPH radical scavenging activity measurement.

Superoxide dismutase (SOD) and catalase activities

To determine whether *yjcC* deletion induces concomitant alteration in the activity of major antioxidant enzymes, the activities of SOD and catalase were examined. As shown in Fig. 7(A), the expression of Mn-SOD and Fe-SOD in *yjcC* mutant appeared to be increased while compared to that of the wild type strain using an in-gel analysis of the enzyme activity. Total SOD activity measurement also revealed an increase activity by the deletion of *yjcC* suggesting the deletion of *yjcC* increased the SOD activity. By contrast, an increased activity of KatG but decreased activity of KatE was observed in *yjcC* mutant (Fig. 7B). These results imply that YjcC regulates SOD and catalase by two different pathways to counter the oxidative stress.



(A)

(B)

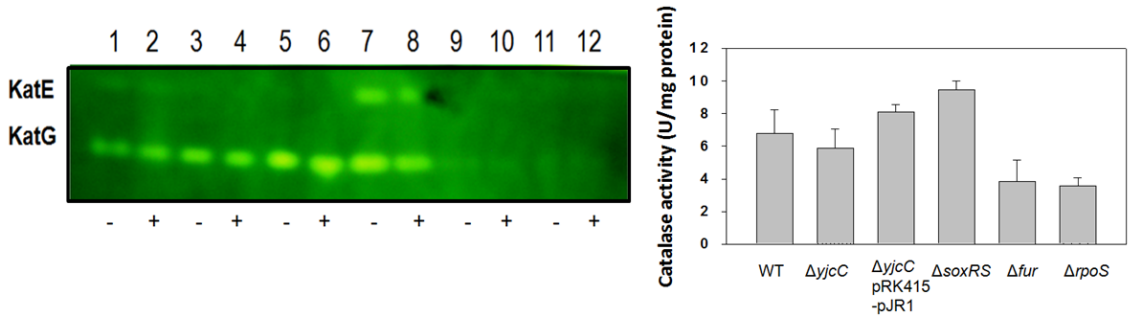


Fig 7. (A) SOD activity determined by in-gel analysis (on left) and quantitative measurement. (B) Catalase activity determined by in-gel analysis (on left) and quantitative measurement. Lanes 1, 2: wild type bacteria; 3, 4: Δ*yjcC*; 5, 6: Δ*yjcC* [pRK415-pJR1]; 7, 8: Δ*soxRS* ; 9, 10: Δ*fur*; 11, 12 : Δ*rpoS* . Lanes 1, 3, 5, 7, 9, and 11 are protein extracts of the bacteria with no stress treatment; 2, 4, 6, 8, and 10 are protein extracts of the bacteria with paraquat (A) or hydrogen peroxide (B) treatment.

The yjcC deletion also decreased the LD50 to mouse and biofilm forming activity

Since *yjcC* is an *ive* gene, a role in establishing an infection has been proposed (1). To measure if *yjcC* affects the virulence of *K. pneumonia* CG43, a mouse peritonitis model was employed. A 10-fold increase of LD50 was found by the deletion of *yjcC*. Complementation analysis using pJR1 and pJR2 will be carried out and the data should help to verify if YjcC plays a virulence role. In addition, biofilm formation analysis was applied to assess biological role of YjcC. As shown in Fig. 8A, increased biofilm formation activity was found for the *yjcC* deletion mutant when compared with the parental strain CG43S3. Since type 3 fimbriae has been reported as a major determinant for *K. pneumoniae* biofilm formation, if YjcC has a negative effect on type 3 fimbriae expression was also investigated. As shown in Fig. 8B, no apparent effect on the major pilin of type 3 fimbriae MrkA expression was observed by the deletion of

yjcC. However, a drastic decrease of MrkA expression was found for CG43S3Δ*yjcC* [pJR1]

suggesting YjcC negatively affects the expression of type 3 fimbriae. The negative effect was no longer observed if the critical residue of the YjcC EAL domain was mutated. This implied the second messenger c-di-GMP plays a role in regulating the expression of type 3 fimbriae.

(A) (B)

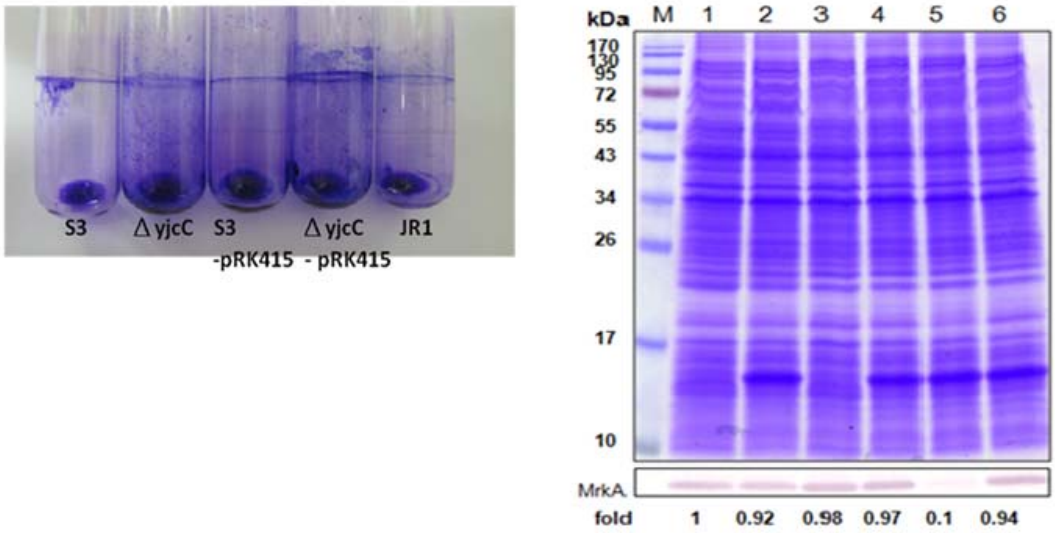


Fig 8. (A) Biofilm formation analysis. (B) Western blotting analysis for the expression of MrkA, the major pilin of type 3 fimbriae. M: pre-stained molecular weight marker. Lanes 1, *K.*

pneumoniae CG43S3; 2, CG43S3 [pRK415]; 3, CG43S3Δ*yjcC*; 4, CG43S3Δ*yjcC*[pRK415];

5, CG43S3Δ*yjcC*[pJR1]; 6, CG43S3Δ*yjcC*[pJR2].

In summary, we have shown that (1) YjcC mRNA as well as several stress response regulators is increased by the treatment of H_2O_2 and paraquat. In addition, the promoter activity is inducible upon treatment with H_2O_2 or paraquat; (2) YjcC is a PDE and the EAL domain is essential for the enzymatic activity; (3) Deletion of *yjcC* increased the susceptibility to paraquat and hydrogen peroxide, and the deficiency could be complemented by transforming into the mutant with pJR1 but not pJR2 indicating that the involvement of YjcC in the oxidative stress response is PDE activity dependent; (4) The intracellular ROS and protein carbonyl groups were increased by the deletion of *yjcC* implying a role of YjcC in modulating the activity of the radical scavenging enzymes. Increasing SOD and KatG activity but decreased KatE activity were observed for the Δ*yjcC* mutant further demonstrating a regulation of YjcC on the activity of some radical scavenging enzymes; (5) Deletion of *yjcC* was also found to affect the bacterial virulence and biofilm formation activity. However, if YjcC directly affects the expression of type 3 fimbriae remained to be investigated.

Discussion

The gene organization analysis revealed *yjcC* gene is next to the major oxidative stress response regulator SoxR and SoxS encoding genes. The cluster gene organization suggested a coordinated expression between *yjcC* and *soxRS*. Nevertheless, deletion of *soxRS* had no apparent effect on the expression of *yjcC*, implied that regulation of *yjcC* may be independent to the SoxRS regulatory pathway.

E.coli W3110 or MG1655 strain has been used as a model system for *trans*-complementation analysis of the changes of motility affected by the level of c-di-GMP (22, 46). The change of swimming activity affected by YjcC (pJR1) expression was not apparent when compared to the bacteria carrying vector only. Nevertheless, an apparent swimming activity difference between the bacteria carrying pJR1 and pJR2 supported that YjcC is a PDE and the E residue is critical for its enzymatic activity. It has been found that many DUF1 (or GGDEF) and DUF2(or EAL) containing proteins have PAS-PAC domain in their N-terminal as a sensory part to receive signal from outside (18, 19). If the N terminal transmembrane region of YjcC plays a similar role remained to be investigated.

When compared to wild type cells, the increased ROS concentration and carbonyl group formation in proteins, which resulted from the amino acid side chain modification induced by ROS, and higher levels of oxidized-proteins were found in the *yjcC* deletion mutant. This implied YjcC is somehow involved in protecting the cell from cytoplasmic oxidation and from oxidants generated under stress. Indeed, YjcC exerted a differential influences on the expression of the superoxide dismutase and catalase (Fig. 7A and B). Several reports have shown that paraquat or hydrogen peroxide was able to increase activity of superoxide dismutase or catalase (65, 76, 86). It is conceivable that SodA, SodB, and KatG activity were increased by the deletion of *yjcC* since YjcC plays a role in the oxidative response. Why the decreased activity of KatE, an RpoS dependent catalase, was observed in the *yjcC* deletion mutant remained to be investigated.

Recent report has shown that *yjcC* expression was able to inhibit *csgD* expression in *Salmonella,* and hence affected the biofilm expression (70, 77). If *K. pneumoniae* YjcC also affected the expression of CsgD thereby negatively regulated the biofilm formation remained to be investigated. *K. pneumoniae* MrkJ protein which carrying an EAL domain containing has been reported a phosphodiesterase activity. Deletion of *mrkJ* was found to decrease the type 3 fimbriae activity in *Klebsiella pneumoniae* (32). The negative effect of YjcC on the biofilm formation activity could be independent of the type 3 fimbriae expression since no apparent change of MrkA expression by the deletion of *yjcC*. Nevertheless, changes of c-di-GMP level apparently influence the MrkA expression.

V. Literature Cited

1. **Acuna, L. G., I. L. Calderon, A. O. Elias, M. E. Castro, and C. C. Vasquez.** 2009. Expression of the yggE gene protects Escherichia coli from potassium tellurite-generated oxidative stress. Arch Microbiol **191:**473-6.
2. **Adedapo, A. A., F. O. Jimoh, S. Koduru, A. J. Afolayan, and P. J. Masika.** 2008. Antibacterial and antioxidant properties of the methanol extracts of the leaves and stems of Calpurnia aurea. BMC Complement Altern Med **8:**53.
3. **Alfaro, C., A. Urios, M. C. Gonzalez, P. Moya, and M. Blanco.** 2003. Screening for metabolites from Penicillium novae-zeelandiae displaying radical-scavenging activity and oxidative mutagenicity: isolation of gentisyl alcohol. Mutat Res **539:**187-94.
4. **Arifuzzaman, M., M. Maeda, A. Itoh, K. Nishikata, C. Takita, R. Saito, T. Ara, K. Nakahigashi, H. C. Huang, A. Hirai, K. Tsuzuki, S. Nakamura, M. Altaf-Ul-Amin, T. Oshima, T. Baba, N. Yamamoto, T. Kawamura, T. Ioka-Nakamichi, M. Kitagawa, M. Tomita, S. Kanaya, C. Wada, and H. Mori.** 2006. Large-scale identification of protein-protein interaction of Escherichia coli K-12. Genome Res **16:**686-91.
5. **Beauchamp, C., and I. Fridovich.** 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem **44:**276-87.
6. **Beier, D., and R. Gross.** 2006. Regulation of bacterial virulence by two-component systems. Curr Opin Microbiol **9:**143-52.
7. **Benov, L. T., and I. Fridovich.** 1994. Escherichia coli expresses a copper- and zinc-containing superoxide dismutase. J Biol Chem **269:**25310-4.
8. **Campos, M. A., M. A. Vargas, V. Regueiro, C. M. Llompart, S. Alberti, and J. A. Bengoechea.** 2004. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. Infect Immun **72:**7107-14.
9. **Chang, H. Y., J. H. Lee, W. L. Deng, T. F. Fu, and H. L. Peng.** 1996. Virulence and outer membrane properties of a galU mutant of Klebsiella pneumoniae CG43. Microb Pathog **20:**255-61.
10. **Chang, W., D. A. Small, F. Toghrol, and W. E. Bentley.** 2006. Global transcriptome analysis of Staphylococcus aureus response to hydrogen peroxide. J Bacteriol **188:**1648-59.
11. **Chang, W., D. A. Small, F. Toghrol, and W. E. Bentley.** 2005. Microarray analysis of Pseudomonas aeruginosa reveals induction of pyocin genes in response to hydrogen peroxide. BMC Genomics **6:**115.
12. **Chen, Y. T., H. Y. Chang, Y. C. Lai, C. C. Pan, S. F. Tsai, and H. L. Peng.** 2004. Sequencing and analysis of the large virulence plasmid pLVPK of Klebsiella pneumoniae CG43. Gene **337:**189-98.
13. **Chuang, Y. P., C. T. Fang, S. Y. Lai, S. C. Chang, and J. T. Wang.** 2006. Genetic determinants of capsular serotype K1 of Klebsiella pneumoniae causing primary pyogenic liver

abscess. J Infect Dis **193:**645-54.

14. **Compan, I., and D. Touati.** 1993. Interaction of six global transcription regulators in expression of manganese superoxide dismutase in Escherichia coli K-12. J Bacteriol **175:**1687-96.
15. **Danielli, A., D. Roncarati, I. Delany, V. Chiarini, R. Rappuoli, and V. Scarlato.** 2006. In vivo dissection of the Helicobacter pylori Fur regulatory circuit by genome-wide location analysis. J Bacteriol **188:**4654-62.
16. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J Bacteriol **172:**6568-72.
17. **Demple, B.** 1991. Regulation of bacterial oxidative stress genes. Annu Rev Genet **25:**315-37.
18. **Dubrac, S., and D. Touati.** 2000. Fur positive regulation of iron superoxide dismutase in Escherichia coli: functional analysis of the sodB promoter. J Bacteriol **182:**3802-8.
19. **Farr, S. B., and T. Kogoma.** 1991. Oxidative stress responses in Escherichia coli and Salmonella typhimurium. Microbiol Rev **55:**561-85.
20. **Fink, R. C., M. R. Evans, S. Porwollik, A. Vazquez-Torres, J. Jones-Carson, B. Troxell, S. J. Libby, M. McClelland, and H. M. Hassan.** 2007. FNR is a global regulator of virulence and anaerobic metabolism in Salmonella enterica serovar Typhimurium (ATCC 14028s). J Bacteriol **189:**2262-73.
21. **Fung, C. P., F. Y. Chang, S. C. Lee, B. S. Hu, B. I. Kuo, C. Y. Liu, M. Ho, and L. K. Siu.** 2002. A global emerging disease of Klebsiella pneumoniae liver abscess: is serotype K1 an important factor for complicated endophthalmitis? Gut **50:**420-4.
22. **Gaudu, P., N. Moon, and B. Weiss.** 1997. Regulation of the soxRS oxidative stress regulon. Reversible oxidation of the Fe-S centers of SoxR in vivo. J Biol Chem **272:**5082-6.
23. **Gilles-Gonzalez, M. A., and G. Gonzalez.** 2004. Signal transduction by heme-containing PAS-domain proteins. J Appl Physiol **96:**774-83.
24. **Han, S. H.** 1995. Review of hepatic abscess from Klebsiella pneumoniae. An association with diabetes mellitus and septic endophthalmitis. West J Med **162:**220-4.
25. **Hassan, H. M., and H. C. Sun.** 1992. Regulatory roles of Fnr, Fur, and Arc in expression of manganese-containing superoxide dismutase in Escherichia coli. Proc Natl Acad Sci U S A **89:**3217-21.
26. **Hengge-Aronis, R.** 2002. Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. Microbiol Mol Biol Rev **66:**373-95, table of contents.
27. **Hisert, K. B., M. MacCoss, M. U. Shiloh, K. H. Darwin, S. Singh, R. A. Jones, S. Ehrt, Z. Zhang, B. L. Gaffney, S. Gandotra, D. W. Holden, D. Murray, and C. Nathan.** 2005. A glutamate-alanine-leucine (EAL) domain protein of Salmonella controls bacterial survival in mice, antioxidant defence and killing of macrophages: role of cyclic diGMP. Mol Microbiol

56:1234-45.

28. **Horsburgh, M. J., E. Ingham, and S. J. Foster.** 2001. In Staphylococcus aureus, fur is an interactive regulator with PerR, contributes to virulence, and Is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. J Bacteriol **183:**468-75.
29. **Huang, B., C. B. Whitchurch, and J. S. Mattick.** 2003. FimX, a multidomain protein connecting environmental signals to twitching motility in Pseudomonas aeruginosa. J Bacteriol **185:**7068-76.
30. **Huang, Y. J., H. W. Liao, C. C. Wu, and H. L. Peng.** 2009. MrkF is a component of type 3 fimbriae in *Klebsiella pneumoniae*. Res Microbiol **160:**71-9.
31. **Imlay, J. A.** 2008. Cellular defenses against superoxide and hydrogen peroxide. Annu Rev Biochem **77:**755-76.
32. **Imlay, J. A.** 2003. Pathways of oxidative damage. Annu Rev Microbiol **57:**395-418.
33. **Johnson, J. G., and S. Clegg.** 2011. Role of MrkJ, a phosphodiesterase, in type 3 fimbrial expression and biofilm formation in Klebsiella pneumoniae. J Bacteriol **192:**3944-50.
34. **Jonas, K., A. N. Edwards, R. Simm, T. Romeo, U. Romling, and O. Melefors.** 2008. The RNA binding protein CsrA controls cyclic di-GMP metabolism by directly regulating the expression of GGDEF proteins. Mol Microbiol **70:**236-57.
35. **Jong, G. M., T. R. Hsiue, C. R. Chen, H. Y. Chang, and C. W. Chen.** 1995. Rapidly fatal outcome of bacteremic Klebsiella pneumoniae pneumonia in alcoholics. Chest **107:**214-7.
36. **Kabha, K., L. Nissimov, A. Athamna, Y. Keisari, H. Parolis, L. A. Parolis, R. M. Grue, J. Schlepper-Schafer, A. R. Ezekowitz, D. E. Ohman, and et al.** 1995. Relationships among capsular structure, phagocytosis, and mouse virulence in Klebsiella pneumoniae. Infect Immun **63:**847-52.
37. **Kawai, S., S. Mori, T. Mukai, W. Hashimoto, and K. Murata.** 2001. Molecular characterization of Escherichia coli NAD kinase. Eur J Biochem **268:**4359-65.
38. **Keynan, Y., J. A. Karlowsky, T. Walus, and E. Rubinstein.** 2007. Pyogenic liver abscess caused by hypermucoviscous Klebsiella pneumoniae. Scand J Infect Dis **39:**828-30.
39. **Lacey, M. M., J. D. Partridge, and J. Green.** 2010. Escherichia coli K-12 YfgF is an anaerobic cyclic di-GMP phosphodiesterase with roles in cell surface remodelling and the oxidative stress response. Microbiology **156:**2873-86.
40. **Lai, Y. C., G. T. Lin, S. L. Yang, H. Y. Chang, and H. L. Peng.** 2003. Identification and characterization of KvgAS, a two-component system in Klebsiella pneumoniae CG43. FEMS Microbiol Lett **218:**121-6.
41. **Lai, Y. C., H. L. Peng, and H. Y. Chang.** 2001. Identification of genes induced in vivo during Klebsiella pneumoniae CG43 infection. Infect Immun **69:**7140-5.
42. **Lai, Y. C., H. L. Peng, and H. Y. Chang.** 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.

43. **Lau, Y. J., B. S. Hu, W. L. Wu, Y. H. Lin, H. Y. Chang, and Z. Y. Shi.** 2000. Identification of a major cluster of Klebsiella pneumoniae isolates from patients with liver abscess in Taiwan. J Clin Microbiol **38:**412-4.
44. **Lee, H. C., Y. C. Chuang, W. L. Yu, N. Y. Lee, C. M. Chang, N. Y. Ko, L. R. Wang, and W. C. Ko.** 2006. Clinical implications of hypermucoviscosity phenotype in Klebsiella pneumoniae isolates: association with invasive syndrome in patients with community-acquired bacteraemia. J Intern Med **259:**606-14.
45. **Lee, H. J., K. J. Park, A. Y. Lee, S. G. Park, B. C. Park, K. H. Lee, and S. J. Park.** 2003. Regulation of fur expression by RpoS and fur in Vibrio vulnificus. J Bacteriol **185:**5891-6.
46. **Lee, P. Y., W. N. Chang, C. H. Lu, M. W. Lin, B. C. Cheng, C. C. Chien, C. J. Chang, and H. W. Chang.** 2003. Clinical features and in vitro antimicrobial susceptibilities of community-acquired Klebsiella pneumoniae meningitis in Taiwan. J Antimicrob Chemother **51:**957-62.
47. **Li, X., and S. C. Ricke.** 2003. Characterization of an Escherichia coli lysA insertion targeted mutant using phenotype arrays. Bioresour Technol **89:**249-53.
48. **Lin, C. T., T. Y. Huang, W. C. Liang, and H. L. Peng.** 2006. Homologous response regulators KvgA, KvhA and KvhR regulate the synthesis of capsular polysaccharide in *Klebsiella pneumoniae* CG43 in a coordinated manner. J Biochem **140:**429-38.
49. **Lin, C. T., Y. J. Huang, P. H. Chu, J. L. Hsu, C. H. Huang, and H. L. Peng.** 2006. Identification of an HptB-mediated multi-step phosphorelay in Pseudomonas aeruginosa PAO1. Res Microbiol **157:**169-75.
50. **Lin, C. T., and H. L. Peng.** 2006. Regulation of the homologous two-component systems KvgAS and KvhAS in Klebsiella pneumoniae CG43. J Biochem **140:**639-48.
51. **Lin, J. C., F. Y. Chang, C. P. Fung, J. Z. Xu, H. P. Cheng, J. J. Wang, L. Y. Huang, and L. K. Siu.** 2004. High prevalence of phagocytic-resistant capsular serotypes of Klebsiella pneumoniae in liver abscess. Microbes Infect **6:**1191-8.
52. **Liyana-Pathirana, C. M., and F. Shahidi.** 2005. Antioxidant activity of commercial soft and hard wheat (Triticum aestivum L.) as affected by gastric pH conditions. J Agric Food Chem **53:**2433-40.
53. **Lushchak, V. I.** 2001. Oxidative stress and mechanisms of protection against it in bacteria. Biochemistry (Mosc) **66:**476-89.
54. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem **244:**6049-55.
55. **Membrillo-Hernandez, J., S. O. Kim, G. M. Cook, and R. K. Poole.** 1997. Paraquat regulation of hmp (flavohemoglobin) gene expression in Escherichia coli K-12 is SoxRS independent but modulated by sigma S. J Bacteriol **179:**3164-70.
56. **Mendez-Ortiz, M. M., M. Hyodo, Y. Hayakawa, and J. Membrillo-Hernandez.** 2006. Genome-wide transcriptional profile of Escherichia coli in response to high levels of the second

messenger 3',5'-cyclic diguanylic acid. J Biol Chem **281:**8090-9.

57. **Mey, A. R., E. E. Wyckoff, V. Kanukurthy, C. R. Fisher, and S. M. Payne.** 2005. Iron and fur regulation in Vibrio cholerae and the role of fur in virulence. Infect Immun **73:**8167-78.
58. **Miller, J. H.** 1972. Experiments in Moliecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
59. **MUENCH, L. J. R. A. H.** 1937. A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS. THE AMERICAN JOURNAL OF HYGIENE **27:**493-497.
60. **Park, K. J., M. J. Kang, S. H. Kim, H. J. Lee, J. K. Lim, S. H. Choi, S. J. Park, and K. H. Lee.** 2004. Isolation and characterization of rpoS from a pathogenic bacterium, Vibrio vulnificus: role of sigmaS in survival of exponential-phase cells under oxidative stress. J Bacteriol **186:**3304-12.
61. **Perez, J. M., F. A. Arenas, G. A. Pradenas, J. M. Sandoval, and C. C. Vasquez.** 2008. Escherichia coli YqhD exhibits aldehyde reductase activity and protects from the harmful effect of lipid peroxidation-derived aldehydes. J Biol Chem **283:**7346-53.
62. **Perez, J. M., I. L. Calderon, F. A. Arenas, D. E. Fuentes, G. A. Pradenas, E. L. Fuentes, J. M. Sandoval, M. E. Castro, A. O. Elias, and C. C. Vasquez.** 2007. Bacterial toxicity of potassium tellurite: unveiling an ancient enigma. PLoS One **2:**e211.
63. **Pesavento, C., G. Becker, N. Sommerfeldt, A. Possling, N. Tschowri, A. Mehlis, and R. Hengge.** 2008. Inverse regulatory coordination of motility and curli-mediated adhesion in Escherichia coli. Genes Dev **22:**2434-46.
64. **Peterson, C. N., V. J. Carabetta, T. Chowdhury, and T. J. Silhavy.** 2006. LrhA regulates rpoS translation in response to the Rcs phosphorelay system in Escherichia coli. J Bacteriol **188:**3175-81.
65. **Podschun, R., and U. Ullmann.** 1998. Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev **11:**589-603.
66. **Privalle, C. T., and I. Fridovich.** 1993. Iron specificity of the Fur-dependent regulation of the biosynthesis of the manganese-containing superoxide dismutase in Escherichia coli. J Biol Chem **268:**5178-81.
67. **Reddy, P., M. Malczynski, A. Obias, S. Reiner, N. Jin, J. Huang, G. A. Noskin, and T. Zembower.** 2007. Screening for extended-spectrum beta-lactamase-producing Enterobacteriaceae among high-risk patients and rates of subsequent bacteremia. Clin Infect Dis **45:**846-52.
68. **Regueiro, V., M. A. Campos, J. Pons, S. Alberti, and J. A. Bengoechea.** 2006. The uptake of a Klebsiella pneumoniae capsule polysaccharide mutant triggers an inflammatory response by human airway epithelial cells. Microbiology **152:**555-66.
69. **Romling, U.** 2009. Rationalizing the evolution of EAL domain-based cyclic di-GMP-specific phosphodiesterases. J Bacteriol **191:**4697-700.
70. **Romling, U., M. Gomelsky, and M. Y. Galperin.** 2005. C-di-GMP: the dawning of a novel

bacterial signalling system. Mol Microbiol **57:**629-39.

71. **Rouf, S. F., I. Ahmad, N. Anwar, S. K. Vodnala, A. Kader, U. Romling, and M. Rhen.** 2011. Opposing contributions of polynucleotide phosphorylase and the membrane protein NlpI to biofilm formation by Salmonella enterica serovar Typhimurium. J Bacteriol **193:**580-2.
72. **Ryan, R. P., Y. Fouhy, J. F. Lucey, and J. M. Dow.** 2006. Cyclic di-GMP signaling in bacteria: recent advances and new puzzles. J Bacteriol **188:**8327-34.
73. **Sambandamurthy, V. K., S. C. Derrick, K. V. Jalapathy, B. Chen, R. G. Russell, S. L. Morris, and W. R. Jacobs, Jr.** 2005. Long-term protection against tuberculosis following vaccination with a severely attenuated double lysine and pantothenate auxotroph of Mycobacterium tuberculosis. Infect Immun **73:**1196-203.
74. **Schmidt, A. J., D. A. Ryjenkov, and M. Gomelsky.** 2005. The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. J Bacteriol **187:**4774-81.
75. **Sebastian, S., S. Agarwal, J. R. Murphy, and C. A. Genco.** 2002. The gonococcal fur regulon: identification of additional genes involved in major catabolic, recombination, and secretory pathways. J Bacteriol **184:**3965-74.
76. **Seib, K. L., H. J. Wu, S. P. Kidd, M. A. Apicella, M. P. Jennings, and A. G. McEwan.** 2006. Defenses against oxidative stress in Neisseria gonorrhoeae: a system tailored for a challenging environment. Microbiol Mol Biol Rev **70:**344-61.
77. **Semchyshyn, H., T. Bagnyukova, K. Storey, and V. Lushchak.** 2005. Hydrogen peroxide increases the activities of soxRS regulon enzymes and the levels of oxidized proteins and lipids in Escherichia coli. Cell Biol Int **29:**898-902.
78. **Simm, R., J. D. Fetherston, A. Kader, U. Romling, and R. D. Perry.** 2005. Phenotypic convergence mediated by GGDEF-domain-containing proteins. J Bacteriol **187:**6816-23.
79. **Simm, R., U. Remminghorst, I. Ahmad, K. Zakikhany, and U. Romling.** 2009. A role for the EAL-like protein STM1344 in regulation of CsgD expression and motility in Salmonella enterica serovar Typhimurium. J Bacteriol **191:**3928-37.
80. **Skorupski, K., and R. K. Taylor.** 1996. Positive selection vectors for allelic exchange. Gene **169:**47-52.
81. **Smirnova, G. V., Z. Y. Samoylova, N. G. Muzyka, and O. N. Oktyabrsky.** 2009. Influence of polyphenols on Escherichia coli resistance to oxidative stress. Free Radic Biol Med **46:**759-68.
82. **Sommerfeldt, N., A. Possling, G. Becker, C. Pesavento, N. Tschowri, and R. Hengge.** 2009. Gene expression patterns and differential input into curli fimbriae regulation of all GGDEF/EAL domain proteins in Escherichia coli. Microbiology **155:**1318-31.
83. **Storz, G., and S. Altuvia.** 1994. OxyR regulon. Methods Enzymol **234:**217-23.
84. **Storz, G., and J. A. Imlay.** 1999. Oxidative stress. Curr Opin Microbiol **2:**188-94.
85. **Sun, Y., J. H. Elwell, and L. W. Oberley.** 1988. A simultaneous visualization of the antioxidant enzymes glutathione peroxidase and catalase on polyacrylamide gels. Free Radic

Res Commun **5:**67-75.

86. **Touati, D.** 2000. Sensing and protecting against superoxide stress in Escherichia coli--how many ways are there to trigger soxRS response? Redox Rep **5:**287-93.
87. **Visick, J. E., and S. Clarke.** 1997. RpoS- and OxyR-independent induction of HPI catalase at stationary phase in Escherichia coli and identification of rpoS mutations in common laboratory strains. J Bacteriol **179:**4158-63.
88. **Wang, J. H., Y. C. Liu, S. S. Lee, M. Y. Yen, Y. S. Chen, S. R. Wann, and H. H. Lin.** 1998. Primary liver abscess due to Klebsiella pneumoniae in Taiwan. Clin Infect Dis **26:**1434-8.
89. **Weber, H., T. Polen, J. Heuveling, V. F. Wendisch, and R. Hengge.** 2005. Genome-wide analysis of the general stress response network in Escherichia coli: sigmaS-dependent genes, promoters, and sigma factor selectivity. J Bacteriol **187:**1591-603.
90. **Wiener, J., J. P. Quinn, P. A. Bradford, R. V. Goering, C. Nathan, K. Bush, and R. A. Weinstein.** 1999. Multiple antibiotic-resistant Klebsiella and Escherichia coli in nursing homes. JAMA **281:**517-23.
91. **Wu, C. C., Y. J. Huang, C. P. Fung, and H. L. Peng.** 2010. Regulation of the *Klebsiella pneumoniae* Kpc fimbriae by the site-specific recombinase KpcI. Microbiology **156:**1983-92.
92. **Zheng, M., B. Doan, T. D. Schneider, and G. Storz.** 1999. OxyR and SoxRS regulation of fur. J Bacteriol **181:**4639-43.

國科會補助專題研究計畫自我評量表

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□ 未達成目標（請說明，以 100 字為限）

□ 實驗失敗

□ 因故實驗中斷

□ 其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 □未發表之文稿 ■撰寫中 □無

專利：□已獲得 □申請中 □無

技轉：□已技轉 □洽談中 □無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

We have carried out the experiments as planned and three manuscripts (1~3) are being prepared to be submitted for publication. Many interesting findings including (1) YjcC mRNA as well as several stress response regulators is increased by the treatment of H_2O_2 and paraquat. In addition, the promoter activity is inducible upon treatment with H_2O_2 or paraquat; (2) YjcC is a PDE and the EAL domain is essential for the enzymatic activity; (3) Deletion of *yjcC* increased the susceptibility to paraquat and hydrogen peroxide, and the deficiency could be complemented by transforming into the mutant with pJR1 but not pJR2 indicating that the involvement of YjcC in the oxidative stress response is PDE activity dependent; (4) The intracellular ROS and protein carbonyl groups were increased by the deletion of *yjcC* implying a role of YjcC in modulating the activity of the radical scavenging enzymes. Increasing SOD and KatG activity but decreased KatE activity were observed for the Δ*yjcC* mutant further demonstrating a regulation of YjcC on the activity of some radical scavenging enzymes; (5) Deletion of *yjcC* was also found to affect the bacterial virulence and biofilm formation activity. However, if YjcC directly affects the expression of type 3 fimbriae remained to be investigated.

1. Huang CJ, Wang ZC and HL Peng (2011) Role of the *in vivo* expression protein YjcC in the oxidative stress responses in *Klebsiella pneumoniae* CG43.
2. Huang CJ, Wang ZC and HL Peng (2011) The YjcC-mediated regulation on type 3 fimbriae expression in *Klebsiella pneumoniae* CG43.
3. Huang CJ, Wu WY and HLing Peng (2011) Involvement of the second messenger c-di-GMP in the oxidative stress response in *Klebsiella pneumoniae* CG43.

國科會補助專題研究計畫項下出席國際學術會議心得報告-1

計畫編號	NSC 97-2320-B-009-001-MY3		
計畫名稱	**克雷白氏肺炎桿菌之活體表現蛋白YjcC在過氧化逆境中扮演的角色**		
出國人員姓名	彭慧玲	服務機構及職稱	交通大學生物科技學系
會議時間	97 年 8 月 5 日至 97 年 8 月 15 日	會議地點	土耳其-伊斯坦堡
會議名稱	(中文)**國際微生物學會聯合會** (英文) **International Union of Microbiological Societies, IMUS**		
發表論文題目	(中文)**克雷白氏肺炎桿菌之活體表現蛋白 YjcC 在過氧化逆境中扮演的角色** (英文)**Functional characterization of the YjcC in *Klebsiella pneumoniae* CG43**		

日期：97 年月___日

一、參加會議經過：**這個三年一次的國際性會議在土耳其伊斯坦堡舉行，其中細菌和應用微生物組會議在八月五號至九號舉行，病毒組則排在八月十號至十五號。可能因伊斯坦堡城區剛發生爆炸事件，與會人數並不多，而自台灣來參與的學界朋友們因此可以天天聚聚討論、交換心得。五天的會議，平均每天有七至十個講題，中午約一個半小時點的壁報討論，會議於早上九開始下午五點半左右結束，每個演講四十鐘，是個議程緊湊但內容極為豐富的學術研究會議。這個會議共二十個議題，不同的議題在同一時段不同會議廳進行。**

二、與會心得：**如附件**

三、考察參觀活動(無是項活動者略)：**無**

四、建議：**鼓勵推動專題性的小型會議**

五、攜回資料名稱及內容：**此會議鼓勵最新發現及尚未發表的研究結果，所以，除了會議流程外，並沒有摘要集也嚴禁照相。**

國科會補助專題研究計畫項下出席國際學術會議心得報告-2

計畫編號	NSC 97-2320-B-009-001-MY3		
計畫名稱	**克雷白氏肺炎桿菌之活體表現蛋白YjcC在過氧化逆境中扮演的角色**		
出國人員姓名	彭慧玲	服務機構及職稱	交通大學生物科技學系
會議時間	99 年 7 月 5 日至 99 年 7 月 8 日	會議地點	英國–**Brighton**
會議名稱	(中文)應用微生物學年會 (英文) Society for applied microbiology		
發表論文題目	(中文)**克雷白氏肺炎桿菌之活體表現蛋白 YjcC 在過氧化逆境中扮演的角色** (英文)**Functional characterization of the YjcC in *Klebsiella pneumoniae* CG43**		

日期：99 年月___日

一、參加會議經過：**在執行計劃中發現過氧化逆境會影響第三型纖毛的表現，而第三型纖毛的表現與生物膜的形成息息相關，因此希望藉參與此會議吸收與過氧化逆境－生物膜形成的相關資訊。我們張貼的壁報摘要節錄於報告最後一頁。這個會議涵蓋三個主要議題（一）李斯特菌的新興感染；（二）噬菌體的應用（三）生物膜的形成和控制。這個大約一百人的小型會議於早上九點開始至傍晚五點半左右結束，平均每天有十一個三十五分鐘的演講；第三天早上十點半至十二點為壁報討論時段，有別於我參與過的學術研討會，這是個應用導向的會議，有不少生技公司的產品開發的介紹，然而，有關生物膜的講題仍偏重基礎學術性的研究。**

二、與會心得：**如附件**

三、考察參觀活動(無是項活動者略)：**無**

四、建議：**鼓勵推動專題性的小型會議**

五、攜回資料名稱及內容：**此會議鼓勵最新發現及尚未發表的研究結果，所以，除了會議流程外，並沒有摘要集也嚴禁照相。**