Abstract

This study shows that the expression of yjcC, an in vivo expression (IVE) gene, and the stress response regulatory genes soxR, soxS, and rpoS are parapatric inductible in Klebsiella pneumoniae CG43. The deletion of rpoS or soxRS decreased yjcC expression, implying an RpoS- or SoxRS-dependent control. After paraquat or H2O2 treatment, the deletion of yjcC reduced bacterial survival. These effects could be complemented by introducing the ΔyjcC mutant with the YjcC-expression plasmid pJR1. The recombinant protein containing only the YjcC-EAL domain exhibited phosphodiesterase (PDE) activity; overexpression of yjcC has lower levels of cyclic di-GMP. The yjcC deletion mutant also exhibited increased reactive oxygen species (ROS) formation, oxidation damage, and oxidative stress scavenging activity. In addition, the yjcC deletion reduced capsular polysaccharide production in the bacteria, but increased the LD50 in mice, biofilm formation, and type 3 fimbriation major pilin MrkA production. Finally, a comparative transcriptome analysis showed 34 upregulated and 29 downregulated genes with the increased production of YjcC. The activated gene products include glutaredoxin I, thioredoxin, heat shock proteins, chaperone, and MrkHl, and proteins for energy metabolism (transporters, cell surface structure, and transcriptional regulation). In conclusion, the results of this study suggest that YjcC positively regulates the oxidative stress response and mouse virulence but negatively affects the biofilm formation and type 3 fimbriation expression by altering the c-di-GMP levels after receiving oxidative stress signaling inputs.

Introduction

During infection, pathogens protect themselves from the oxidative burst of phagocytic cells and the challenging oxidative environments within cellular and extracellular compartments. Upon exposure to oxidative stress such as tellurite, paraparquat or hydrogen peroxide, E. coli exhibits an increase in the intracellular ROS and the content of protein carbonyls groups [1–3]. Reactive oxygen species (ROS), including superoxide anion (O2−), hydrogen peroxide (H2O2), and hydroxyl radicals (HO•), may damage DNA, proteins, and cell membranes and often lead to cell death [4,5]. The bacterial defense mechanism includes sensing, avoiding, and removing the ROS [6]. In general, SodA, SodB, and SodC remove superoxide, whereas catalases (KatE and KatG) and peroxidases (AhpC and GST) remove hydrogen peroxide [7,8]. These various stress defenses are controlled by regulators that respond to superoxide and redox-cycling drugs (e.g., SoxRS), hydrogen peroxide (e.g., OxyR), iron (e.g., Fur), or oxygen tension (e.g., FNR and ArcA) [8–11]. Diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) regulate the levels of bacterial second messenger cyclic di-GMP (c-di-GMP) by catalyzing molecular synthesis and hydrolysis, respectively [12,13]. The regulatory roles of c-di-GMP appear in numerous bacteria in various cellular functions, including cell surface remodeling [14], cellulose synthesis [15], virulence [16], motility [17], and biofilm formation [18–20]. E. coli YfgF, which exhibits PDE activity, regulates not only surface cell remodeling but also the oxidative stress response by modulating c-di-GMP levels [11]. The disruption of Salmonella enterica Var. typhimurium cdgR, which encodes a PDE protein, also decreases bacterial resistance to hydrogen peroxide and accelerates death by macrophages [21]. Klebsiella pneumoniae pyogenic liver abscess isolates often carry heavy capsular polysaccharides (CPS) to avoid phagocytosis or death by serum factors [22,23]. This thick and viscous structure also helps regulate the bacterial colonization and biofilm formation at the infection site [24]. Several regulators, such as RcsB, RmpA, RmpA2, KvhR, KvgA, and KvhA, help control the CPS biosynthesis by regulating the cpx transcription in K. pneumoniae [25,26]. An increase in CPS synthesis protects K. pneumoniae from oxidative stress [27–29]. However, whether the modulation of c-di-GMP affects CPS synthesis remains unclear.

The expression of yjcC, an IVE gene isolated from the liver abscess isolate K. pneumoniae CG43, is inducible in the presence of 10 μM paraparquat [30]. Sequence analysis of YjcC shows a signal
peptide followed by 2 transmembrane domains and a CSS motif at the N-terminal region, whereas the C-terminal contains a conserved EAL domain of the PDE enzyme [31]. In addition, the encoding gene \( yjcC \) is cluster-located with \( soxRS \) genes, suggesting that it plays a role in the oxidative stress response. This study investigates whether \( yjcC \) plays a role in oxidative stress defenses and if \( yjcC \) uses PDE activity to execute its regulation.

### Results

#### The \( yjcC \) expression is paraquat inducible, and \( soxRS \) and \( RpoS \) dependent

To confirm the previously reported paraquat-induced expression phenotype [30], the IVN DNA containing the 5′ non-coding region and part of the coding sequence of \( yjcC \) was isolated from \( K. pneumoniae \) CG43S3 and cloned in front of the promoterless lacZ gene of \( p\text{LaCZ15} \) [26]. The resulting plasmid was called \( p\text{P}_{\text{lacZ15}} \).

The sequence analysis of \( p\text{P}_{\text{lacZ15}} \) shows a conserved Fur box \( TGTTGA-N_2-TCACA \) [32] centered approximately 400-bp upstream of the \( yjcC \) start codon. This process also generated recombinant plasmids \( p\text{P}_{\text{ lacZ15}} \) and \( p\text{P}_{\text{ lacZ15}} \) carrying truncated forms of \( yjcC \). These plasmids respectively removed the putative Fur box and the small stem-loop sequence of the 33-bp coding region (Fig. 1A). As Fig. 1B shows, the bacteria containing \( p\text{P}_{\text{ lacZ15}} \) exhibited the highest level of \( \beta \)-galactosidase activity, whereas \( CG43S3[p\text{P}_{\text{ lacZ15}}] \) had the lowest activity. In addition, the activity of \( p\text{P}_{\text{ lacZ15}} \), but not \( p\text{P}_{\text{ lacZ15}} \) nor \( p\text{P}_{\text{ lacZ15}} \) increased after added 10 \( \mu \)M paraquat to the culture medium. This paraquat-induced characteristic also appeared when the concentration increased to 30 \( \mu \)M, further enhancing the activity of \( p\text{P}_{\text{ lacZ15}} \).

As Fig. 1C shows, the addition 30 \( \mu \)M paraquat to the bacterial culture significantly increased the \( yjcC \) mRNA level. Compared to the expression of the well-characterized stress response regulators \( soxS \), \( soxR \), \( RpoS \), and \( Fur \), the \( yjcC \) gene expression was more responsive to paraquat than to hydrogen peroxide exposure. This study also investigates whether \( yjcC \) is subjected to regulation by \( soxRS \) or \( RpoS \). As Fig. 1D shows, the deletion of \( soxR \), \( soxS \), or \( rpoS \) reduces the \( yjcC \) expression, implying that \( soxRS \) and \( RpoS \) play a positive role in the oxidative stress response.

#### \( yjcC \) plays a positive role in the oxidative stress response

Paragout is a superoxide ion generator. Thus, the paraquat-inducible expression suggests that \( yjcC \) plays a role in the oxidative stress response. To investigate this possibility, an \( yjcC \) deletion mutant was generated through an allelic exchange strategy. As Fig. 2A shows, the \( yjcC \) deletion mutant was more sensitive to paraquat and hydrogen peroxide when compared to the wild type bacteria \( K. pneumoniae \) CG43S3. The deletion effect could be complemented by transforming the \( yjcC \) expression plasmid \( p\text{R}1 \) into the mutant. However, introducing the mutant \( p\text{R}2 \), which expresses the mutant form of \( yjcC \) with the conserved E residue of the EAL domain replaced by A or \( p\text{R}3 \) (which carries the coding region of the \( YjcC \) EAL domain), had no complementation effect. Neither of the two EAL-domain protein encoding plasmids \( p\text{mK}7 \) and \( p\text{mK}8 \), which carry PDE activity, could complement the \( yjcC \) deletion effect. These results suggest that the stress response is \( YjcC \) dependent and both the N-terminal signaling receiving region and the EAL domain of \( YjcC \) are required and specific for an oxidative stress response.

To determine if the \( YjcC \)-EAL domain exhibits PDE activity, the recombinant expression plasmid containing the DNA coding for the EAL domain of \( YjcC \) or the AAL coding region of \( p\text{R}2 \) was constructed and overexpressed in \( E. coli \), and the recombinant proteins were purified. Figure 2B shows that the purified EAL domain protein exhibits PDE activity towards pNpp. This activity is lower than the level of the recombinant \( M\text{R}k \) [33], but considerably higher than the activity of the recombinant protein AAL\(_{yjcC} \). As Fig. 2C shows, the c-di-GMP level of \( CG43S3\Delta yjcC[p\text{R}1] \) was significantly lower than those of \( CG43S3[p\text{R}1] \), \( CG43S3\Delta yjcC[p\text{R}2] \), or \( CG43S3\Delta yjcC[p\text{R}3] \). This suggests that \( YjcC \) in vivo functions as a PDE enzyme capable of reducing the intracellular c-di-GMP levels. The deletion of \( yjcC \) gene from \( CG43S3 \) increased the c-di-GMP amounts and the difference between the levels was much more apparent after the bacteria exposure to 30 \( \mu \)M paraquat (Fig. 2D). This also suggests that \( YjcC \) is able to degrade c-di-GMP and the catalytic activity could be enhanced by oxidative stress.

#### Deletion of \( yjcC \) places bacteria in an oxidative stress state

As Figs. 3A and 3B show, the deletion of \( yjcC \) after treatment of \( H_2O_2 \) or paraquat significantly raised the levels of the fluorescent probe H2DCFDA (used to monitor the formation of ROS) and carbonyl proteins. The introduction of \( p\text{R}1 \) into \( CG43S3\Delta yjcC \) mutant appeared to reduce the levels of ROS and the carbonyl proteins, showing that \( YjcC \) is involved in the removal of ROS or damaged molecules. Thus, this study also investigates the anti-oxidant activity of \( YjcC \). As Fig. 3C shows, the deletion of \( yjcC \) reduced the oxidant scavenging activity, as assessed by the absorbance change at 517 nm for the decolorization degree of the purple color, supporting the possibility that \( YjcC \) modulates anti-oxidant activity in a certain manner. Numerous studies have shown that \( Fur \) and \( RpoS \) affect and regulate numerous SODs and catalases [7,34–37]. Figure 3D shows that zymogel analysis and total activity measurement exhibit significant changes in the SOD or catalase activity after the deletion of \( fur \) or \( rpoS \). However, the deletion of \( yjcC \) has no apparent influence on SOD or catalase activity, suggesting that the \( YjcC \)-dependent anti-oxidant enzyme remains to be identified.

#### \( yjcC \) plays a regulatory role in the virulence, CPS production, biofilm formation, and type 3 fimbriae expression

\( YjcC \), previously identified as an IVN gene product, is likely involved in infection [30]. To investigate whether \( YjcC \) is a virulence factor for the bacteria to establish infection, a mouse peritonitis model was employed. As Table 1 shows, the LD50 to Balb/c mice increased approximately 10-fold after \( yjcC \) deletion; introducing \( \Delta yjcC \) with \( p\text{R}1 \), but not \( p\text{R}2 \) or \( p\text{R}3 \), could restore the LD50. This indicates that \( YjcC \) expression at a certain stage is required for mouse infection. It is interesting to note that the \( \Delta yjcC \) colony is smaller and less mucoid, as determined by a string test [25], than its parental strain on LB agar plate. Therefore, sedimentation analysis and glucuronic acid content measurement are carried out to determine the CPS production. As Fig. 4A shows the deletion of \( yjcC \) reduces CPS production. The CPS deficient phenotype can be fully complemented with the transformation of \( p\text{R}1 \) into the \( \Delta yjcC \) mutant. However, transforming the mutant with \( p\text{R}2 \) or \( p\text{R}3 \) partially restores glucuronic acid production.

The second messenger c-di-GMP plays an important role in bacterial biofilm formation [14-16]. Figure 4B shows that the biofilm formation activity of \( \Delta yjcC \) appears to increase compared to the parental strain, whereas that transformed with \( p\text{R}1 \) decreases biofilm formation. This can be attributed to the level changes of c-di-GMP (Fig. 2C), which indicates that the \( YjcC \)
Figure 1. The yjcC is paraquat inducible, and SoxRS and RpoS dependent
(A) The putative promoters respectively containing 525 bp (P_yjcC1), 385 bp (P_yjcC2) and 415 bp (P_yjcC0) of yjcC were isolated and cloned into the LacZ reporter plasmid placZ15 (22). (B) The recombinant plasmids placZ15, pP_yjcC1, pP_yjcC2 and pP_yjcC0 were then transformed to K. pneumoniae CG43Z01 and the β-galactosidase activities of the transformants grown to log-phase in LB broth were determined. The results are shown as an average of triplicate samples. Error bars indicate standard deviations. (C) Total RNA of K. pneumoniae CG43S3 was isolated after the bacteria were grown in 2 mM H2O2 or 30 μM of paraquat. Specific primer pairs used to detect the expression of soxR, soxS, rpoS, and yjcC are listed in Table S2. Relative fold expression was compared with the non-induced condition and determined by the 2^−ΔΔCt method (60). Error bars indicate standard deviation of the mean. Data are representative of three independent experiments, *, P<0.001.

YjcC Regulation of Oxidative Stress

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expression of pJR1 significantly reduces the c-di-GMP level, thus reducing biofilm forming activity. Moreover, type 3 fimbriae is a major determinant of biofilm formation in K. pneumoniae [38]. Therefore, this study also investigates the deletion effect on type 3 fimbriae expression. As Fig. 4C shows, the western blot hybridization with anti-MrkA antibody shows that the yjcC deletion also increased the major pilin MrkA production of type 3 fimbriae.

Effects of YjcC overexpression assessed using a transcriptome study

This study uses comparative transcriptome analysis between CG43S3[pJR415] and CG43S3[pJR1] to gain further insights into how YjcC executes its regulation. Analysis of the genome annotation of liver abscess isolate K. pneumoniae NTHU-K2044 [39] shows that the increased expression of yjcC significantly enhances the expression of 34 genes. As Table 2 shows, the YjcC-activated genes can be categorized into 12 functional groups. These include the oxidative stress response genes grxA, ybbN, dnaJ, pinB, and xfp1, which are involved in anti-oxidation [40,41] or DNA repair [41], the heat shock chaperone protein encoding genes hspB, hspA, htpG, and dnaK, which are generally induced in stress conditions [42], and the genes coding for chaperone ClpB and PspB to protect protein from aggregation and help maintain protein motive force (PMF) to counteract stress conditions [43,44]. Increasing the expression of yjcC also enhanced the expression of PilZ domain protein MrkH and the LuxR-type transcription factor MrkI. Conversely, 29 genes whose expressions were significantly repressed by the increase of YjcC expression include fimB, which is regulated by fur under limited oxygen and anaerobic conditions [45]. Other YjcC negatively affected genes are metabolite transporter genes and genes coding for permease and energy metabolism involved in the synthesis of amino acids (Table 3).

As assessed by qRT-PCR analysis, Fig. 5 shows that the mRNA level of mrkH and mrkI, respectively increased 2.87- and 3.24-fold in CG43S3[pJR1] compared to that of CG43S3[pRK415]. In contrast, the mrkI transcript levels dropped to approximately 1/3 of that of CG43S3[pRK415].

Discussion

In E. coli, all the described genes inducible by paraquat are a part of the SoxRS regulon [46–48]. The expression of yjcC is RpoS-dependent in E. coli [49]. Consistent with this finding, the yjcC expression in K. pneumoniae is affected by RpoS and by SoxRS at the transcriptional level. No SoxRS or RpoS binding box appears within the putative promoter sequence, suggesting the possibility of an indirect control. In E. coli, the FNR regulator controls the transition between aerobic and anaerobic growth at the transcriptional level [50]. The conserved fur binding box present in the upstream non-coding region of yjcC implies an FNR dependent control of YjcC expression. Thus, the yjcC regulation by FNR likely occurs in poorly oxygenated environments.

The K. pneumoniae NTHU K2044 genome contains 27 genes encoding GGDEF-, EAL-, and GGDEF-EAL-domain proteins of potential DGC and PDE enzyme activity [51]. The yjcC encoding gene is also identified as member of the protein family. The family regulation specificity is determined by the sensory domain of the DGC or PDE proteins. Figure 2A shows that the PDE expression plasmids pJMK and pMK22G, which contain the respective coding region with putative promoter, failed to complement yjcC deletion. This may be due to fimK and mrkJ genes are not induced in comparison to yjcC in the presence of paraquat. There is also the possibility that the N-terminal peptide of approximately 300 aa of YjcC plays a role in the oxidative stress response besides signal sensing. Various sensory domains can bind to small molecular signals, and through this connection, modulate the levels of c-di-GMP [52,53]. Although the signal for the CSS-motif remains unknown, we speculate that YjcC-mediated signaling sensing may occur in the periplasmic space because of the signal peptide and the transmembrane domain at the N-terminal region. The purified recombinant EAL domain of YjcC exhibits PDE activity. However, the amount of c-di-GMP in CG43S3[yjcC][pJR3] is approximately the same as that in CG43S3[yjcC][pRK415] and CG43S3[yjcC][pJR2], suggesting that the EAL domain has extremely low levels of PDE activity. The N terminal region of YjcC likely requires receiving some signal from outside, and the interaction of the sensory domain with signaling molecules activates the PDE activity. This is supported by Fig. 2D showing that the deletion of yjcC gene from CG43S3 raised the c-di-GMP levels and the influence was much more apparent after the bacteria exposure to 30 μM paraquat.

The deletion of yjcC decreases the CPS production and the virulence attenuation, indicating that YjcC is required to avoid the damage of oxidative stress. In bacteria, superoxide dismutase and catalase are common antioxidant enzymes that scavenge ROS from oxidative stress. The redox proteins (including GrxA and VhsN) are required for maintaining redox status in bacteria are also protect bacteria from oxidative stress [54,55]. The deletion of yjcC has no apparent effect on the SOD and catalase activity, but appears to increase the transcription of grxA and ybbN. This suggests that YjcC is involved in regulating the redox levels in bacteria after oxidative stress. The chaperone protein ClpB stabilizes protein and suppresses the protein aggregation induced by heat or other stresses [43,56]. The phage shock proteins, PspB and PspC, act as positive regulators to transduce stress signal(s) to PspA through protein–protein interaction, maintaining the proton-motive force under extracytoplasmic stress conditions [44]. Transcriptome analysis shows that an increase in YjcC induces the expression of several heat shock proteins and chaperones, suggesting that YjcC is involved in regulating anti-stress responses. The fumarase gene fumB, which is expressed under anaerobic cell growth conditions, is regulated by Fur and ArcA [57,58]. The result of yjcC expression reduced the fumB transcripts implies that YjcC is a component of the Fur and ArcA regulatory pathway.

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In *K. pneumoniae*, the second messenger c-di-GMP activates type 3 fimbriae expression through MrkHI activation [18]. However, the increased synthesis of *mrkH* and *mrkI* transcripts by the overexpression of *yjcC* remains unclear. We have found that *mrkHI* expression is barely detected under LB culture (unpublished observation). Under oxidative stress pressure, the N-terminal
region of YjcC may turn on the expression of MrkHI. Thus, the N-terminal region likely plays a determinant role in YjcC-dependent regulation. E. coli YdeH has c-di-GMP cyclase activity [59]. The transcriptome analysis as shown in Table S3 revealed that the transcript levels of ydeH, ydeK, and ydeI in CG43S[pRK415-ydeH], with c-di-GMP level of 23.1 fmole/mg−1 (data not shown), significantly increased compared to those of CG43S3[pJR1]. This also indicates that ydeHI expression is c-di-GMP level-dependent and the N-terminal part of YjcC plays a positive regulatory role in the expression of ydeH.

Overall, these results indicate that the YjcC-mediated regulatory system is considerably more complex than expected. During infection, the transition from aerobic to microaerobic conditions or the transition from a microaerobic to oxidative stress environment, YjcC may be activated through sensory regulatory systems on the N-terminal region. Thereafter, YjcC modulates the levels of c-di-GMP to affect the expression of the downstream regulatory pathways. In conclusion, YjcC regulates the oxidative stress response, mouse virulence, CPS synthesis, biofilm formation, and type 3 fimbriae expression. This most likely occurs through the adjustment of c-di-GMP levels after receiving outside signals.

Materials and Methods

Ethics Statement

All animal experiments were performed in strict accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals of the National Laboratory Animal Center (Taiwan), and the protocol was approved by the Animal Experimental Center of National Chiao Tung University (Permit number: 009990006). All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

Plasmids, bacterial strain, and growth conditions

Table S1 presents the bacterial strains and plasmids used in this study. E. coli and K. pneumoniae CG43 [25,26] and its derivatives were propagated at 37°C in Luria-Bertani (LB) broth. The antibiotics used include ampicillin (100 μg/mL), chloramphenicol (35 μg/mL), kanamycin (25 μg/mL), streptomycin (500 μg/mL), and tetracycline (12.5 μg/mL). Table S2 presents the primers used in this study.

Measurement of promoter activity

The promoter reporter plasmids were individually mobilized from E.coli S17-1α pK to K. pneumoniae strains by conjugation. The β-galactosidase activity was measured as described previously [26]. The bacteria were grown to the log phase in the LB medium (OD600 of 0.6–0.7) and 100 μL of the culture was mixed with 900 μL of Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, and 50 mM β-mercaptoethanol), 17 μL of 0.1% sodium dodecyl sulfate (SDS), and 35 μL of chloroform, followed by vigorous shaking. After incubation at 30°C for 10 min, 200 μL of a 4-mg/mL concentration of o-nitrophenyl-β-D galactopyranoside (ONPG; Sigma-Aldrich, Milwaukee, WI) was added to the mixture to initiate the reaction. When yellow coloration appeared, the reaction was stopped by adding 500 μL of 1 M Na2CO3 to the mixture. The absorbance at OD420 was recorded, and the activity was expressed as Miller units. Each sample was assayed in triplicate, and at least 3 independent experiments were conducted. The data shown were calculated from one representative experiment, and are presented as the means and standard deviations from triplicate samples.

Real-time PCR analysis

Total RNA was isolated from bacteria using High Pure RNA isolation Kit (Roche, Germany), and the residual DNA was eliminated with RNase-free DNase I (Roche, Germany). The cDNAs used for PCR were synthesized from 1.5 μg RNA using random hexamer primer from RevertAid™ H Minus First strand cDNA synthesis Kit (Fermentas, Canada). An ABI Prism 7000 Detection system was used to perform PCR following the manufacturer instructions, and the products were detected using SYBR Green RCR Master Mix (Roche, Germany). The RNA samples were normalized to the level of 23S rRNA. PCR analysis was performed in triplicate in a reaction volume of 25 μL containing 12.5 μL of SYBR Green RCR Master Mix, 300 nM of primer pairs, 9.5 μL of distilled H2O, and 1 μL of cDNA. Samples were heated for 10 min at 95°C and amplified for 40 cycles for 15 s at 95°C and 60 s at 60°C. Quantification was performed using the 2−ΔΔCt method [60].

Construction of the gene deletion mutants and complementation plasmids

Specific gene deletion was introduced into K. pneumoniae CG43 using an allelic-exchange strategy as previously described [26]. Two DNA fragments of approximately 1000-bp flanking both sides of the deleted region were cloned into pKAS46 [25]. The plasmid pKAS46 is a suicide vector containing pKD46, which allows positive selection with streptomycin for vector loss. The resulting plasmids were mobilized from E.coli S17-1α pK to K. pneumoniae CG43S3 or CG43S3Δmac by conjugation, respectively. The transconjugants, with the plasmid integrated into the chromosome...
### Table 2. Significantly upregulated genes by \textit{yjcC} overexpression.

| Proposed function                                      | Gene name | Fold\(^a\) expression | ORF\(^b\) ID  |
|--------------------------------------------------------|-----------|------------------------|---------------|
| **Oxidative response/repair/sos responsive**           | GrxA      | 2.6                    | KP1\_1843     |
|                                                        | YbbN      | 2.4                    | KP1\_1349     |
|                                                        | Dnl       | 2.1                    | KP1\_2061     |
| **Heat shock response/chaperones/protein modification**| IbpB      | 5.9                    | KP1\_5467     |
|                                                        | Ibpa      | 5.6                    | KP1\_2622     |
|                                                        | HtpG      | 2.9                    | KP1\_1331     |
|                                                        | DnaK      | 3.5                    | KP1\_0835     |
|                                                        | ClpB      | 3.2                    | KP1\_4170     |
| **Phage shock protein B**                              | PspB      | 2.1                    | KP1\_2344     |
| **Cl-di GMP metabolite**                               | YjcC      | 6.3                    | KP1\_0324     |
|                                                        | MrkH      | 3.2                    | KP1\_4551     |
| **Energy/intermediary metabolism**                     | CarA      | 3.2                    | KP1\_0853     |
|                                                        | Ascorbate specific PTS family enzyme | 3.1 | KP1\_2793     |
|                                                        | D-arabinitol dehydrogenase | 2.9 | KP1\_3760     |
|                                                        | Allulose-6-phosphate 3-epimerase | 2.3 | KP1\_2791     |
|                                                        | Xylulokinase | 2   | KP1\_3759     |
| **Transporter**                                        | YdjN      | 2                      | KP1\_2272     |
| **Amino acid biosynthesis**                            | CysD      | 2.4                    | KP1\_4384     |
| **Nucleotide biosynthesis and metabolism**             | MsrA      | 2.5                    | KP1\_0489     |
| **DNA replication/recombination/repair**               | PriB      | 2.1                    | KP1\_0469     |
|                                                        | StpA      | 2.6                    | KP1\_4260     |
| **Cell surface structures**                            | YcgZ      | 2.6                    | KP1\_1728     |
|                                                        | PspD      | 2.3                    | KP1\_0998     |
| **Regulators**                                         | NhaR      | 2.1                    | KP1\_0838     |
|                                                        | Mrl      | 2.6                    | KP1\_4552     |
|                                                        | Hha       | 2.6                    | KP1\_1317     |
|                                                        | PspC      | 2.1                    | KP1\_2344     |
| **Hypothetical proteins**                              | PspD      | 3.3                    | KP1\_2152     |
|                                                        | Formylglycine generating sulfatase | 2.6 | KP1\_3378     |
|                                                        | Membrane anchored protein | 2    | KP1\_2896     |

\(^a\)Log\(_2\) fold change represents the log\(_2\) ratio of mRNA transcript levels of CG43S3[pJR1] to CG43S3[pRK415].

\(^b\)Open reading frame (ORF) ID is as annotated from \textit{K. pneumoniae} NTUH K2044.

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through homologous recombination, were selected with ampicillin and kanamycin on M9 agar plates. Several of the colonies were grown overnight in LB broth at 37°C and then spread onto an LB agar plate containing 500 mg of streptomycin/mL. Streptomycin-resistant and kanamycin-sensitive colonies were selected, and the deletion was verified by PCR. Table S1 presents the resulting mutant strains. To obtain the complementation plasmids, DNA fragments containing the yjcC and soxRS loci were PCR amplified using the primer pairs pjr1-F/pjr1-R, pjr2-F/pjr2-R, and pjr3-F/pjr3-R (Table S2). The PCR products were cloned in pRK415 to generate pJR1(pRK415-yjcC), pJR2(pRK415-yjcC-AAL), pJR3(pRK415-EAL domain only of yjcC), pmrkJ, and pfimK respectively (Table S1).

Site-directed mutagenesis

Site-directed mutagenesis was performed on the plasmid pJR1 to substitute the critical residue E with A in the EAL domain of YjcC using a QuickChange site-directed mutagenesis kit and following the manufacturer protocols (Stratagene). The resulting PCR product contained one point mutation, corresponding to the E303-to-A303 change in the active EAL site. The resulting PCR

| Proposed function | Gene name | Fold expression | ORF ID |
|-------------------|-----------|-----------------|--------|
| Anaerobic response protein | fumB | −5.7 | KP1_2562 |
| Transporter | mglC | −4.8 | KP1_0277 |
| Putative ABC transport system component | −4.6 | KP1_3175 |
| Molybdate ABC transporter system | −4.4 | KP1_3995 |
| Maltose/maltodextrin transporter ATP binding protein | −4.2 | KP1_0276 |
| Sugar ABC transport system permease component | −4.1 | KP1_1424 |
| Putative ABC transporter | −3.7 | KP1_1423 |
| Putative rhiizopine uptake ABC transporter | −3.7 | KP1_1422 |
| Permease | −6.5 | KP1_0760 |
| Putative PTS permease | −4.3 | KP1_1204 |
| Amino acid biosynthesis | −4.5 | KP1_2499 |
| Acetylornithine transaminase | −5.0 | KP1_2498 |
| Putative glutamine synthetase | −3.3 | KP1_2006 |
| Energy/intermediary metabolism | −6.4 | KP1_2563 |
| Succinate antipporter | −5.3 | KP1_2502 |
| Glucose-6-phosphate dehydrogenase | −6.0 | KP1_0764 |
| NADH: flavin oxidoreductase | −5.8 | KP1_2565 |
| Phospho-beta glucosidase | −5.2 | KP1_3803 |
| Acety-CoA synthetase | −4.3 | KP1_0342 |
| Succinylglutamate desuccinylase | −4.0 | KP1_2256 |
| Phosphomannomutase | −3.4 | KP1_3702 |
| Putative monoxygenase subunit | −3.1 | KP1_1996 |
| Putative acid phosphatase | −3.1 | KP1_3725 |
| Regulators | −4.5 | KP1_5297 |
| DNA binding transcriptional repressor | −4.8 | KP1_0277 |
| Cell surface structures | | | |

*Log2 fold change represents the log2 ratio of mRNA transcript levels of CG43S3[pJR1] to CG43S3[pRK415].

bOpen reading frame (ORF) ID is as annotated from K. pneumoniae NTUH K2044.

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Condensation with PEG 20000. The purity was determined by chromatography using His-Bind resin (Novagen). The purified proteins were then dialyzed against 1 TBS (Tris-buffered saline; pH 7.4) containing 10% glycerol at 4°C overnight, followed by condensation with PEG 20000. The purity was determined by SDS-PAGE.

Phosphodiesterase activity assay

The recombinant plasmids pET30b-EAL, pET30b-AAL, and pET30b-mrkJ and pET30b-mrkI were transformed into E. coli BL21(DE3), and the protein induced expression using 0.5 mM IPTG at 22°C for 12 h. The PDE activity was determined using the synthetic chromogenic substrate bis-p-nitrophenyl phosphate (bis-pNPP) (Sigma-Aldrich) as previously described [62,63]. The specific PDE activity was determined using purified proteins and by measuring the release of p-nitrophenol (pNP) at 405 nm. The calculations in this study use an extinction coefficient of 1.78*104/M*cm for p-nitrophenol. Control BSA without extracts was included to account for any non-enzymatic bis-pNP hydrolysis and MrkJ, which carries PDE activity as a positive control [33].

In vivo ci-di GMP content

To measure the ci-di-GMP contents, cellular extracts were prepared as described [64]. The cultured bacteria were collected and treated with formaldehyde (0.19% final concentration) and then pelleted by centrifugation. The pellet was suspended in water and heated to 95°C for 10 min before the nucleotides were extracted by 65% ethanol. The lyophilized samples were then resuspended in water and this suspension was used for c-di-GMP detection with a cyclic diguanylate ELISA kit (Wuhan EIAab Science co., Ltd). The ci-di GMP activity of crude extracts (1 mg of total protein/mL) containing WT-pRK415 vector only, pJR1, and pJR2 in ΔyjcC was also assayed as described above.

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 13000 g, washed with 10 mM potassium phosphate (pH 7.0) buffer (pH 7.0) buffer (Buffer A), and then suspended in 500 μL of the same buffer, which contained 10 μM 2',7'-dihydrodichlorofluorescein diacetate (H2DCFDA). After shaking for defined periods in darkness at room temperature, cells were centrifuged as mentioned 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 13000 g, aliquots of 100 μL supernatants were used to determine fluorescence intensity (excitation 490 nm and emission 519 nm) as described [1,2].

Oxidation of cytoplasmic proteins

Bacteria was grown to an OD600 of 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 and then disrupted by sonication. After centrifugation at 13000 g, 4 aliquots 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 13000 g for 5 min. The precipitate was washed 3 times with a mixture of ethanol:ethyl acetate (1:1). Finally, the sediment was dissolved in 450 μL of 6M guanidine hydrochloride/dithiothreitol and carbonyl concentration was determined spectrophotometrically at 370 nm (ε = 22000 M⁻¹ cm⁻¹) [2,3].

Evaluation of antioxidant activity with DPPH assay

DPPH radical scavenging activity was estimated using the method of Liyanage-Pathirana and Adeedapo [65,66]. When DPPH accepts an electron donated by an antioxidant compound, the free radical is decolorized from a stable purple color to yellow which

Figure 5. qRT-PCR analysis of the expression of mrkA, mrkH, and mrkI. Total RNA of K. pneumoniae CG43S3[pRK415] and CG43S3[pJR1] were isolated after the bacteria were grown overnight in LB supplemented with 12.5 μg/mL tetracycline. Specific primer pairs used to detect the expression of mrkA, mrkH, and mrkI are listed in Table S2. Relative fold expression was compared with the non-induced condition and determined by the 2-uDD method (60). Error bars indicate standard deviation of the mean. Data are representative of three independent experiments. doi:10.1371/journal.pone.0066740.g005

Product was digested with BamH I and HindIII and ligated into BamH I/HindIII-digested plasmid pRK415-pJR2.

Paraquat and H2O2 survival assessment

One hundred microliter of bacteria grown overnight were inoculated in LB and incubated at 37°C to OD600 of 0.6–0.7. An aliquot of the bacteria was collected by centrifugation and then resuspended in 10 mM of paraquat and 10 mM H2O2, respectively, and then subjected to 37°C incubation for 35 min. The colony-forming unit (CFU) of the bacteria was counted after the stress treatment, and the survival rate was determined by the CFU ratio. This study presents the representative data of at least 3 independent experiments. Every sample was assayed in triplicate, and this study presents the average activity and standard deviation.

Cloning, expression, and purification of the recombinant proteins

The coding regions of the EAL or AAL domains of yjcC were PCR amplified with the primer sets yEAL-F/yEAL-R and yAAL-F/yAAL-R (Table S2) and cloned into the NdeI/XhoI site in pET30b (Novagen, Madison, WI). This process generated pET30b-EAL or pET30b-AAL with a carboxyl-terminus His tag (RcsB-His6). The resulting plasmids pEAL (pET30b-EAL of yjcC) and pAAL (pET30b-AAL of yjcC) were individually transformed into E. coli BL21(DE3)/pLysS (Invitrogen), and the overproduction of the recombinant protein was induced by adding 0.5 mM IPTG for 4 h at 37°C. The recombinant proteins were then purified from the soluble fraction of the total cell lysate by affinity chromatography using His-Bind resin (Novagen). The purified proteins were then dialyzed against 1 TBS (Tris-buffered saline; pH 7.4) containing 10% glycerol at 4°C overnight, followed by condensation with PEG 20000. The purity was determined by SDS-PAGE.
can be quantitatively measured from the changes in absorbance. 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 with a spectrophotometer at 517 nm, with ascorbic acid and BHT as references. The scavenging ability to remove DPPH radicals was calculated using the following equation: DPPH radical scavenging activity (%$=\frac{(\text{Abs control}-\text{Abs sample})}{\text{Abs control}}\times100$, where Abs control is the absorbance of DPPH radical+methanol, and Abs sample is the absorbance of DPPH radical+sample extract/standard.

**Determination of SOD and catalase enzyme activity**

Cell-free extracts were harvested (15000 rpm, 4°C, 20 min) from the exponential phased bacteria (OD600=0.7–0.8) and suspended in ice-cold potassium phosphate buffer (50 mM, pH 7). After cells were disrupted by ultra-sonication, cell debris was removed by 12000 rpm centrifugation for 10 min at 4°C, and the supernatant was collected. The extraction of total proteins was conducted on ice and the concentrations were estimated using the Bradford method [67]. Aliquots of the extracted proteins were individually loaded onto 10% native polyacrylamide gels and the proteins were separated at a constant voltage of 150 V for 2 h. The gels were then removed and stained for SOD and CAT activity using the methods of Beauchamp and Fridovich [68] and Woodbury et al. [69], respectively. The SODs were localized by soaking gels in 2.45 mM nitro blue tetrazolium for 20 min, followed by immersion in a solution of 50 mM phosphate buffer (pH 7.0), 0.028 mM riboflavin, and 0.028 M TEMED ($\text{N},\text{N},\text{N}$-,tetrachloroethyleneenediamine). The gels were then removed from the solution and exposed to light for approximately 20 min. The SOD activity produced achrornatic zones in the 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 transferring the gels to a solution of 1% ferric chloride and 1% potassium ferricyanide for 10 min. The localized-CAT produced colorless bands on a dark green background.

The SOD activity was determined using a spectrophotometer at 25°C using the xanthine oxidase-cytochrome C method [68]. The assay mixture in 0.7 mL contained 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 50 mM xanthine, 1.7 mM xanthine oxidase, and 10 mM cytochrome C. The reduction of cytochrome C was measured at A550. One unit (U) of activity was defined as the amount of enzyme that catalyses the oxidation of 1 mmol H2O2 min$^{-1}$ under assay conditions.

**Mouse lethality assay**

The bacterial virulence in mice was determined as described [25]. Female BALB/c mice (aged 4 to 5 wk) were obtained from the National Laboratory Animal Center and acclimatized in an animal house for 7 d. The tested bacterial strains were cultured overnight in LB medium at 37°C. Four mice in each group were injected intraperitoneally with 0.2 mL of bacterial suspension in saline in 10-fold graded doses. The LD50 values were calculated using the Reed and Muench method [70] on the number of survivors after 14 d.

**Biofilm formation assay**

The ability of bacteria to form biofilm was analyzed as described, with a minor modification. Bacteria were diluted 1/100 in LB broth supplemented with the appropriate antibiotics, and this dilution was inoculated into each well of a 96-well microtitre 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 to stain the attached cells for 30 min. Unattached dye was rinsed by washing 3 times with distilled water, and the stained biomass was solubilized in 1% (w/v) SDS. The absorbance was determined at 595 nm, and relative bacterial biofilm-forming activities were observed.

**Western blot analysis**

*K. pneumoniae* CG43S3 and its derived mutants were grown in LB broth with agitation at 37°C. The bacterial total protein, approximately 3 μg per lane, was then subjected to western blot analysis using MrkA antiserum. Aliquots of total cellular lysates were resolved by SDS-PAGE, and the proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After incubation with 5% skim milk at room temperature for 1 h, the membrane was washed 3 times with 1× PBS. The membrane was subsequently incubated with diluted anti-GAPDH (GeneTex Inc.) and anti-MrkA serum at room temperature for 1 h. After 3 washes with 1× PBS, a 5000-fold diluted alkaline phosphatase-conjugated anti-rabbit immunoglobulin G was added and incubated for 1 h. The blot was washed again and the bound antibodies were detected using the chromogenic reagents BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (Nitro blue tetrazolium).

**Transcriptome analysis**

Total RNA from CG43S3[pJR1] and CG43S3[pRK415] were isolated using Trizol reagent (Invitrogen), subsequently purified using RNAeasy MinElute Cleanup Kit (Qiagen), and eluted in RNase-free water. The RNA samples were sequenced on Illumina’s sequencing instrument. In the data analysis pipeline, we used the FASTX-Toolkit to remove or trim deep sequencing reads containing low quality bases or adaptor sequences. To estimate expression levels of genes, all the remaining reads were mapped to the *Klebsiella pneumoniae* NTUH-K2044 genome [39] using TopHat and determined using Cufflinks package. The genes were identified as significantly transcript abundance changed in CG43S3[pJR1] as compared to that in CG43S3[pRK415] if the log2 fold change was greater than 2 (up and down). Finally, functional annotation tools, such as DAVID,
can be used to illustrate the biological regulation role from Gene Ontology or KEGG pathway database.

Supporting Information

Table S1 Strains and plasmids used in this study.

Table S2 Oligonucleotide primers used in this study.

References

1. Acuna LG, Calderon IL, Elias AO, Castro ME, Vasquez CC (2009) Expression of the yggE gene protects Escherichia coli from potassium tellurite-generated oxidative stress. Arch Microbiol 191: 473–476.
2. Perez JM, Arenas FA, Pradosa GA, Sandoval JM, Vasquez CC (2008) Escherichia coli YqHDC exhibits aldehyde reductase activity and protects from the harmful effect of lipid peroxidation-derived aldehydes. J Biol Chem 283: 7346–7353.
3. Perez JM, Calderon IL, Arenas FA, Fuentes DE, Pradosa GA, et al. (2007) Bacterial toxicity of potassium tellurite: unveiling an ancient enigma. PLoS One 2: e211.
4. Imlay JA (2003) Oxidative stress and mechanisms of protection against it in bacteria. Biochemistry (Moscow) 68: 476–489.
5. Niederhoffer EG, Naranjan CM, Bradley KL, Fee JA (1999) Control of Escherichia coli superoxide dismutase (sodA and sodB) genes by the ferric uptake regulation (fur) locus. J Bacteriol 171: 1930–1938.
6. Furuba S, Koga T (1991) Oxidative stress responses in Escherichia coli and Salmonella typhimurium. Microbiol Rev 55: 561–585.
7. Semchyshyn H, Banakhukova T, Storey K, Lushchak V (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: 988–992.
8. Zuchelli M, Doan B, Schneider TD, Storr G (1999) OxyR and SoxRS regulation of fur. J Bacteriol 181: 4639–4643.
9. Chattopadhyay S, Wu Y, Dutta P (1997) Involvement of Fur and ArcA in anaerobic expression of the tdc operon of Escherichia coli. J Bacteriol 179: 4069–4073.
10. Romling U, Gomelsky M, Galperin MY, et al. (2005) C-di-GMP: the dawning of a novel bacterial signalling system. Mol Microbiol 60: 1026–1043.
11. Wilksch JJ, Yang J, Clerenws A, Gabbe JL, Short KR, et al. (2010) MkH, a novel c-di-GMP-dependent transcriptional activator, controls Klebsiella pneumoniae biofilm formation by regulating t3 dimarina expression. PLoS Pathog 6: e1000284.
12. Liu TN, Chien KH, Liu JH, Wang AH, Chou SH (2009) Xanthomonas campestris adopts a PIIZ domain-like structure without a c-di-GMP switch. Proteins 75: 202–208.
13. Krasna TV, Feng JC, Shih YJ, Sehgal S, Navarro MV, et al. (2010) Vhbo cholerae VpT regulates matrix production and motility by directly sensing cyclic di-GMP. Science 327: 866–868.
14. Hsien KB, Mao JH, Shih HJ, Lee YH, Wu GJ, et al. (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: 1234–1245.
15. Campos MA, Vargas MA, Reguero V, Llopart CM, Alberi S, et al. (2004) Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. J Immunol 72: 7107–7114.

Table S3 RNA sequencing analysis of expression of mrkA, mrkH and mrkJ.

Author Contributions

Conceived and designed the experiments: CJH HLP. Performed the experiments: CJH ZCW. Analyzed the data: CJH HLP. Contributed reagents/materials/analysis tools: ZCW HYH HDH. Wrote the paper: CJH HLP.

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47. Koh YS, Roe JH (1995) Isolation of a novel paraquat-inducible (pqi) gene regulated by the soxRS locus in Escherichia coli. J Bacteriol 177: 2673–2678.
48. Liochev SI, Hausladen A, Beyer WF Jr, Fridovich I (1994) NADPH: ferredoxin oxidoreductase acts as a paraquat diaphorase and is a member of the soxRS regulon. Proc Natl Acad Sci U S A 91: 1328–1331.
49. Sommerfeld N, Possling A, Becker G, Perausgen C, Tschesnuk N, et al. (2009) Gene expression patterns and differential input into curli fimbriae regulation of all GGDEF/EAL domain proteins in Escherichia coli. Microbiology 155: 1318–1331.
50. Tolla DA, Savageau MA (2010) Regulation of aerobic-to-anaerobic transitions by the FNR cycle in Escherichia coli. J Mol Biol 397: 893–903.
51. Cruz DP, Huertas MG, Lozano M, Zarate L, Zambrano MM (2012) Comparative analysis of diguanylate cyclase and phosphodiesterase genes in Klebsiella pneumoniae. BMC Microbiol 12: 139.
52. Zhulin IB, Nikolskaya AN, Galperin MY (2003) Common extracellular sensory domains in transmembrane receptors for diverse signal transduction pathways in bacteria and archaea. J Bacteriol 185: 285–294.
53. Huang B, Whitchurch CB, Mattick JS (2003) FimX, a multidomain protein connecting environmental signals to twitching motility in Pseudomonas aeruginosa. J Bacteriol 185: 7068–7076.
54. Meyer Y, Buchanan BB, Vignols F, Reichheld JP (2009) Thioredoxins and glutaredoxins: unifying elements in redox biology. Annu Rev Genet 43: 335–367.
55. Caldas T, Malki A, Kern R, Abdallah J, Richarme G (2006) The Escherichia coli thioredoxin homolog YbbN/Trxsc is a chaperone and a weak protein oxidoreductase. Biochem Biophys Res Commun 343: 780–786.
56. Thomas JG, Baneyx F (2000) ClpB and HtpG facilitate de novo protein folding in stressed Escherichia coli cells. Mol Microbiol 36: 1360–1370.
57. Tseng CP (1997) Regulation of fumarase (fumB) gene expression in response to oxygen, iron and heme availability: role of the arcA, fur, and hemA gene products. J Bacteriol 189: 4774–4781.
58. Cheng HY, Chen YS, Wu CY, Chang HY, Lai YC, et al. (2010) RmpA regulation of capsular polysaccharide biosynthesis in Klebsiella pneumoniae CG43. J Bacteriol 192: 3144–3158.