行政院國家科學委員會補助專題研究計畫 □成果報告 ■期中進度報告

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

計畫類別：■ 個別型計畫　□ 整合型計畫
計畫編號： NSC 97－2320－B－009－001-MY3
執行期間：　98 年 8 月 1 日至 99 年 7 月 31 日

計畫主持人：彭慧玲
共同主持人：
計畫參與人員：黃靜柔

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：國立交通大學生物科技學院生物科技研究所

中　華　民　國　九十九年五月二十七日

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，近一年的工作如下:

1. 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*）的表現都會增加，而 *yjcC* 的表現增加量比 *rpoS* 較為明顯，更支持去年實驗的結果顯示 *yjcC* 基因表現與抗氧化壓力有關。
2. 我們利用 trans-complementation 方法證明 YjcC 蛋白具有 phoshodiesterase 活性，而 YjcC 的 EAL motif 中 E 殘基定點突變只輕微降低其 phoshodiesterase 活性，此結果顯示 C 端的 EAL motif 不是決定 YjcC 蛋白 phoshodiesterase 活性的主要區域，此外其 N 端區域的兩個 transmembrane domain 也可能影響此酵素的活化，進而影響其二級訊息傳遞分子 cyclic-di-GMP 的濃度來調控細菌抗氧化壓力的反應。
3. 細菌在對抗氧化壓力時，會產生抗氧化酵素如 superoxide dismutase (SOD)和 catalase 來清除自由基的傷害。我們分別利用原態膠電泳和定量分析來測定 SOD 與 catalase 酵素活性是否受 *yjcC* 基因缺損的影響，結果顯示 *yjcC* 基因缺損會誘導 Mn-SOD 和 Fe-SOD 的活性，卻降低 catalase 酵素 HPII-1 的活性。

I. 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 (3), was found to be inducible by 10 μM paraquat. Analysis of the gene organization revealed two oxidative stress regulators SoxR and SoxS encoding genes located next to *yjcC* gene. Deletion of *yjcC* was found to decrease the bacterial survival under paraquat treatment which supporting the involvement of YjcC in the oxidative stress defense. For further investigation of the biological role of *yjcC*, the accomplished works in the past year are following:

1. Using real-time PCR analysis of the *K. pneumoniae* CG43 under the treatment of 500 μM paraquat or 10 mM H2O2, we have found an increased expression of *yjcC* as well as the regulator genes including *soxRS, oxyR, RpoS, fur,* and *fnr* for the resistance to oxidative stress. In addition, the induced expression level of *yjcC* appeared to be higher than that of *rpoS*. This supports the previous speculation that YjcC plays a role in anti-oxidative stress response.
2. We have also used trans-complementation assay to demonstrate that YjcC carries a phosphodiesterase activity. Site-directed muatgenesis employed to substitute the active residue of the C terminal EAL domain revealed that the YjcC-AAL mutant only showed a slightly decrease of phosphodiesterase activity. This implied that the EAL motif may not be the major active sites for the enzymatic activity. Nevertheless, this does not preclude that the two transmembrane domains at the N-terminal region of YjcC may also play a role in activating the enzyme activity leading to the control of the second messenger cyclic di-GMP level which in turn to regulate the anti-oxidative stress response.
3. Bacteria in general produce antioxidant enzymes such as superoxide dismutase (SOD) and catalase to prevent from the damages of superoxide or other free radicals. The analysis including native gel detection and enzyme measurements to assess the *yjcC* deletion effect revealed that both Mn-SOD and Fe-SOD were induced-expressed in the *yjcC* deletion mutant. On the other hand, activity of the catalase HPII-1 was decreased.

II. Background and Significance

In *Escherichia coli* and *Salmonella*, there are many global regulators involved in oxidative stress, such as SoxRS, OxyR, RpoS, Fur and Fnr (8, 9, 11, 12). *E. coli*, widely used as a model organism for the study of oxidative stress response, possess two primary cellular responses respectively controlled by OxyR and SoxRS (Pomposiello and Demple, 2002). The OxyR activates gene whose products protect cells against peroxide radicals, whereas SoxRS system activates set of genes in response to superoxide radicals. Hydrogen peroxide at high concentration could trigger the SoxRS system (Manchado *et al.*, 2000). At stationary phase, RpoS activates the expression of *katE* to prevent from the oxidative stress. Evidences have shown that Fur (8), which is a ferric uptake regulator, also regulates the expression of the key enzymes in the defense against oxygen toxicity manganese and iron superoxide dismutase (MnSOD and FeSOD). In *E. coli*, Fur is also under regulated by soxRS and OxyR FNR which is an O_2 sensor is a major regulator involved in the physiological switch between aerobic and anaerobic growth conditions. FNR is also involved in the oxidative stress response since *sodA* (Mn-SOD) expression in anaerobic condition is controlled by FNR (15).

The defense mechanisms, which play an important role in determining the bacterial virulence, include sensing, avoiding, and removing the oxidants (10, 13, 14). As shown in Fig. 1, superoxide is removed by SODs (SodA, SodB and SodC) (6), while the generating hydrogen peroxide removed by catalases (KatE, KatG) and peroxidases (AhpC) in *E.coli*. The transcriptome analysis of *Pseudomonas aeruginosa* (1) or *Staphylococcus aureus* (2) response to H_2O_2 revealed many more induced expression genes which include virulence genes, the genes encoding products involved in DNA repair and anaerobic metabolism,the induced-expression of more than 100 genes in responding to either H_2O_2 or paraquat suggests a complexity of the antioxidant strategies (16).

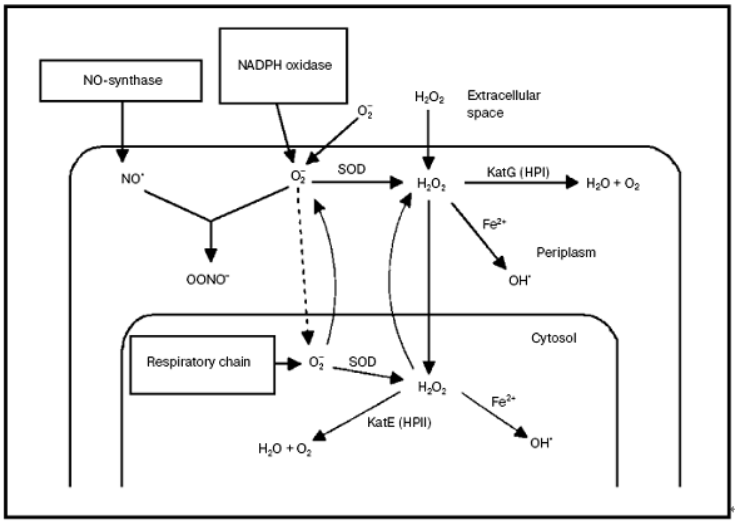


Fig. 1. Bacterial cell responses to different oxidative stresses. External reactive oxygen species enter the periplasm and then can either be inactivated or enter the cell. In the cell, they again can either be inactivated or result in a cell response. Some of the ROS entering the cell or generated in the cell may leave the cell.

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. The C-terminal sequences containing EAL domain appeared to be highly conserved in three bacteria. Multiple sequence alignment using Clastal W has classified YjcC as an active EAL domain containing protein.

Increasing evidences have shown that cyclic diguanylate (c-di-GMP), a bacterial second messenger, acts to modulate the expression of virulence genes for the infection of bacterial pathogen during the transition from environmental reservoirs to the human host (4, 5). 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. 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 under high level of c-di-GMP.

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 (17). 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 cycalse (DGC) and phosphodiesterase (PDE). Many of the sensory proteins contain N-terminal cytoplasmic sensor domains that affect the enzymatic activities of the downstream domains. However, the ligands of most sensory domains remain obscure.

III. Materials and methods

Real-Time PCR analysis Total RNA was isolated from fresh bacterial cultures (OD_{600}= 0.6~0.7). Three micrograms of the DNase I-treated total RNA extracted from *K. pneumoniae* CG43 or the derived strains treated with 500 μM paraquat and 10 mM H_2O_2 was reverse transcribed to generate cDNA using AMV Reverse Transcriptase and random primer pDN (6) according to the manufacturer's instructions (Roche). Real-time PCR (RT-PCR) analysis of the transcription of *rpoS*, *oxyR*, *fur*, *yjcC*, *soxR*, *soxS* and 23S rRNA with each of the 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 is 23S rRNA.

Trans-complementation for the assessment of phosphodiesterase activity

(a) 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.

(b) Site-directed mutagenesis

Site directed mutagenesis was performed on the plasmids pJR1 according to the QuickChange site-directed mutagenesis kit following the manufacturer's protocol (Stratagene). to substitute critical residue in the EAL domain of YjcC . The generated PCR product contained one point mutation corresponding to E303A to A303A 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) Preparation of cell-free extracts. To measure specific activities of SOD and catalase, cell-free extracts were harvested (15000 rpm, 4℃, 20 min) in OD_{600}= 0.7~0.8

exponential phase of growth and suspended in ice-cold 50 mM potassium phosphate buffer, pH 7. Cells were disrupted by ultra-sonication, Cell debris was removed by centrifugation for 10 min at 12000 rpm and 4℃, and collected the supernatant on ice. The extraction of total proteins was made at 4 ℃. Protein concentrations were estimated according to Bradford (1976) Bradford's method using bovine serum albumin as standard.

(b) Native-PAGE and gel staining for enzymatic activity

Cell lysates, each with the same quantity of protein, were loaded onto 10% native polyacrylamide gels and the proteins separated at a constant voltage of 150 V for 2 h. Subsequently, the gels were removed and stained for either SOD or CAT activity according to the methods of Beauchamp and Fridovich (1971) and Woodbury et al. (1971), respectively. 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. An attempt was also made to identify the expression of CAT activity soaking gels in 10 mM hydrogen peroxide for 30 min with gentle shaking to localize CAT. The gels were transferred to a solution of 1% ferric chloride and 1% potassium ferricyanide for 10 min the localized-CAT activity gave rise to colorless bands on a dark green background.

(c) SOD specific activity determination

SOD activity was determined spectrophotometrically at 25℃ by the xanthine oxidase–cytochrome C method (7). The 0.7 ml assay mixture contained: 50mM potassium phosphate, pH 7.8; 0.1mM EDTA; 50 mM xanthine; 1.7 mU xanthine oxidase ; 10 mM cytochrome C. The reduction of cytochrome C was followed by measuring 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%.

(d) Catalase specific activity determination.

Catalase activity was determined spectrophotometrically at 25℃ (1 cm cuvette, JascoV-550 spectrophotometer) by monitoring the decrease in A240 of 10mM H_2O_2 in 50mM 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 1mmol H_2O_2 min^{-1} under the assay conditions. Protein concentrations were determined by the method of Bradford (1976) using standard reagents (Bio-Rad) and bovine serum albumin as standard.

IV. Progress Report and Discussion

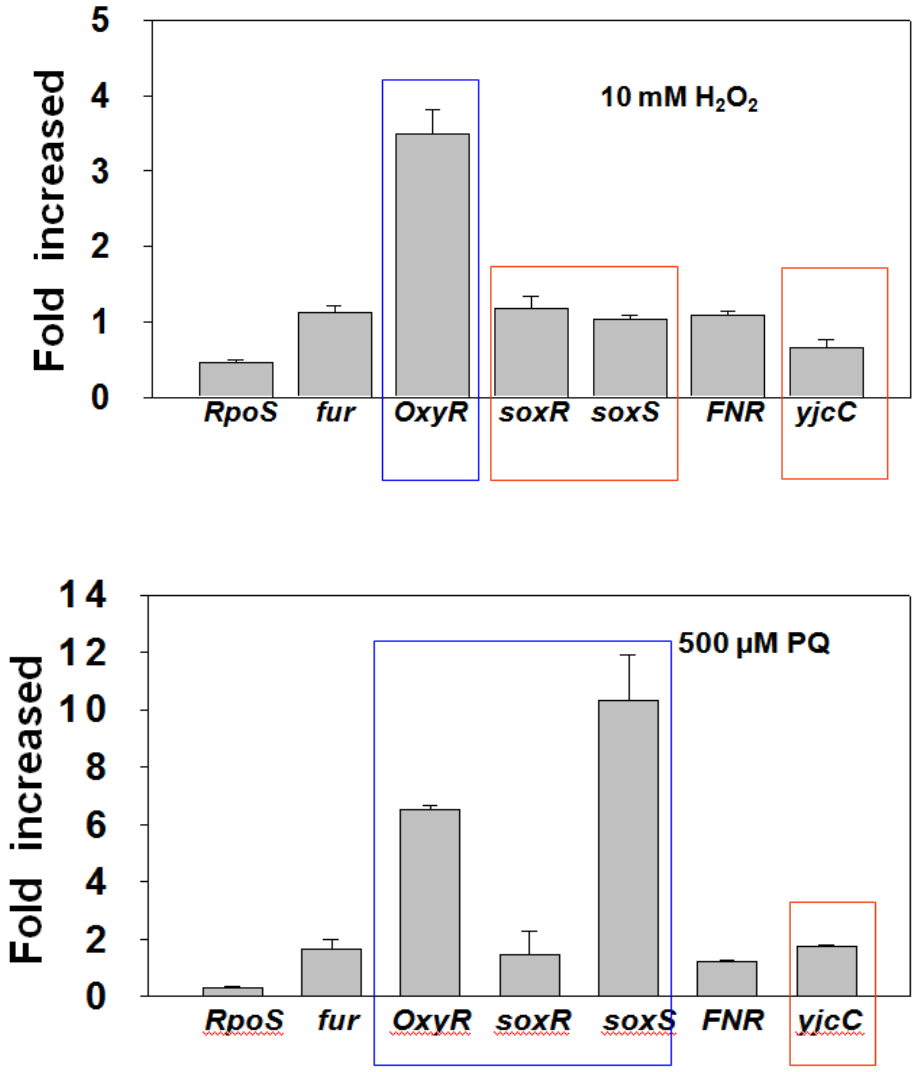
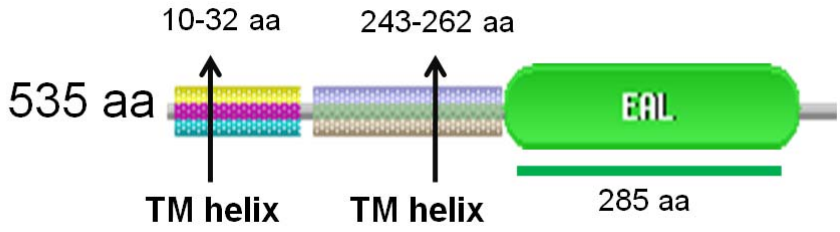


Fig. 2. The *yjcC* expression is inducible by hydrogen peroxide and paraquat. Q-PCR was employed for the measurement of the inducing effect of 10 mM hydrogen peroxide or 500 μM praquat on the expression of oxidative stress genes in *Klebsiella pneumoniae*.

(A)



(B)

```
Dos-Eco          -------RLVLGAALKEAISNNQLKLVYQPQIFAE-----TGELYGIEALARWHDPLHGH  48
KP-yjcC          ------QYHSPRNMLQRALSCRQLRLHYQPIIDIK-----NNRCVGAEALLRWPGFDGPV  49
yahA-Eco         ----------TPEAISLALENHEFKPWIQPVFCAQ-----TGVLTGCEVLVRWEHPQTGM  45
yhjH-sa          MIKQVIQQLRVPDAGIENLQERRYWLQCERAYTYQPIYQTDGRLMTVELLTAVTHPDN--  58
                                :. ..     :     :      .     * *

Dos-Eco          VPPSRFIPLAEEIGEIENIGRWVIAEACRQLAEWRSQN-IHIPALSVNLSALHFRSNQLP 107
KP-yjcC          MNPAEFIPLAENEGMIAQVTDYVVDELFYEMGEFLASH-PQL-YIAINLSASDFHSARLI 107
yahA-Eco         IPPDQFIPLAESSGLIVIMTRQLMKQTADILMPVKHLL-PDNFHIGINVSAGCFLAAGFE 104
yhjH-sa          -PSRRIAPDRYFAELAVRHRIDVVKEQLHQLEQKADFFTRHRLLASVNVDGPTLIAMRQQ 117
                   . .: *               :: :    :          .    .:*:..  : :

Dos-Eco          NQVSDAMHAWGIDGHQLTVEITESMMMEHDTEIFKRIQILRDMGVGLSVDDFGTGFSGLS 167
KP-yjcC          SQISEKAHSYAVCIGQIKIEVTERGFIDVP-KTTPVIQAFREAGYEIAIDDFGTGYSNLH 166
yahA-Eco         KECLNLVKKLGNDKIKLVLELTERNPIPVTPEARAIFDSLHQHNITFALDDFGTGYATYR 164
yhjH-sa          PDILAAMERLP----WLRFELVEHIRLPKDSSFASMCEFVP-----LWLDDFGTGMANFS 168
                  :      .         : .*:.*   :     .     : .       : :****** :

Dos-Eco          RLVSLPVTEIKIDKSFVDRCLTEKRILALLEAITSIGQSLNLTVVAEGVETKEQFEMLRK 227
KP-yjcC          NLHALNVDILKIDKTFVDTLTTNNTSHLIAEHIIEMARGLRLKTIAEGVETPEQVSWLYK 226
yahA-Eco         YLQAFPVDFIKIDKSFVQMASVDEISGHIVDNIVELARKPGLSIVAEGVETQEQADLMIG 224
yhjH-sa          ALSEVRYDYIKVARELFVMLRQSPEGRNLFILLLQLMNRYCRGVIVEGVETLEEWRDVQR 228
                  *  .     :*: : :.      .      :    : .: .       :.***** *:    :

Dos-Eco          IHCRVIQGYFFSRPLPAEEIPGWMSSVLPLKI 259
KP-yjcC          RGVQYCQGWLFAKA------------------ 240
yahA-Eco         KGVHFLQGYLYSPPVPGNKFISEWVMKAGG-- 254
yhjH-sa          SPAFAAQGYFLSRPVPLISLEEVILTL----- 255
                       **.. .
```

Fig. 3. YjcC contains an enzymatically active EAL domain. (A) Pfam analysis of YjcC (http://pfam.sanger.ac.uk/) (B) Sequence alignment of the C-terminus of YjcC with the EAL proteins grouped into active phosphodiesterase activity base on the EAL signature.

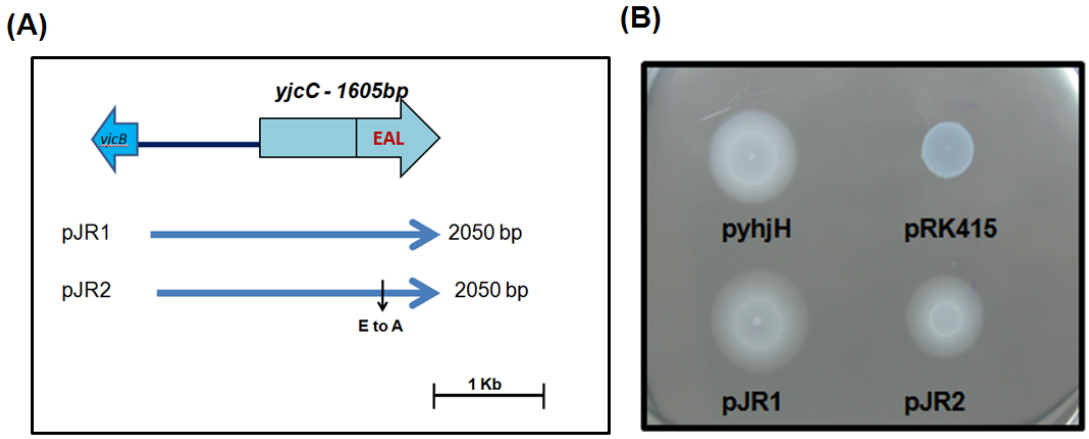
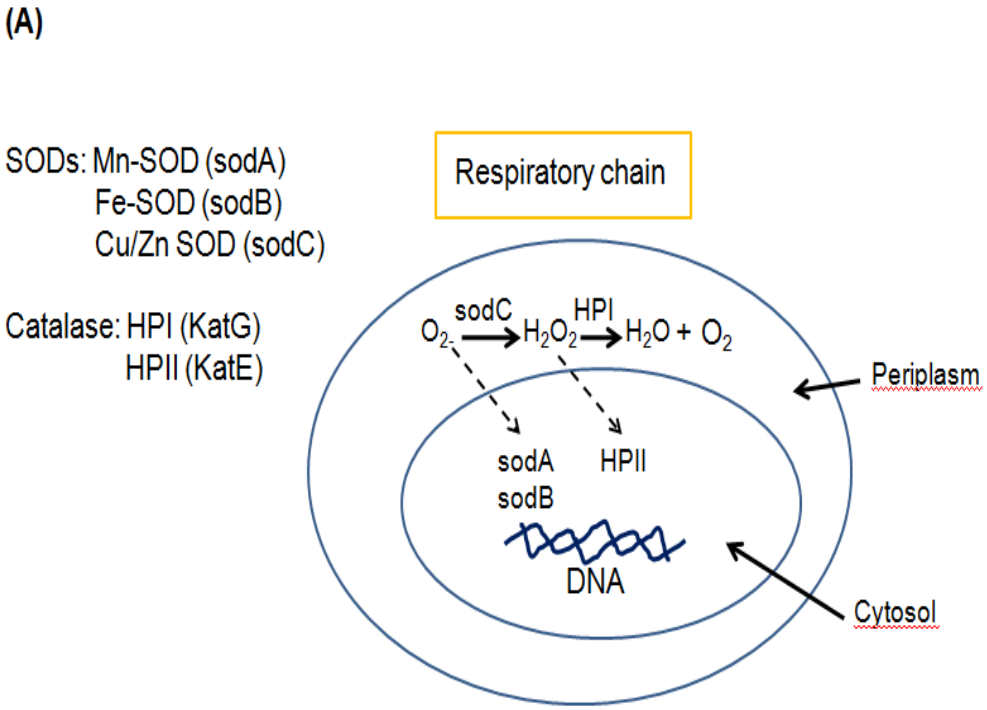


Fig. 4. YjcC carries a c-di-GMP phosphodiesterase activity. (A) The *yjcC* expression plasmid pJR1 and the derived single residue (E to A) mutant pJR2. (B) Swimming activity analysis for the assessment of the phoshodiesterase activity. The bacteria respectively carry pYhjH (encoding an active phosphodiesetrase), pRK415 (a broad host range vector), pJR1 (*yjcC*-pRK415), and pJR2 (*yjcC* mutant-pRK415) were spot onto the swimming plate, incubated for 16 h at 30°C, and the photo taken.



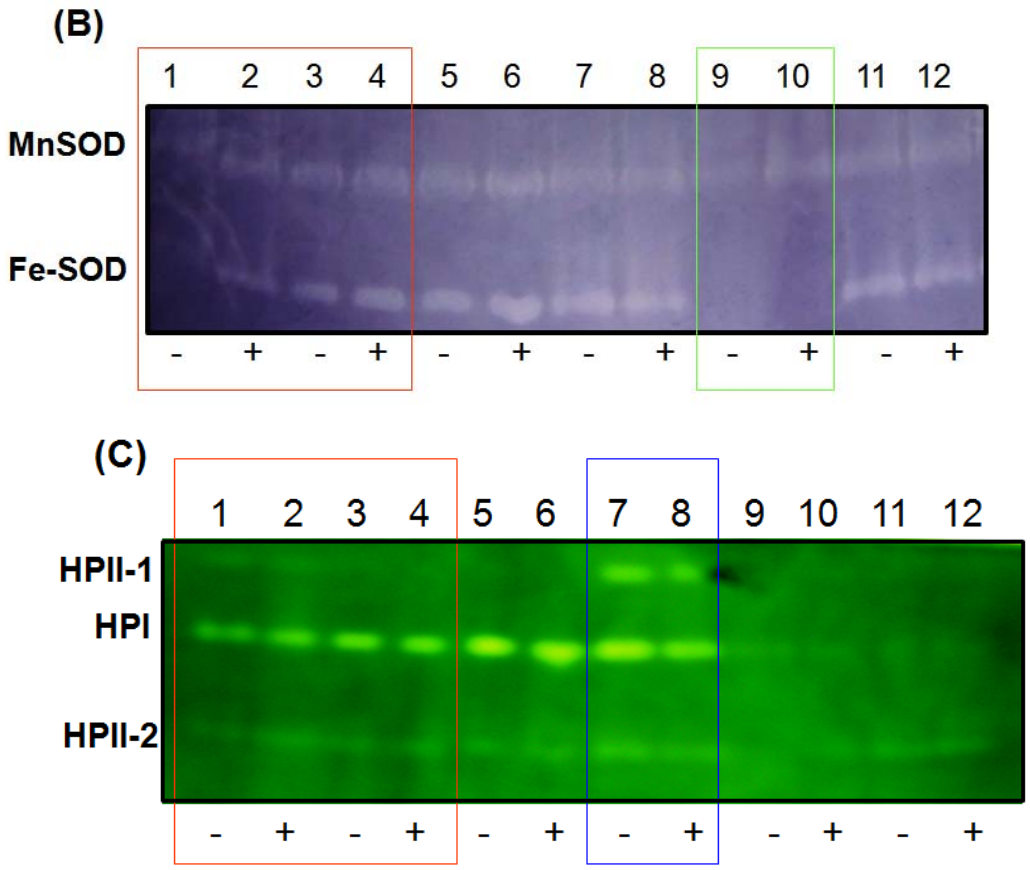


Fig. 5. (A) Schematic representation of the antioxidant enzymes to protect from oxidative stress. (B), (C) Effects of 500 μM paraquat(+) and non treatment(-) on SOD and catalase activities on 10% and 8% native gel. Strains 1, 2: WT; 3, 4: Δ*yjcC*; 5, 6:Δ*yjcC* /pRK415-pJR1; 7, 8: Δ*soxRS* ; 9, 10: Δ*fur* and 11, 12: Δ*rpoS*. HPII-1: catalase E (KatE); HPII-2: *rpoS*-dependent hydroperoxidase (KatF); HPI: catalase G (KatG).

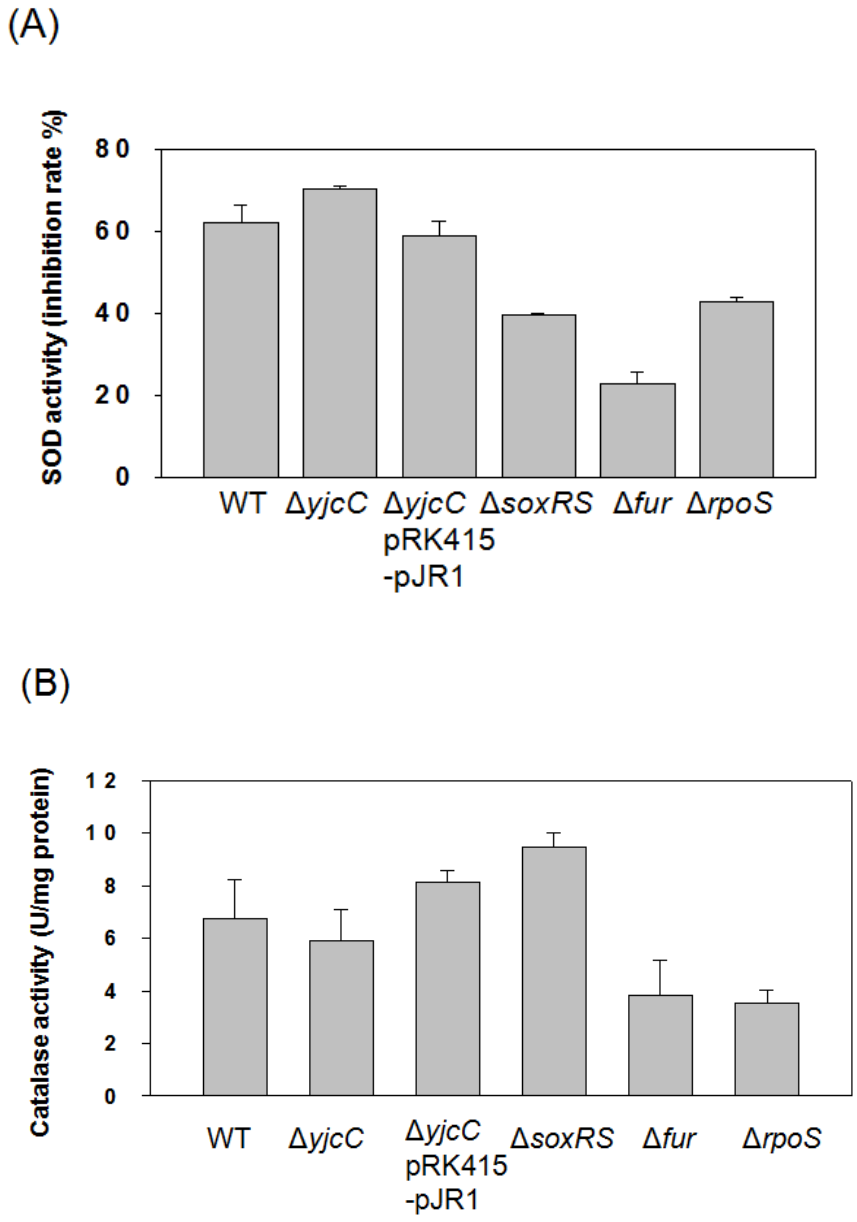


Fig. 6. SOD (A) and catalase (B) activities in *K. pneumoniae* CG43. The enzyme activities were determined in *K. pneumoniae* WT, Δ*yjcC*, Δ*yjcC*/pRK415-pJR1, Δ *soxRS*, Δ *fur* and Δ *rpoS* cells grown in the presence of 500 μM paraqquat.

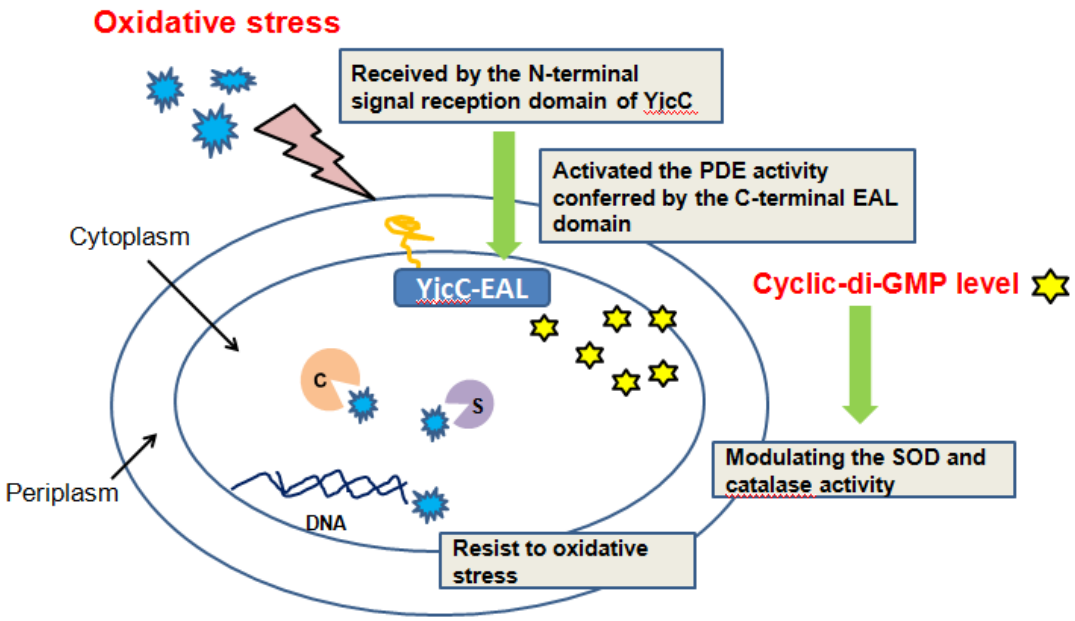


Fig. 7. The proposed model for functional role of YjcC.

V. Discussion

Several of the oxidative stress responsive genes including *soxR, soxS, RpoS, fur and OxyR*, as well as *yjcC* appeared to be inducible by H_2O_2 or paraquat using real-time PCR analysis. Sequence analysis showed that YjcC has C-terminal EAL domain and two transmembrane domains at N-terminal part. The *in-trans* complementation using swimming activity analysis suggested YjcC is a c-di-GMP phoshodiesterase. Specific residue substitution of the EAL motif resulted in a slightly decreased of the swimming activity. This implied that the EAL motif may not be the major active sites for the enzymatic activity. Nevertheless, this does not preclude that the two transmembrane domains at the N terminal region of YjcC may also play a role in activating the enzyme activity. If *yjcC* received the signal by its transmembrane domain from N-terminal and activate PDE activity to modulate cyclic di-GMP to resist oxidative stress remains to be determined.In addition, the analysis including native gel detection and enzymatic measurements to assess the *yjcC* deletion effect revealed that both Mn-SOD and Fe-SOD were induced expressed in the *yjcC* deletion mutant. On the other hand, the *yjcC* deletion decreased the catalase HPII-1 activity. How YjcC exerts its regulation to affect expression of the SOD or KatE will be investigated.

VI. Literature Cited

1. Chang W, Small DA., Toghrol F, Bentley WE. 2005. Microarray analysis of *Pseudomonas aeruginosa* reveals induction of pyocin genes in response to hydrogen peroxide. BMC Genomics 6:115.
2. Chang W, Small DA, Toghrol F, Bentley WE. 2006. Global transcriptome analysis of *Staphylococcus aureus* response to hydrogen peroxide. J Bacteriol 188:1648-1659.
3. Lai YC, Peng HL, Chang HY. 2001. Identification of genes induced in vivo during *Klebsiella pneimoniae* CG43 infection. Infect Immun 69:7140-5.
4. Romling U, Gomelsky M, Galperin MY. 2005. C-di-GMP: the dawning of a novel bacterial signaling system. Mol Microbiol 57:629-39.
5. Schmidt AJ, Ryjenkov DA, Gomelsky M. 2005. The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. J Bacteriol 187:4774-81.
6. Benov, L. T., and I. Fridovich. 1994. *Escherichia coli* express a copper- and zinc-containing superoxide dismutase. J. Biol. Chem. 269:25310–25314.
7. McCord, J. M., and I. Fridovich**.** 1969. Superoxide dismutase, an enzymatic function for rythrocuprein (hemocuprein). J. Biol. Chem. 244:6049–6055.
8. Zheng, M., B. Doan, T. D. Schneider, and G. Storz. 1999. OxyR and SoxRS regulation of *fur***.** J Bacteriol, Aug. p. 4639–4643.
9. 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. 272:5082–5086.
10. Storz, G., and J. A. Imlay. 1999. Oxidative stress. Curr. Opin. Microbiol. **2:**188–194.
11. M-H´Ndez, 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 σ^s. J Bacteriol. p. 3164–3170.
12. Touati, D. 2000. Sensing and protecting against superoxide stress in *Escherichia coli*- how many ways are there to trigger *soxRS* response? Redox Report 5, No. 5.
13. Imlay, J. A. 2003. Pathways of oxidative damage. Annu. Rev. Microbiol. 57:395–418.
14. Imlay, J. A. 2008. Cellular defenses against superoxide and hydrogen peroxide. Annu. Rev. Biochem. 77:755–76
15. 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: 2262–2273.
16. Lushchak V. I. 2001. Oxidative stress and mechanisms of protection against it in

bacteria. Biochemistry (Moscow), Vol. 66: 476-489.

17. Simm R, Fetherston JD, Kader A, Romling U, Perry RD. 2005. Phenotypic convergence mediated by GGDEF-domain-containing proteins. J Bacteriol 187:6816-23.