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

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中　華　民　國　九十八　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，
過氧化逆境反應，小鼠毒性。

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。我們在過去一年中除了建立 *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，而在定量 PCR 的實驗當中，初步結果顯示加入不同濃度的巴拉刮後，*yjcC* 轉錄作用表現的趨勢與 RpoS 和 Fur 的趨勢相同；另外，分別建構了不同片段的 *yjcC*-expression plasmids 以 200 μM 的巴拉刮或 10 mM 的過氧化氫進行存活率測試，發現將 *yjcC* 基因的 C 端帶有 EAL domain 的區域剔除掉以及將麩安酸(E:glutamic acid)突變之後則對於抗氧化逆境的能力比原先互補 *yjcC* 的質體下降，暗示著 EAL domain 在功能上能幫助 YjcC 對抗氧化逆境。最後，利用小鼠腹膜炎實驗發現：相較於野株，*yjcC* 基因剔除的突變株毒性減弱約 10 倍，因此推測 YjcC 蛋白功能與其感染致病相關。

I. Abstract

Ib. Abstract in English

Key words: *Klebsiella pneumoniae* CG43; IVE *yjcC*; functional EAL domain, oxidative stress responses and virulence determination using mouse peritonitis model.

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 M 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 the involvement of YjcC in the oxidative stress defense. We have carried out the studies in the past year to demonstrate that the expression of *yjcC* is paraquat inducible assessed using a LacZ reporter system. The subsequent real-time PCR analysis under the treatment with paraquat or H_2O_2 revealed similar expression patterns of *yjcC* with those of *rpoS* and *fur* gene. Transformation of the *yjcC* deletion mutant with the plasmid pC2050 that carries *yjcC* encoding gene restored the bacterial growth upon the treatment with 200 μM of paraquat or 10 mM H_2O_2, further supporting a role of YjcC in the oxidative stress response. In addition, the plasmid pC1250, carrying a truncated *yjcC* removed of EAL domain encoding region, or pC2050A, carrying a site-directed-mutation *yjcC* gene coding for $YjcC_{E303A}$, has also been introduced into the mutant. Nevertheless, neither plasmid could complement the yjcC deletion effect suggesting that the EAL domain is required for a functional YjcC. Finally, the *yjcC* deletion mutant exerted a 10-fold decrease of LD50 in mouse peritonitis model further potentiates a role of YjcC in the bacterial infection.

II. Background and Significance

***Klebsiella pneumoniae yjcC*, an IVE (*in vivo* expression) gene**

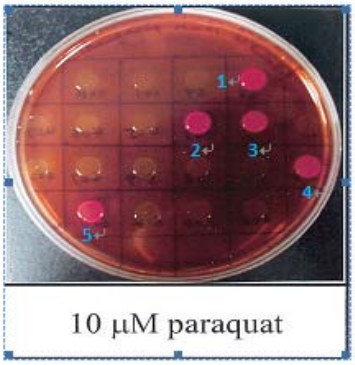

Fig. 1. Phenotypes of IVE clones on MacConkey-galactose agar containing 10 μM paraquat (1). 1: *lysA* , 2: *yfjB*, 3: *yjcC* , 4 and 5:unknown function.

As shown in Fig. 1, 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 (2). *Mycobacterium tuberculosis lysA* deletion mutant has been demonstrated to be attenuated and safe as vaccine candidate in mice (3).

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 (4). While analysis of the yet known function YjcC revealed similar gene organization and significant sequence homology with that of the *E. coli* and *S. typhimurium* homologs.

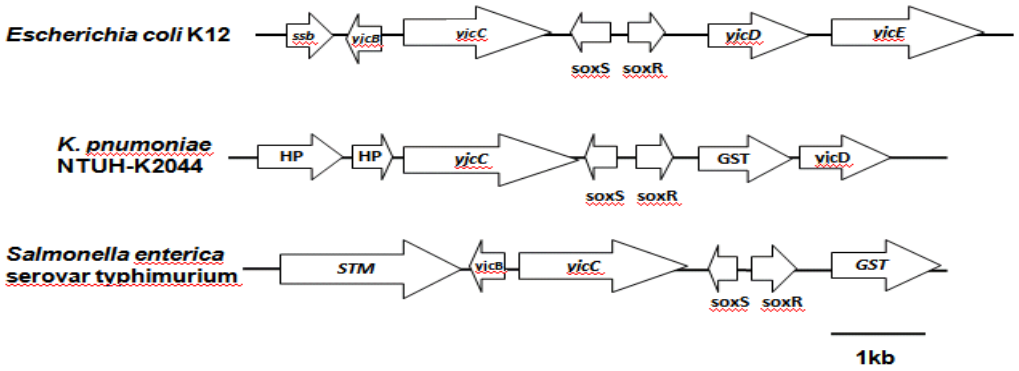

Fig. 2. The *yjcC* containing gene clusters of *E. coli* K12, *K. pneumoniae* NTUHK2044, and *Salmonella enterica* serovar Typhimurium. As shown in Fig. 2, the global oxidative stress regulators SoxR and SoxS encoding genes are upstream located suggesting a role of YjcC in the response to oxidative stress and hence *yjcC* was chosen for further study.

As shown in Fig. 3(A), 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 (Fig. 3A) could be identified. In contrast, the C-terminal sequences containing EAL domain appeared to be highly conserved in three bacteria (Fig. 3B).

(A)

(B)

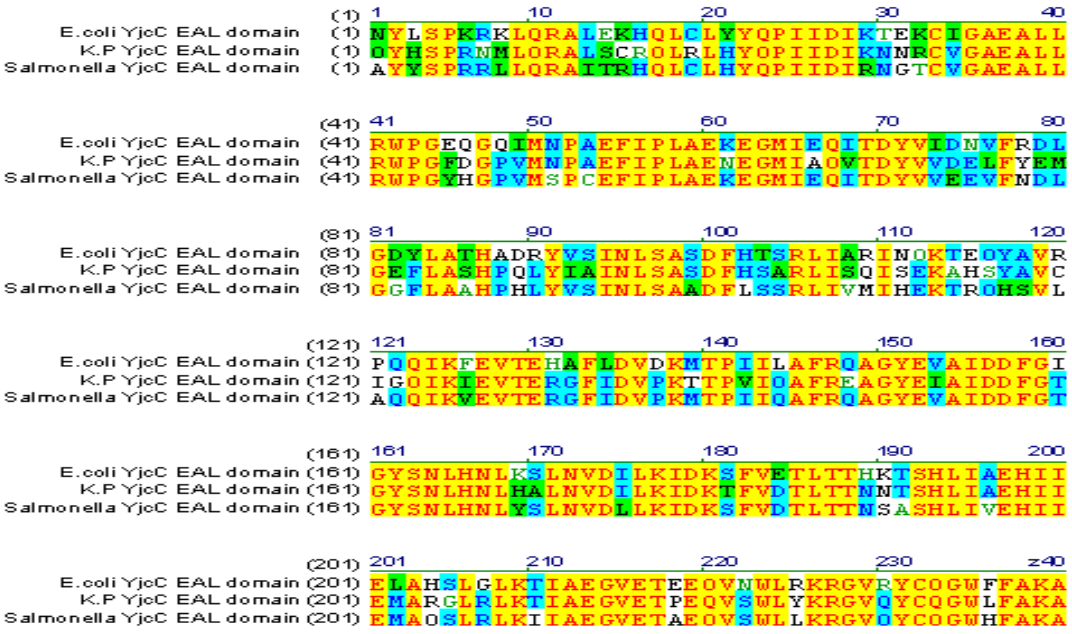

Fig. 3. Alignments of YjcC (A) N-terminal sequences and (B) EAL domain containing sequences of *E. coli* K12, *K. penumoniae* NTUH-K2004 and *S.* Typhi.

Pilot study To investigate if YjcC plays a role in oxidative stress response, the *yjcC* deletion mutant was obtained by allelic exchange method and the susceptibility to paraquat was determined.

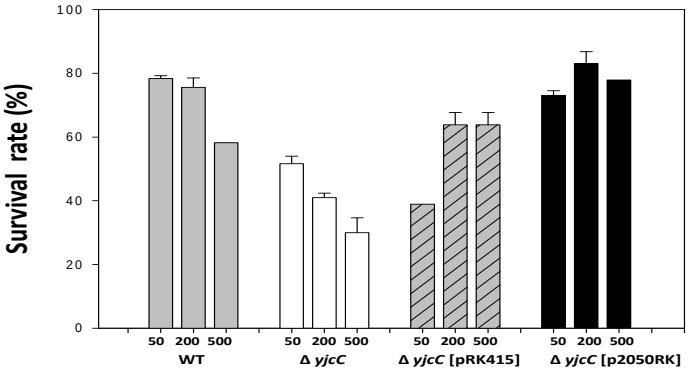

Fig. 4. Paraquat sensitivity assay. The overnight cultures were refreshed grown in LB until OD600 of 0.6~0.7. Aliquots of 50, 200,500 μM of paraquat were then added and the incubation continued for 35 min at 37℃. Finally, the cultures were plated onto LB plates for colony formation and the relative survival was determined as ratio of the viable count to the colony number of untreated-inoculum.

As shown in Fig. 4, 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 500 μM), supporting a role of YjcC in the bacterial resistance to oxidative stress. Specific aims of the proposed studies as shown in Fig. 5 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.

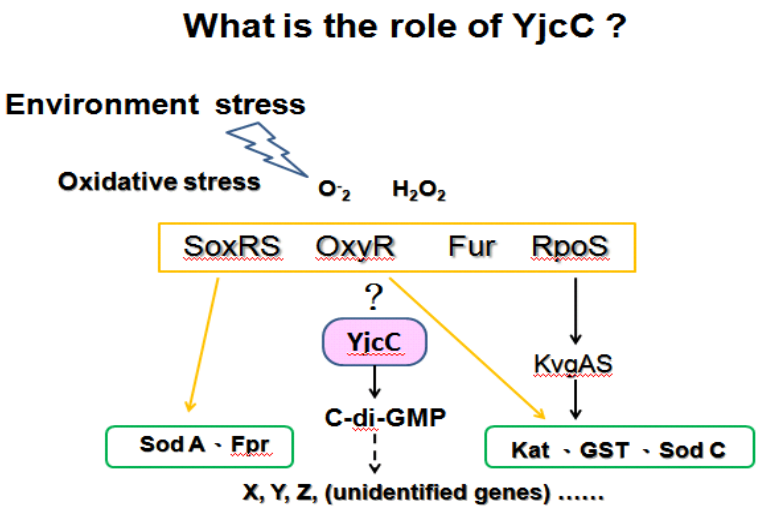

Fig. 5. A proposed study for the functional role of YjcC in *K. pneumoniae* CG43.

III. Materials and methods

Paraquat and H_2O_2 survival assessment One-hundredth overninght grown bacteria were inoculated to LB and incubated at 37℃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℃ 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.

Construction of $YjcC_{E303A}$ expression plasmid by site-directed mutagenesis Site-directed mutagenesis was carried out essentially as described by QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA). Glu303 in the EAL domain of YjcC is responsible for phosphodiestrase enzyme activity, which was changed in to alanine in pC2050, and the resulting plasmids was named pC2050A ($YjcCE_{303A}$).

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 (5) 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(6) 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.

IV. Progress Report and Discussion

Promoter activity analysis using LacZ as a reporter indicated P_{yjcC} is a paraquat-inducible promoter

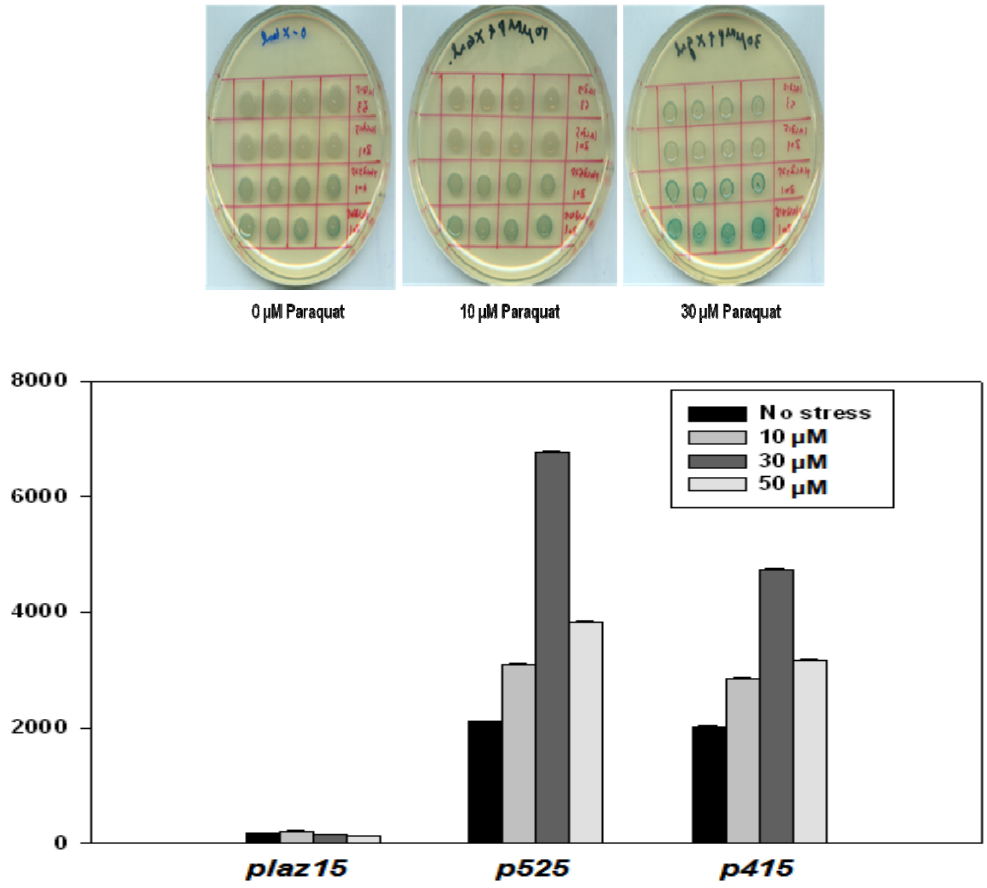

Fig. 6. Measurement of the *yjcC* promoter activity. The putative promoters which respectively contain 415 bp and 525 bp of the non-coding region upstream of *yjcC* were isolated and cloned into the promoterless *lacZ* reporter plasmid placZ15. The recombinant plasmids were then transformed to *K. pneumoniae* CG43Z01 and the β-galactosidase activity of the transformants measured.

Real-time PCR analysis under the treatment with paraquat (PQ) or H_2O_2 revealed similar expression patterns of *yjcC* with those of *rpoS* and *fur* gene

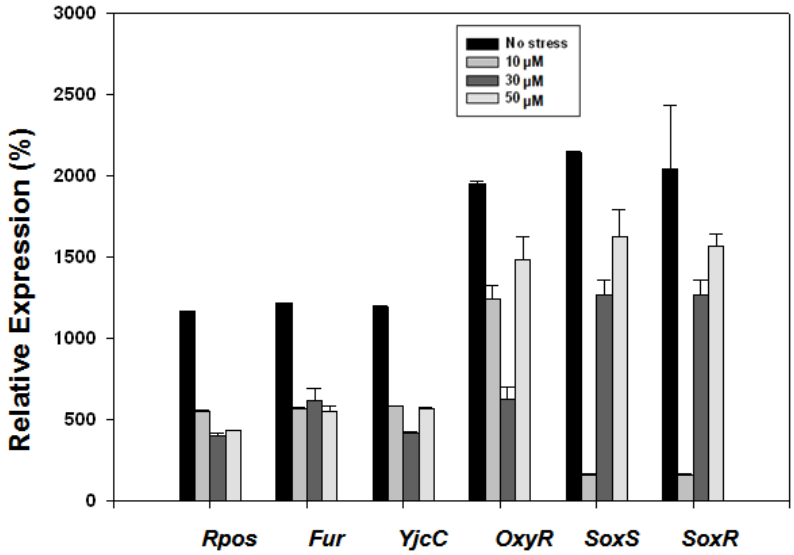

Fig. 7. Real –Time PCR analysis. The overnight cultures were collected, refreshed grown in LB until OD600 of 0.6~0.7 and incubated with 10, 30, or 50 μM PQ or without PQ for 35 min. The target genes for RT-PCR analysis included *rpoS*, *fur*, *yjcC*, *oxyR*, *soxS* and *soxR*. 100% expression is defined as the expression of wild-type strain

Construction of the recombinant plasmids for the complementation analysis

The recombinant plasmids are pC2050, the plasmid carries a *yjcC* encoding gene; pC1250, the plasmid carries a truncated *yjcC* removal of the EAL domain coding region; pC2050A, carries a site-directed-mutation *yjcC* coding for $YjcC_{E303A}$.

The *yjcC* deletion increased the bacterial susceptibility to paraquat and H_2O_2, and the complementation analysis indicated a functional role of the EAL domain

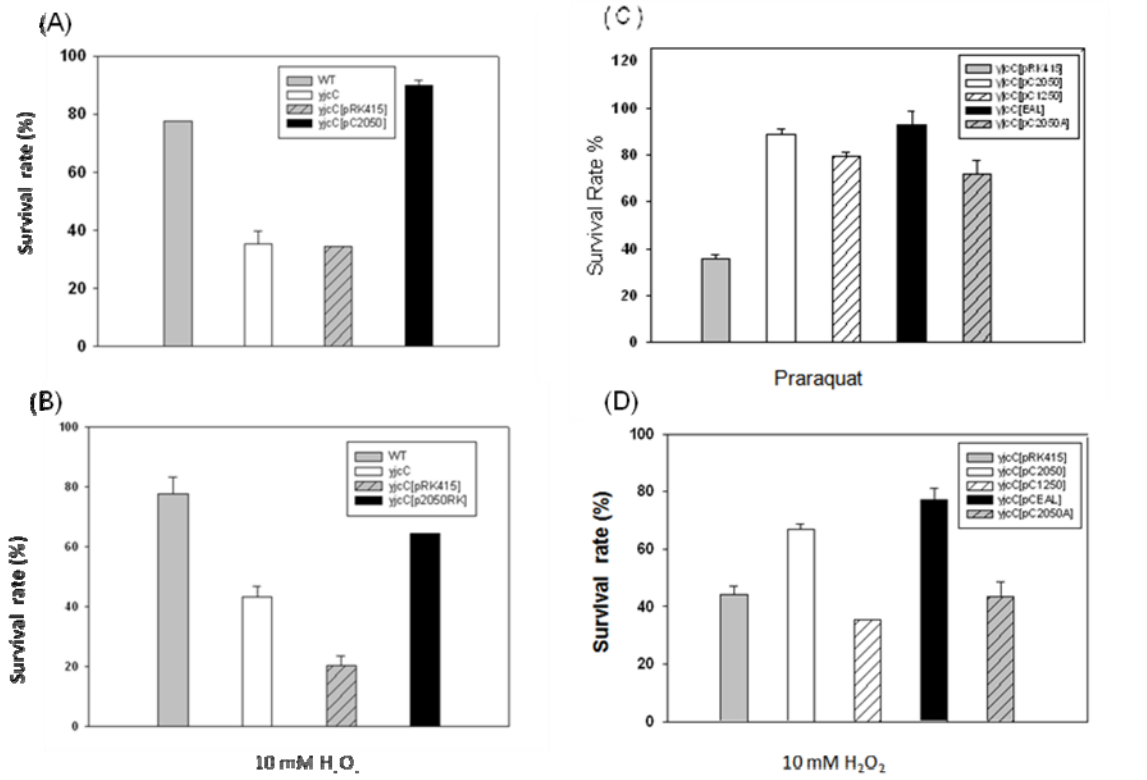

Fig. 8. Paraquat and H_2O_2 sensitivity assay. The overnight cultures were collected, refreshed in LB, and the cultures continued until OD600 of 0.6~0.7 Subsequently, aliquots of 200 μM of paraquat (A and C) or 10 mM H_2O_2 (B and D)were respectively added and the cultures continued for 45 min at 37℃. Finally, the cultures were plated onto LB plates for colony formation.

Mouse lethality assay revealed a virulence role of YjcC

Strain	LD 50 (CFU)
CG43S3	1×10^4
ΔyjcC	1×10^5
C2050	2.74 x 106

Table 1. Mouse lethality assay . The tested bacterial strains were cultured in LB medium at 37°C overnight. Four mice of a group were injected intraperitoneally with bacteria suspended in 0.2 ml of PBS in 10-fold-graded doses. The LD50, based on the number of survivors after one week, was calculated by the method of Reed and Muench and expressed as CFU.

V. Discussion

Sequence analysis of the putative promoter of *yjcC* revealed no conserved RpoS- or Fur-binding elements. However, the promoter activity measurement and the real-time PCR analysis suggested that YjcC is a component of the RpoS or Fur regulon. If *yjcC* is under the control of RpoS or Fur is currently under investigation. The *yjcC* deletion mutant revealed an increased susceptibility to paraquat and hydrogen peroxide, and the deficiency could be complemented by transferring into the mutant with a YjcC-expression plasmid. This indicated an involvement of YjcC in the anti-oxidative stress response. The paraquat resistance activity was not able to be restored while in trans supplying the mutant with pC1250 or pC2050A implying the EAL domain plays a role in the oxidative stress response. Moreover, the deletion of *yjcC* resulted in an increase of LD50 suggesting a role of YjcC in regulation of the bacterial virulence. To demonstrate that *yjcC* plays a role in anti-phagocytosis during the infection, CG43-derived *wza-yjcC* double mutant has been generated.

VI. Literature Cited

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