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Characterization of DicB Inhibitory Activity in Cell Division under Stress Conditions

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Abstract

It was previously shown that DicB interacts with MinC and functions to inhibit cell division. Here in we created various N-terminal truncation constructs in which up to ~36 residues were removed but retained binding ability to MinC. We investigated the role of DicB in different physiological environments by monitoring the growth of *E. coli* in high salt environment and in the presence of an acylampicillin antibiotic (Azlocillin). Our results revealed that DicB helps *E. coli* to cope with the high salt stress by impeding cell division. Under alkaline conditions, the inhibitory effect of DicB/MinC-DicB on *E. coli* cell division was also enhanced. Taken together, our work has shed further lights on the function of DicB which has largely remained elusive.

Keywords: Prophage; DicB; Cell division inhibition; Stress; pH

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Introduction

A prophage is a DNA sequence that can be integrated into the host genome, usually in a non-infected state in the cell [1-3]. Many bacterial genomes contain phage DNA, which can help host cells survive in the changing environments [4]. However, it is noteworthy that phage-mediated transformation also increases the risk of bacterial virulence [5]. It was previously found that phages applied lysogeny (incorporation into the chromosome) was helpful in understanding the relationship between bacteria and ahage [6]. The fact that prophage DNA would induce a selective disadvantage to the host has been known for decades, yet much is still unknown about prophage in general. Studies have shown that prophages are inserted in the chromosome of the host which gradually decline and become inactive bacteriophage particle formation and cell lysis [7,8].

Cryptic prophage Kim (Qin) is one of the prophages resides in *E. coli* chromosome, *dicB* gene is a fragment of Qin [9,10]. It encodes a small protein containing only 62 amino acids. Under normal conditions, the expression of *dicB* is strongly restrained. Once DicB is expressed, cell division immediately ceases [11,12] and this division restraint is rely on MinC [9,13,14]. *minC* belongs to the Min system, including *minC, minD and minE*[15]. Min oscillation system is a regulator for cell division which involves MinC, MinD and MinE. The three proteins orchestrate to impel the Z ring formation in the mid cell, which is a polymer composed of FtsZ subunits and normally situated at the mid-site of cells undergoing division [16,17]. Nevertheless, without Min system, the Z ring can be formed in the polar regions of cells which leads unequal fission [18]. Under normal circumstances, both Min proteins and the Z ring are required to cooperate in *E. coli* cell division. MinC is a key protein that can cause cell division inhibition [19]. It has been known that either MinD or an apparently unrelated protein DicB, cooperates with MinC to regulate cell division. DicB can compete with MinD for binding to MinC. DicB,

like MinD, stimulates the disassembly of Z rings by MinC *in vivo* [14,20]. Studies suggest that DicB interacts directly with MinC and that the DicB/MinC complex functions as a repressor for the septal ring formation. In addition, it was found that DicB/MinC-mediated division inhibition does not require MinD and MinE [21,22]. However, it is unclear under what circumstances, MinC/DicB will affect the inhibition of cell division; such knowledge will not only help to understand the biochemical function of DicB but also help to comprehend the role of cryptic prophages.

In our previous studies, the intrinsic cytotoxicity of DicB hindered the study of its function but the difficulty was overcome by MBP-DicB fusion strategy which effectively reduced toxicity. Taking advantage of the successful expression of MBP-DicB fusion protein which renders DicB cellular studies possible, herein we further investigated DicB and MinC interactions using different DicB constructs and found that the first 36 amino acids of the N-terminus of DicB is responsible for DicB's interaction with MinC. In addition, we examined the morphologies of the cells, the results of which support the observation that MinC is essential for DicB's function *in vivo*. More importantly, we studied the regulatory function of DicB in various stress environments including probing the growth of *E. coli* in high salt and in the presence of Azlocillin. An increased inhibitory effect of DicB/MinC-DicB on *E. coli* cell division was also shown under alkaline conditions.

Materials and Methods

Strains and plasmids

All the strains and plasmids used in this study are listed in the supplementary information **(Table 1)**. Bacterial TOP10 cell was used for plasmids construction, amplification and permanent maintenance. BL21 (RP) cell was employed for protein expression, pull-down assay, RT-PCR and environment stress experiments. Strains of *E. coli* BW25113 (wild-type), MinC-KO (knock out) and DicB-KO were used for the scanning electron microscopy (SEM) experiments and stress experiments.

Pull-down assay

Protein expression and purification are described previously [23]. MBP-DicB or its truncations and MinC were co-lysed by sonication in lysis buffer (20 mM Tris-HCl, 0.2 M NaCl, 1 mM DTT, 1 mM EDTA, pH 7.4) and centrifugation. After the protein was incubated with maltose binding protein beads (amylose resin), 10 mM maltose was added to lysis buffer to elute the protein. Eluted samples were evaluated using SDS-PAGE and visualized by CBB staining. For western blotting, protein samples were transferred onto PVDF membrane and then probed with a mouse monoclonal anti-His-HRP-conjugated antibody (Tianjin Sungene Biotech). Band intensity was measured using software Image J.

Scanning electron microscopy

To study bacterial morphology induced by Minc and DicB, scanning electron microscopy (SEM) sample preparation was carried out as previously described [23]. The SEM images of cells were captured by SU8010 Scanning Electron Microscope (Hitachi). More than 50 cells from each sample were randomly selected for statistical analysis of cell length by Graphpad Prism 6 (Graphpad Software, Inc.).

Table 1 Strains and plasmids used in this study.

Strain or Plasmid	Genotype	Source, reference or Construction
Strains		
BL21-RP	F^- ompT hsdS (rb^-mb^-)dcm$^+$Tetr gal λ (DE3) endA The [argU proL Cam^r]	TIANGEN Biotech (Beijing)Co.,Ltd.
BW25113	F-, Δ (araD-araB)567, ΔlacZ4787 (::rrnB-3), rph-1, Δ (rhaD-rhaB)568, hsdR514	Keio Collection
BW25113ΔMinc	F-, Δ (araD-araB)567, ΔlacZ4787 (::rrnB-3), λ-, ΔminC765::kan, rph-1, Δ (rhaD-rhaB)568, hsdR514	Keio Collection
BW25113ΔDicB	F-, Δ (araD-araB)567, ΔlacZ4787 (::rrnB-3), λ-, ΔDicB::kan, rph-1, Δ (rhaD-rhaB)568, hsdR514	Keio Collection
Plasmids		
pET-22b-MinC	C-His, Ampicillin	This study
pBAD33-DicB	C-His, Chloramphenicol	This study
pET-28b-MBP-DicB	C-His, Kanamycin	(25)
pET-28b-GFP-DicB	C-His, Kanamycin	This study
pET-22b-RFP-MinC	C-His, Ampicillin	This study
pET-28b-MBP-DicB-derivative	C-His, Kanamycin	This study

Stress experiments

Strains were cultured overnight in Luria-Bertani (LB) at 37°C and 0.5 mM IPTG or 1 mM arabinose were added to cultures of each strain at 16°C and incubated for 20 hours. All strains were diluted to the same OD_{600} and constantly serial diluted (100:1). After that, the diluted strains were respectively cultured on LB plates (pH 4.5-10.5, 0.5 each interval) to determine the cfu/ml. Results are shown as mean values of at least three separate samples.

RNA isolation and real-time PCR

The BL21 (RP) strain was grown in LB until OD_{600} reached 0.8; 0.5 mL strain was added to 5 mL fresh LB (pH 5.0, 6.0, 7.0, 7.5, 8.0, 9.0, 10.0) and harvested post-incubation for 12 h at 37°C. Total RNA was extracted from 5 mL cells using RNAprep pure Cell/Bacteria Kit (TIANGEN). For each sample 20 µL standardized total RNA (as estimated from A_{260}) was used as template and reversely transcribed into cDNA using a BeyoRT™ II First Strand cDNA Synthesis Kit (Beyotime Biotechnology). For the RT-PCR experiment, gene expression of *dicB* and *minC* under different stress conditions was assessed using BeyoFast™ SYBR Green qPCR Mix (2X, Low ROX, Beyotime Biotechnology) and a 7500 real-time instrument (Applied Biosystems). PCR conditions were 95°C/30 seconds, 95°C/5 seconds and 60°C/34 seconds for 40 cycles. Relative quantification was performed using the comparative threshold cycle (CT) method [24]. The 16S rRNA gene was used as

the reference for comparison with the genes of interest. Primers specific to individual *dicB*, *minC* or 16S rRNA gene are listed in **Table 2**. The relative quantification changes in transcript levels and standard deviations were calculated as the means of the results of three independent experiments.

3D Fluorescence confocal microscope

DicB or MinC was fused to the C-terminus of fluorescent protein GFP or RFP respectively, as shown in **Table 3**. The vector containing the fusion fluorescent protein was transformed into the BL21 (RP) *E. coli* strains individually or in combination. In the case of co-transformation, the vectors have different antibiotic resistance. 100 µL of the transformed strains was taken and coated on a solid LB plate medium containing the corresponding antibiotics and cultured overnight at 37°C. Monoclonal colonies were picked from the above plates and inoculated into 5 mL of LB liquid medium containing antibiotics (0.1%) and then cultured at 37°C for 10-12 hours. Bacterial liquid was diluted to the same OD_{600} in different media at 150 rpm and cultured at 37°C for ~10 hours. The cells were induced with IPTG at a final concentration of 0.5 mM and cultured with shaking at 37°C for 4 hours. LB medium containing 2% (0.02 g/mL) agarose was dropped on a microscope slide to prepare a thin plate agarose medium membrane. 5 µL of the above bacterial strains was dropped on a thin plate agar medium and covered with a microscope observation sample coverslip before being observed using a 3D fluorescence confocal microscope.

Table 2 Primers used in RT-PCR.

Oligonucleotides	Sequence (5'-3')
minC For	ATCACTCAGCCAGTATTCACC
minC Rev	CAGCGTATTTATGCTCCACA
dicB For	GGTGTCACTATCAGTAACCCAG
dicB Rev	CGTAAACGAGCCAGCATT
16S RTFor	ATGGCTCAGATTGAACGC
16S RTRev	GGCAGTTTCCCAGACATTAC

Table 3 Primers used in this study.

Primer	Sequence	Enzyme cutting site
MinC-5F-Nde1	GGAATTCCATATGTCAAACACGCCAATCG	*Nde1*
MinC-3R-Xho1	CCGCTCGAGATTTAACGGTTGAACGGTCAAA	*Xho1*
DicB-5F-Nde1	GGAATTCCATATGAAAACGTTATTACCAAACGTTAA	*Nde1*
DicB-3R-Xho1	CCGCTCGAGTTGTGTACATCCTTTTGGCATC	*Xho1*
MBP-DicB-5F-Nde1	GGAATTCCATATGATGAAAATCGAAGAAGGTAA	*Nde1*
MBP-DicB-3R-Xho1	CCGCTCGAGTTGTGTACATCCTTT	*Xho1*
DicB-ΔN30-5F-Nde1	GGAATTCCATATGATTAACAAGAGAAAACAAG	*Nde1*
DicB-ΔN30-3R-Xho1	CCGCTCGAGTTGTGTACATCCTTTTGGCATC	*Xho1*
DicB-ΔN33-5F-Nde1	GGAATTCCATATGAGAAAACAAGAACGG	*Nde1*
DicB-ΔN33-3R-Xho1	CCGCTCGAGCTTGTTAATGGCATCTTCAG	*Xho1*
DicB-ΔN34-5F-Nde1	GGAATTCCATATGAAACAAGAACGGGAG	*Nde1*
DicB-ΔN34-3R-Xho1	CCGCTCGAGTCTCTTGTTAATGGCATCTTCA	*Xho1*
DicB-ΔN35-5F-Nde1	GGAATTCCATATGCAAGAACGGGAG	*Nde1*
DicB-ΔN35-3R-Xho1	CCGCTCGAGTTGTGTACATCCTTTTGGCATC	*Xho1*
DicB-ΔN36-5F-Nde1	GGAATTCCATATGGAACGGGAGCTATTA	*Nde1*
DicB-ΔN36-3R-Xho1	CCGCTCGAGTTGTGTACATCCTTTTGGCATC	*Xho1*
DicB-N36-5F-Nde1	GGAATTCCATATGAAAACGTTATTACCAAACGTTAA	*Nde1*
DicB-N36-3R-Xho1	CCGCTCGAGTTGTTTTCTCTTGTTAATGG	*Xho1*

Cell growth conditions

Strains were cultured overnight in Luria-Bertani (LB) at 37°C then diluted to the same OD_{600}. IPTG was added to induce the protein expression with a 0.5 mM final concentration. After the cultures were shaken at 37°C on 96-well plates for 12 hours, cell growth was evaluated by OD_{600} hourly. All samples were prepared in triplicate.

Results and Discussion

Pull-down experiments *invitro*

Previous studies have shown that an unspecified N-terminal region of DicB interacts with MinC [23]. However, the precise fragment is unknown. In order to reveal the exact sequences responsible for the interaction, we constructed a series of N-terminal truncation derivatives of MBP-DicB (ΔN30, ΔN32, ΔN34, ΔN36, ΔN37, ΔN38; number signifies the removed residues at DicB's N-terminus) for pull-down experiments **(Figure 1A)**. We used these constructs in pull-down experiment to assess DicB's binding affinity to MinC using both CBB staining and Western blotting. Most deletion constructs were able to pull down MinC, although smaller amounts than the full-length DicB. In the SDS CBB staining gel, compared with MBP-DicB, it appears that ΔN34 and ΔN36 still weakly interacts with MinC **(Figure 1A)**. In the Western blotting, except for ΔN37 and ΔN38 all other deletion derivatives seem to weakly bind to MinC **(Figure 1B)**. We estimated the band intensity of the gel **(Figure 1A)** and calculated the band intensity ratio. The intensities of MBP-DicB, MBP-DicBΔN30, MBP-DicBΔN32, MBP-DicBΔN34 and MBP-DicBΔN36 are 2.58, 2.74.3.14, 3.44, 3.64 folds of corresponding MinC respectively. These results indicate the weakened binding of the DicB truncation mutants with MinC. Previously, we showed that ΔN6, ΔN7, ΔN13, ΔN26 and N26 could all pull down MinC to varying degrees[23]. Taken together, it appears that most residues in the N-terminal half of DicB collectively contribute to MinC binding, instead of a few specific residues or shorter fragments. On the base of the predicted secondary structure of DicB **(Figure S3)**, we believe that the random coil and β-sheet at the N-terminal half of DicB are involved in the binding to MinC.

MinC is essential for DicB's function

We used BW25113 strains to investigate the role of MinC in the presence of DicB in cell division. We transformed MBP-DicB into BW25113 and MinC-KO cells, respectively. When MBP-DicB was introduced into BW25113, the cell body length was longer than 22 µm, while the length of the wild-type cells was ~3 µm **(Figures 2A and 2B)**. However, when MBP-DicB was transformed into MinC-KO, the cell morphology of the transformant was not affected with cell length similar to MinC-KO **(Figures 2C and 2D)**. Comparing the cell morphology of BW25113 (MBP-DicB) with BW25113-MinC KO (MBP-DicB), only in the presence of *minC*, DicB resulted in the elongation of the cell body. As expected, MinC complementation was able to restore the cell morphology

(Figure S1). In the control experiments, MBP alone did not affect *E. coli* morphology **(Figure S1)**. By observing the morphological changes of *E. coli* through SEM **(Figure 2)**, we demonstrated that DicB cannot function alone and MinC is obligatory for DicB's function. Since MinC is a cell division inhibitor, abnormal division could occur when MinC is not present in *E. coli*, leading to abnormal cell morphology in MinC-KO cells **(Figures 2B and 2D)**.

DicB increases resistance to Azlocillin and helps cope with high salt

DicB enhances the inhibition of MinC on FtsZ polymerization, however, it is not known under what conditions DicB would be triggered to activate MinC. To investigate this problem, we carried out a series of stress experiments. Interestingly, there are preliminary evidences indicating that DicB could help *E. coli* survival in Azlocillin (an acylampicillin antibiotic) and high salt environments [6]. To quantify these effects, we further studied the cell growth behavior in the presence of Azlocillin and salt of different concentrations using BW25113 strains. Our results **(Figure 3)** show that the *dicB* knockout (DicB-KO) cells grew slightly slower than BW25113 (wild type). In the presence of Azlocillin, pBAD33-DicB was transformed into DicB-KO, compared with the control (without pBAD33-DicB plasmid)，with the DicB induction by arabinose the strains grew significantly faster, in

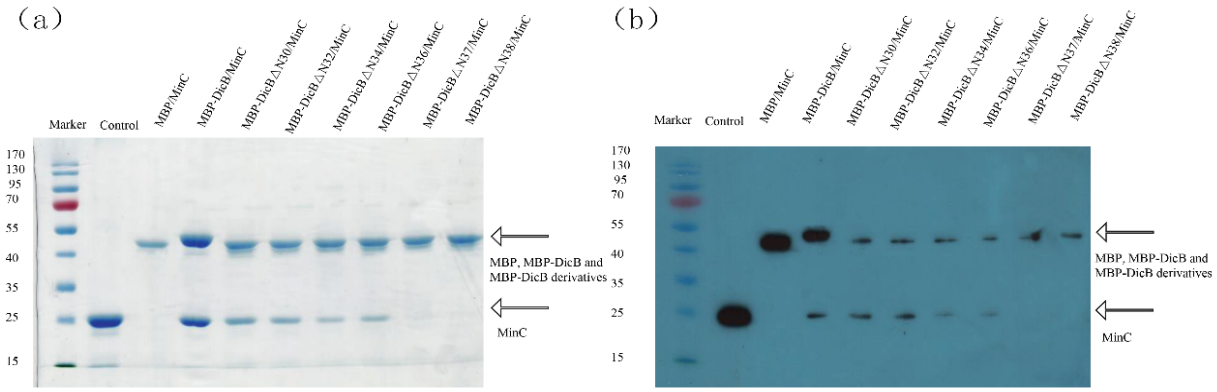

Figure 1 Pull-down experiments between MBP-DicB (with His tag) N-terminal truncation derivatives and MinC (with His tag) using SDS-PAGE and Western blotting. (a) SDS-PAGE of MBP-DicB N-terminal truncation derivatives and MinC purified by MBP column. (b) Western blotting (anti-His-tag) of MBP-DicB N-terminal truncation derivatives and MinC. The control experiment used MinC which was purified by nickel column, the MBP/MinC sample confirmed that MinC has no nonspecific binding to amylose column.

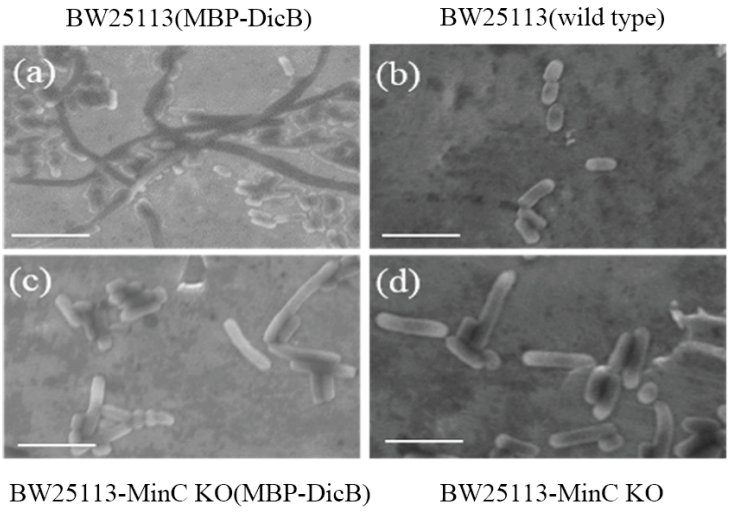

Figure 2 Scanning electron micrographs (SEM) of DicB expression in BW25113. (a) BW25113 (MBP-DicB); (b) BW25113; (c) BW25113-MinC KO (MBP-DicB); (d) BW25113-MinC KO. Cells of BW25113 (MBP-DicB) exhibited much longer cell morphology than BW25113 (wild type) while cell morphology of BW25113-MinC KO (MBP-DicB) was similar to BW25113-MinC KO, demonstrating that the effects of DicB on cell division is dependent on the existence of MinC. Scale bars, 5 μm.

fact even higher than BW25113 (wild type). With the increase of Azlocillin concentration, the ability with which that DicB could help *E. coli* cope with this stress environment became more obvious **(Figure 3A, 3B and 3C)**. Likely due to high concentrations of the antibiotic and long growth time, BW25113 (wild type) had a bacteriolytic phenomenon and in the end of the experiment, the bacteria density decreased slightly. Under high salt stress, although the growth was similar in the first 8 hours, after which OD_{600} of BW25113-DicB KO (DicB) rapidly increased **(Figure 3D)**. Taken together, DicB appears to help the survival of *E. coli* in high salt and increase resistance to Azlocillin, thus suggesting the prophage can help bacteria overcome certain extreme surroundings.

DicB and pH stress

To further probe stress response, we explored whether DicB would influence cell survival under different pH conditions **(Figure 4)**. We transformed MinC, MBP-DicB, MBP and MinC/MBP-DicB into BL21 (RP) (wild type) respectively, with 0.5 mM IPTG induction. The colonies on LB plates under different pH conditions were counted. As expected, when MinC, MBP-DicB and MinC/MBP-DicB were transformed into RP cells, the number of clones decreased significantly by about four orders of magnitude **(Figure 4A)**. In the case of MinC expression, *E. coli* cells could survive between pH 4.5-9.0, compared with the survival of WT between pH 4.5-10.5 **(Figures 4A and 4B)**. When DicB was expressed, the survival pH of cells was between 4.5-10, similarly to WT. Nonetheless, once pH exceeded 7, the number of clones of DicB began to decrease **(Figure 4A)**. When MinC/MBP-DicB were co-transformed into BL21 (RP), the survival range narrowed to pH 4.5-8.5. Similarly, once pH went beyond 7, the number of clones reduced sharply **(Figure 4A)**. As a control, MBP was transformed into BL21 (RP) which showed no participation of MBP in the inhibition of the cell division of *E. coli* **(Figure 4B)**. When MinC and DicB was co-expressed in BL21 (RP), the number of colonies on the LB plate drastically reduced compared with that of the individual MinC or DicB expression **(Figure 4A)**, which indicates that DicB inhibits the cell division of *E. coli* in alkaline ambience. We thus suggest that DicB can enhance cell division inhibition of MinC in alkaline environment. Our results of real-time fluorescent quantitative experiments (RT-PCR) show that the RNA expression of MinC and DicB in the alkaline environment was higher than in the acidic environment **(Figure S3)**.

3D Fluorescence confocal microscopic studies of MinC and DicB

To further assess the distribution of MinC and DicB in *E. coli*, we varied the LB medium conditions of cell culture, including NaCl concentrations of 400 mM and 600 mM, pH of 5.0, 6.0 and 8.0 (the normal LB medium contains ~170 mM NaCl, with pH 7.0). After incubating the cells in the various media aforementioned and normal LB (normal), we used 3D fluorescence confocal microscopy to observe the distribution. Green fluorescent protein (GFP) and red fluorescent protein (RFP) were fused with DicB and MinC respectively, the constructs of which are shown in **Table 3**. The results show that GFP-DicB was distributed throughout the cell and a small amount aggregated on the cell membrane **(Figure 5 A1-F1)**, whereas RFP-MinC distributed discontinuously in the cell, much like the tiger's tail **(Figure 5 A2-F2)**. In comparison, in GFP-DicB and RFP-MinC co-expression cells

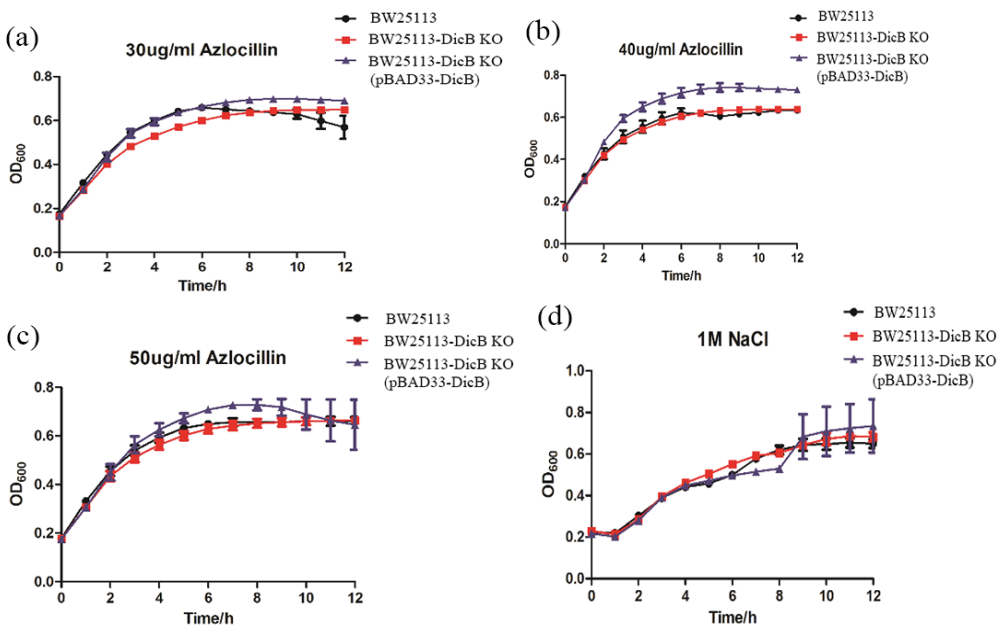

Figure 3 DicB influences BW25113 cell growth in the presence of Azlocillin or NaCl. BW25113 (wild type) (black), BW25113-DicB KO (red), BW25113-DicB KO(pBAD-DicB) (blue). (a) 30 μg·ml^{-1} Azlocillin; (b) 40 μg·ml^{-1}Azlocillin; (c) 50 μg·ml^{-1} Azlocillin; (d) 1 M NaCl. All experiments were completed in triplicate and performed at three times. Error bars represent standard error of the mean.

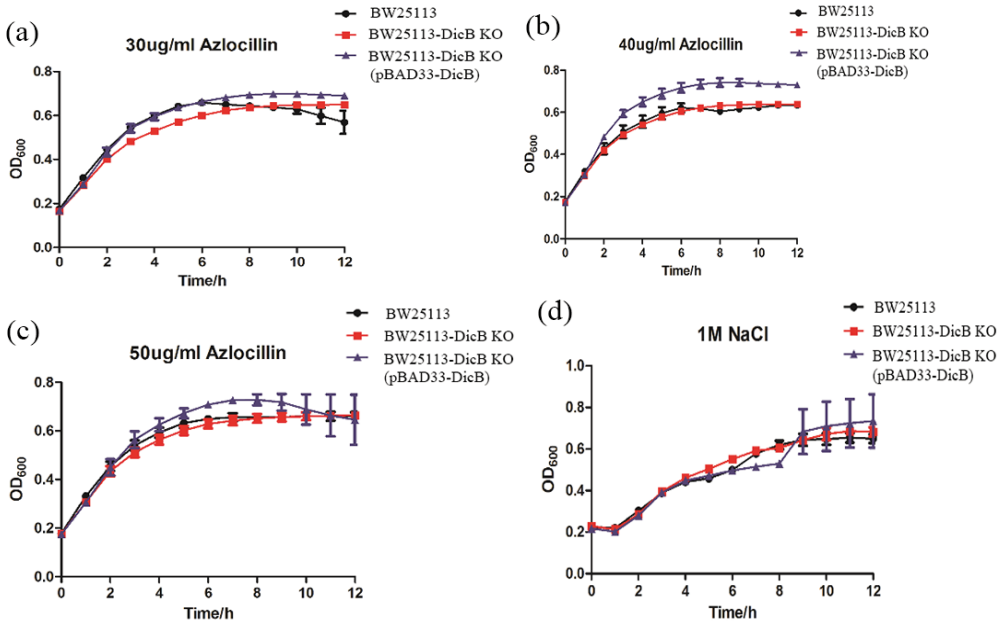

Figure 4 DicB's function in the cell growth under different pH. (a) MBP-DicB (black), MinC (blue), MinC/MBP-DicB (red); (b) MBP (black), as a control, BL21 (RP) (wild type) (red). All experiments were completed in triplicates and performed at three times. Error bars represent standard error of the mean.

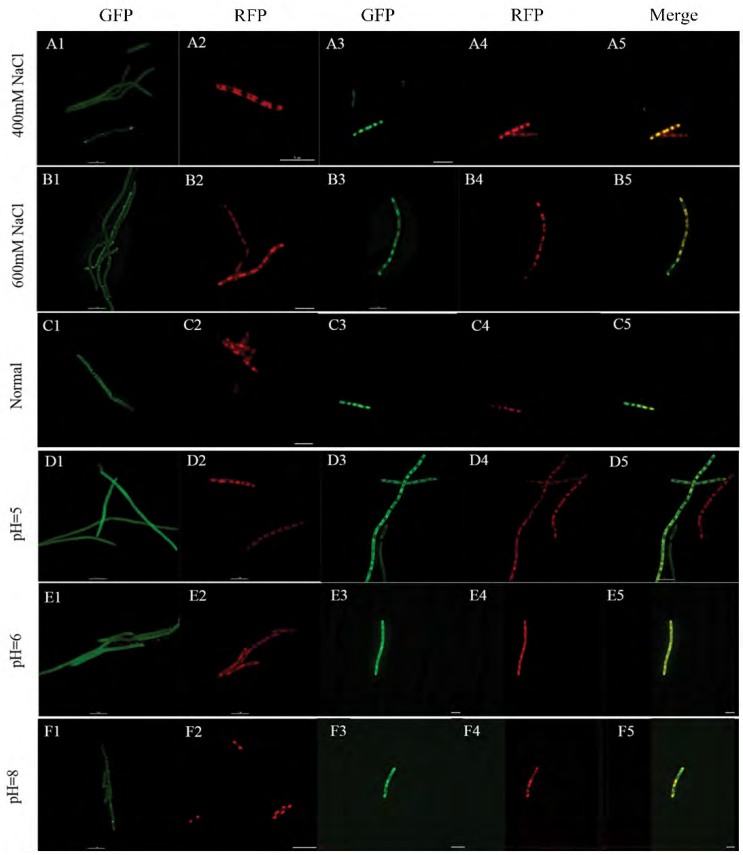

Figure 5 3D Fluorescence confocal microscope of MinC and DicB. (A1-F1) GFP-DicB, whole cell distribution; (A2-F2) RFP-MinC, interval distribution, spaced like a tiger's tail; (A3-F3) Co-expression of GFP-DicB and RFP-MinC (A3-F5), GFP-DicB and RFP-MinC overlap colocalization (yellow) and the distribution change of GFP-DicB induced by RFP-MinC. Scale bars, 5 μm.

(Figure 5 A3-F3), RFP-MinC would induce an interval distribution pattern of GFP-DicB in the cell, indicating that MinC seems to dictate the distribution of DicB. Varying salt and pH conditions did not change the distribution pattern.

Conclusion

As part of host physiology, functions of cryptic prophage genes have recently gained attentions, especially under the conditions where the cells encounter stresses such as high salt, acid and alkali. DicB is such a prophage gene, although its function is still elusive. Through a series of stress experiments, we have observed that DicB can help *E. coli* to better survive stress conditions in the face of high salt and antibiotic assaults. Under alkaline conditions, DicB promotes the inhibitory activity of MinC in cell division. OWe have also revealed that the entire N-terminal half of DicB participates in MinC binding. In summary, our work has provided further evidence to elucidate DicB's regulatory role, along with MinC, in cell division inhibition.

Author Contributions

J. M. Zheng and Z. C. Jia conceived and supervised the study, S.N. Han and Z.F. Lu performed experiments and wrote the manuscript, S. Y. Yang, Y. Y. Xu, Q. Y. Wang, H. R. Pei and Q. Z. Yang conceived the study.

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