The MinCDE Cell Division System Participates in the Regulation of Type III Secretion System (T3SS) Genes, Bacterial Virulence, and Motility in Xanthomonas oryzae pv. oryzae

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Abstract: Xanthomonas oryzae pv. oryzae (Xoo) causes bacterial leaf blight (BLB) in rice, which is one of the most severe bacterial diseases in rice in some Asian countries. The type III secretion system (T3SS) of Xoo encoded by the hypersensitive response and pathogenicity (hrp) genes is essential for its pathogenicity in host rice. Here, we identified the Min system (MinC, MinD, and MinE), a negative regulatory system for bacterial cell division encoded by minC, minD, and minE genes, which is involved in negative regulation of hrp genes (hrpB1 and hrpF) in Xoo. We found that the deletion of minC, minD, and minCDE resulted in enhanced hrpB1 and hrpF expression, which is dependent on two key hrp regulators HrpG and HrpX. The minC, minD, and minCDE mutants exhibited elongated cell lengths, and the classic Min system-defective cell morphology including minicells and short filamentations. Mutation of minC in Xoo resulted in significantly impaired virulence in host rice, swimming motility, and enhanced biofilm formation. Our transcriptome profiling also indicated some virulence genes were differentially expressed in the minC mutants. To our knowledge, this is the first report about the Min system participating in the regulation of T3SS expression. It sheds light on the understanding of Xoo virulence mechanisms.

Keywords: Xanthomonas oryzae pv. oryzae; MinCDE system; type III secretion system; virulence; motility

1. Introduction

Xanthomonas is a genus of Gram-negative bacteria that includes numerous species that cause disease in over 400 different plant hosts, including rice, citrus, wheat, cabbage, tomato, cassava, and pepper [1]. Xanthomonas oryzae pv. oryzae (Xoo) is widespread in Southern China, West Africa, and Southeast Asian countries such as Thailand and Vietnam [2]. The phytopathogenic Xoo infects rice, causing bacterial leaf blight (BLB), which induces worldwide output losses of up to 50% [3]. Xoo produces a variety of virulence factors, including lipopolysaccharides (LPS), exopolysaccharides (EPS), extracellular enzymes, toxins, adhesions, and effectors injected into host rice by the type III secretion system (T3SS), and so on [4]. The Xoo T3SS that controls the pathogenicity in susceptible host rice is encoded by a hypersensitive response and pathogenicity (hrp) gene cluster, which contains 27 genes including 10 hrp, 9 hrc (hrp conserved), and 8 hpa (hrp-associated) genes [5].

The expression of hrp genes of Xoo is significantly stimulated in planta, or in minimum medium (XOM3), an artificial hrp-inducing medium, but inhibited in the nutrient-rich medium [6]. The expression of Xoo hrp genes was regulated by two key regulators, HrpG and HrpX. HrpG belongs to the OmpR-family response regulator of two-component regulatory systems. It has a response receiver (RR) domain at the N-terminus and a DNA-binding
motif at the C-terminus [7]. HrpX binds directly to the plant-inducible promoter (PIP) box consensus motif (TTCGC-N15-TTCGC), a cis-regulatory region [8]. HrpG acts as a positive regulator of hrpX expression and is also a crucial regulator in some Xanthomonas species or pathovars. In X. campesstris pv. campesstris (Xcc), HpaS has been demonstrated to act as a sensor kinase for HrpG; however, there is no intact homolog of the hpaS gene of Xcc in the genomes of Xoo strains [9]. In Xcc 8004, the sensor kinase RpfC can regulate hrpX and T3SS genes expression in the nutrient broth and the host environment via the DSF cell–cell communication system [10]. The global transcriptional regulator Clp has been reported to bind to the promoter regions of downstream targets zur, cellulases engXCA, and flrR, hence promoting the production of virulence-associated genes [11]. In X. citri subsp. citri (Xcci) [12], Lon, an ATP-dependent protease, can degrade HrpG protein in the rich medium; however, Lon was phosphorylated and lost its inhibitory impact on HrpG in host plants [13]. Lon inhibits the expression of T3SS and flagellar synthesis and participates in cell division and exopolysaccharide formation [13]. In Xoo, several components, including GntR-family regulator Trh [14], and the two-component systems PhoP/PhoQ [15], have been associated with hrg expression. Moreover, KdgR, a negative regulator of hrg, has been reported to directly bind to the promoter regions of hrg, thereby repressing the transcription of hrg genes [16]. The other upstream regulators of T3SS in Xoo remain unknown and need further investigation.

How a cell finds its middle has been studied for the last 50 years in Escherichia coli [17]. Two negative regulatory systems for cell division have been identified in E. coli. One is the nucleoid occlusion (NO) system that prevents Z-ring formation over the nucleoid, and the other is the Min system encoded by minC, minD, and minE genes, which inhibits the formation of Z-ring at the poles [18]. A current model suggests that the concentration gradient of MinC in a cell regulates the Z-ring position [19]. MinC is an inhibitor of FtsZ and can directly interact with FtsZ, thereby inhibiting its polymerization [20–22]. MinD is an ATPase that can bind and recruit MinC to the membrane [23]. MinE can stimulate the ATPase activity of MinD, and thus detach it from the membrane [24]. As MinE assembles at mid-cell, and cycles back and forth toward the cell poles, the dissociation of the MinC/MinD complex results in the oscillation behavior of Min proteins in cells [19,22,25]. This causes a concentration gradient of MinC/MinD complex to be highest at the cell poles and lowest at mid-cell, thus allowing Z-ring formation at mid-cell in a narrow zone [19]. The Min system-defective mutants share similar phenotypic characteristics: minicells and filamentous cells [17]. The focus of past studies on the role of the Min system was to characterize its oscillation and interaction with divisome-associated proteins.

Some current studies have suggested the involvement of the Min system in cellular processes such as bacterial motility, colonization, and virulence. The minC mutants of Proteus mirabilis and Helicobacter pylori significantly reduced swarming motility [26,27]. Neisseria gonorrhoeae (Ng) mutants without MinD or MinC exhibited decreased adherence to urothelial cells [28]. MinCD complex of E. coli can attach to the membrane and assist in segregating chromosomes [29]. The MinC oscillations from pole to pole also were observed in Xcci, and similar to Min system-defective mutants in E. coli, the Xcc minC mutant could form branching cells with aberrant extension and bulging at both poles [30]. A current finding identified MinD of Xoo as a host-induced protein required for Xoo full virulence in host rice [31], indicating that some mechanisms and pathways of Xanthomonas Min proteins that regulate virulence during infection are unknown.

In this study, we screened two transposon mutants, 8–24 and 24–46, with up-regulated expression of hpf and hprB1, using a Tn5 transposon mutagenesis, in which the transposon was inserted into the Xoo PXO99A minC and minD genes, respectively. The Xoo PXO99A Min system is composed of MinC, MinD, and MinE proteins that are encoded by the minC (PXO_04463), minD (PXO_04464), and minE (PXO_04465) genes, respectively. We revealed the link between the Min system and the T3SS expression of Xoo. We demonstrated the negative effects of the Min system on T3SS expression through the HrpG–HrpX regulatory pathway, and the involvement of the Min system in Xoo cell division, full virulence, swim-
ming motility, and biofilm formation. Our findings propose new indications that the Min system contributes to the virulence regulatory networks of Xoo.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids, and Growth Conditions

*Xanthomonas* wild-type *PXO99A* [32] and other Xoo strains were grown on nutrient-rich NA plates, or in NB medium and minimal XOM3 medium with appropriate antibiotics at 28 °C [6,33]. *E. coli* strains were cultured on LB agar (LA) plates or in Luria-Bertani (LB) medium with appropriate antibiotics at 37 °C. The following final concentrations of antibiotics were used: kanamycin (Km), 50 µg/mL; gentamicin (Gm), 25 µg/mL; spectinomycin (Sp), 100 µg/mL. The bacterial strains and plasmids employed in this study are listed in Table S1.

2.2. Construction of Mutant and Complementation Strains

All primers used in this study are listed in Table S2. We constructed the deletion mutant of the Min system (*minC, minD, and minCDE*) using the suicide vector pKMS1 with *sacB* gene by homologous recombination [34]. The specific primers amplified the upstream, and downstream sequences of *minC, minD, and minCDE* were ligated into the pKMS1 to create the pK-min-system construct. The plasmids were transformed into the *Xoo* wild-type *PXO99A* by electroporation, respectively. The colonies were followed by the selection on NA plates with 10% sucrose. The P∆*min*-system deletion mutants were selected by the sensitivity to Km on the NA medium. The transposon mutant strains 8–24 and 24–46 with highly *hrpF* and *hrpB1* expression were selected, respectively. For complementation of the P∆*min*-system, the fragments containing the *minC, minD, and minCDE* encoding regions were amplified using the primer pairs (Table S2) and cloned into pML123 to obtain the recombinant plasmids pML123-*minC*, pML123-*minD*, and pML123-*minCDE*, respectively. Electroporation of the recombinant plasmids was transformed into the insertion or deletion mutants to obtain the complemented strains CP∆*minC*, CP∆*minD*, and CP∆*minCDE*.

2.3. Synteny Analysis on Chromosomes

To determine whether Min system genes are conserved among *Xanthomonas* strains, we use the SyntTax bioinformatics tool with the ABSYNTE algorithm to perform the synthetic analysis (http://archaea.u-psud.fr/synttax/, accessed on 5 March 2022) [35], employing 10 *Xanthomonas* genomes as a reference, including *Xoo* PXO99A and PXO86, *X. oryzae* pv. *oryzicola* BLS256 and RS105, *X. axonopodis* pv. *commiphorae* LMG26789, *X. vasicola* NCPPB902, *X. citri* subsp. *citri* 49, 29–1, and 306, and *X. campestris* pv. *campestris* 8004.

2.4. Microscopy

Wild-type *PXO99A* and the Min mutant strains were grown on NA plates at 28 °C. Cells were washed twice with PBS and fixed to 1.5 mL tubes with 3% glutaraldehyde overnight at 4 °C. After removing the blocking buffer and washing twice with PBS, the cells were stationary for 1 h with 1% osmic acid at 4 °C. Then, the bacterial cells were dehydrated with ethyl alcohol concentration and placed into a drying oven overnight at 37 °C for CO₂ drying. The microstructures of cells were observed by using scanning electron microscopy (SEM). We utilized the ImageJ software to measure the lengths of the cells. For transmission electron microscope (TEM) analysis, the experiment was employed according to our previous protocol [36]. For fluorescence microscope observation, the *Xoo* strains carrying pHM1-*gfp* with a highly expressed GFP were analyzed according to our previous protocol [36].

2.5. Biofilm Formation Assay

Biofilm formation assay was determined as described previously [36]. *Xoo* strains were grown for 12 h in NB medium and diluted with 1:100 to overnight culture in NB medium. The bacterial cells were collected by centrifugation at 5000 rpm for 3 min. Then,
we adjusted the optical density (OD) from 600 nm to 2.0 and incubated 5 mL bacterial suspension in a test tube at 28 °C. Following three days of incubation, the supernatant was carefully removed, and the adhering bacterial cells were stained with 6 mL 0.1% crystal violet (CV) for 30 min. The CV-stained cells were washed twice with distilled water and dried in a 37 °C incubator to observe the depth of the purple circle formed on the glass tube. The stained cells were solubilized in 95% ethanol. The absorbance of samples at 590 nm was determined using the Spectramax (Molecular Devices, Sunnyvale, CA, USA).

2.6. Swimming Motility Assay
The swimming motility assay of Xoo strains was performed on semi-solid medium plates as previously described [36]. Xoo strains were grown in NB medium overnight at 28 °C, and the OD600 was adjusted to 0.3. Then, 2 µL Xoo bacteria were inoculated in the center of the 0.3% semi-solid medium plates (1 g/L yeast extract, 10 g/L sucrose, 5 g/L bacto peptones, and 3 g/L agar) by pipetting. Tested plates were incubated at 28 °C for 3 days. The diameter of circular zones was measured and evaluated.

2.7. Growth Measurement and Virulence Assay of Xoo
Xoo strains were cultivated in NB medium at 28 °C for 12 h and the OD600 was adjusted to 0.05. Then, the samples were inoculated into fresh NB medium for shaken culture at 28 °C for 14 h. The bacterial OD600 values were evaluated every 2 h. As previous studies described, pathogenicity investigations of Xoo were accomplished in the glasshouse at a temperature of 25–28 °C. Briefly, Xoo strains were cultured in NB medium overnight with appropriate antibiotics and collected by centrifugation. The collected cells were resuspended with distilled water and the OD600 was adjusted to 0.3. Bacterial suspensions were pressure-infiltrated into the leaves of susceptible rice IR24. The water-soaking regions caused by Xoo were quantified using ImageJ software 3 days after infiltration. The OD600 of the suspensions was adjusted to 0.6 and inoculated in the rice IR24 by the leaf-clipping method. Disease lesion lengths were observed to evaluate the virulence of Xoo strains 14 days after inoculation. There were three independent replications of these experiments.

2.8. RNA-Seq and Real-Time Quantitative RT-PCR (qRT-PCR) Analysis
The P∆minC, P∆minD, P∆minCDE, and PXO99A strains were re-suspended with XOM3 medium and shaken cultured at 28 °C for 12 h. Personalbio (Personalbio, Shanghai, China) evaluated RNA-seq on the Illumina Hiseq platform. The expression of Xoo genes was analyzed by qRT-PCR employing the ABI 7500 software and SYBR Green I Mix (TransGen, Beijing, China). cDNAs were amplified using the specific primers (Table S1). The Xoo rpoD and gyrB genes were used to normalize the qRT-PCR results, and the 2ΔΔCT method was used to calculate the gene expression, as previously described. The GOseq R package analyzed DEGs’ Gene Ontology (GO) enrichment. GO terms with a p-value of 0.05 were considered significantly enriched. As previously described, we analyzed the Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.9. Western Blotting Analysis
The protein expression vectors pH1-hrpG::FLAG, pH3-hrpX::FLAG, and pH3-hrpB1::FLAG were constructed in our previous study [6], then were electroporated into the Xoo PXO99A, P∆minC, P∆minD, and P∆minCDE, respectively. Overnight, Xoo strains were grown in NB medium at 28 °C and collected by centrifugation. Bacterial cells were rinsed with sterile
water and resuspended at an OD$_{600}$ of 2.0 in a type III-inducing XOM3 medium. These XOM3 suspensions were incubated in the shaken culture at 28 °C for 12 h. Protein samples were extracted from XOM3 suspension and separated by 10% SDS-PAGE. The proteins were then transferred to a PVDF membrane for immunoblotting using the Flag tag and anti-mouse IgG antibody (TransGen, Beijing, China). The membrane was visualized with the EasySee Western Kit (TransGen, Beijing, China). We used the E. coli RNA polymerase subunit (RNAP) antibody as the loading control.

2.10. GUS Assays

The promoter-probe vectors pHG2-$hrpG$, pHG2-$hrpX$, pHG2-$hrpF$, and pHG3-$hrpB1$ were constructed in our previous studies [6,33,36]. These plasmids were transferred into the mutants $P\Delta minC$, $P\Delta minD$, $P\Delta minCDE$, and PXO99A by electroporation. The reporter strains were grown in NA medium with appropriate antibiotics at 28 °C overnight. Then, the Xoo cells were collected and cultured in the XOM3 medium for $hrp$ induction. The β-glucuronidase (GUS) activities were detected and calculated as previously described [6].

2.11. Southern Blotting Analysis

The deletion mutants $P\Delta minC$, $P\Delta minD$, $P\Delta minCDE$, and transposon mutants were further confirmed by Southern blotting analysis according to our previous operation [36]. Total genomic DNA of the Min mutant strains was extracted using the Bacteria Genomic DNA Kit (TransGen, Beijing, China) as the manufacturer recommended. DNA samples of Xoo were digested aseptically for 6 h at 37 °C with the restriction enzyme BamHI (Takara Bio, Kusatsu, Japan). Separation and transfer to Hybond N+ nylon membrane using electrophoresis were carried out as described previously [36]. The digoxigenin (DIG)-labeled minC, minD, and minCDE probes and the DIG Easy hybridization buffer (Roche, Sweden) were used for membrane hybridization. The membrane was incubated by detection buffer (Roche) and detected using a digital camera.

2.12. Statistical Analysis

All experiments were replicated at least three times independently. The statistical software SPSS v24.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data, before proceeding with Duncan’s test.

3. Results

3.1. Min System Participates in Negative Regulation of T3SS Expression

To identify novel T3SS regulators in Xoo, we selected two representative $hrp$ genes, $hrpF$ and $hrpB1$, to construct the plasmid-borne reporters pHG2-$hrpF$ and pHG3-$hrpB1$, which contain the $hrpF$ and $hrpB1$ promoter-uidA transcriptional fusion, respectively. The $hrpF$ gene was speculated to encode a translocator that transports the T3SE proteins into the host cells [38,39]. The $hrpB1$ gene is the first one in the $hrpB$ operon of the Xoo $hrp$ cluster, the promoter region of which contains a PIP box region that has been demonstrated to bind and activate by HrpX [5]. We screened two mutant 8–24 and 24–46 from ten thousand Tn5 transposon mutants, and found that 8–24 with increased $hrpF$ promoter-driven GUS activity in XOM3, a $hrp$-inducing medium (Figure S1A), had a Tn5 transposon insertion at 541 bp position of the minC gene (Figure 1A), as well as 24–46 with enhanced $hrpB1$ promoter-driven GUS activity (Figure 1E), had a Tn5 transposon insertion at 411 bp position of the minD gene (Figure 1A). In Xoo, the minC and minD genes along with the minC gene are located in an operon, which is highly conserved in xanthomonads such as Xoo, Xcc and Xcci (Figure S2). The minCDE genes encode Min system proteins that have been demonstrated to prevent the formation of septa at cell poles by inhibiting the Z-ring, ensuring that bacterial cell division occurs in the middle of cell, not at cell poles in E. coli, Bacillus subtilis, and Pseudomonas aeruginosa [18,19,40]. Our PCR analysis, based on the cDNA and genomic DNA of the wild-type PXO99A, showed that minC, minD, and minE, with the other two genes, PXO_04462 and PXO_04466, are located in a transcription unit (operon) (Figure S3A).
PXO_04462 encodes a putative Gcn5-related N-acetyltransferase (GNAT)-family protein that includes a large number of members among eukaryotes and prokaryotes [41].

Figure 1. The Xoo Min system negatively regulates hrb1 expression. (A) Genomic location of Min system on the Xoo PXO99\(^{\Delta}\) chromosome. Arrows indicate the Tn5 transposon insertion sites in the nucleotides 541 of minC and the nucleotides 411 of minD, respectively. (B) The hrb1 promoter-driven GUS activity of Xoo wild-type PXO99\(^{A}\), 8–24, C8–24, P\(^{\Delta}\)minC, and CP\(^{\Delta}\)minC in XOM3 at 3 hr post-induction. (C) Expression ratios of hrb1 in 8–24, P\(^{\Delta}\)minC, and CP\(^{\Delta}\)minC compared to that in Xoo wild-type PXO99\(^{A}\) by qRT-PCR. (D) The abundance of HrpB1 proteins in PXO99\(^{A}\), 8–24, and P\(^{\Delta}\)minC by Western blotting. P\(^{\Delta}\)hrpG as a negative control of HrpB1 protein expression. (E) The hrb1 promoter-driven GUS activity of PXO99\(^{A}\), 24–46, P\(^{\Delta}\)minD, and P\(^{\Delta}\)minCDE in XOM3 at 3 hr post-induction. (F) Expression ratios of hrb1 in 24–46, P\(^{\Delta}\)minD, and P\(^{\Delta}\)minCDE compared to that in Xoo wild-type PXO99\(^{A}\) by qRT-PCR. (G) The abundance of HrpB1 proteins in PXO99\(^{A}\), 24–46, P\(^{\Delta}\)minD, and P\(^{\Delta}\)minCDE by Western blotting. P\(^{\Delta}\)hrpG as a negative control of HrpB1 protein expression. The total protein extracts were analyzed by Western blotting using anti-FLAG antibodies. RNAP, RNA polymerase subunit alpha from E. coli was used as a loading control. Relative protein abundance was calculated by ImageJ software. Similar results were observed in two independent experiments. As assessed by Duncan’s test, different letters indicate statistically significant differences, and the same letter displays no significant differences (p < 0.05) between Xoo strains.

To confirm the increase in hrb1 and hrf expression in the transposon mutants, we constructed the minC deletion mutant P\(^{\Delta}\)minC, the minD deletion mutant P\(^{\Delta}\)minD, and the triple mutant P\(^{\Delta}\)minCDE containing the deletion of minC, minD, and minE in the background of Xoo wild-type PXO99\(^{A}\) using the SacB-based markerless knockout technique. The Southern blotting was performed in the mutant strains to prove the deletions.
Similar to the transposon mutant 8–24, PΔminC exhibited a significant increase in GUS activity of hrpB1 and hrpF promoters, hrpB1 mRNA levels, and HrpB1 protein expression levels compared with the wild-type PXO99\textsuperscript{A} in XOM3 (Figures 1B–D and S1A). The enhanced hrpB1 and hrpF expression of PΔminC could be fully restored to the wild-type levels in CPAminC, a complementary strain of PΔminC, which carries a functional minC gene expressed by its native promoter in a low copy number plasmid pML123 (Figures 1B,C and S1A). However, the expression of minC in trans in 8–24 could partially restore hrpB1 expression to wild-type levels (Figure 1B). Similarly, like the transposon mutant 24–46, an increase in hrpB1 and hrpF promoter-driven GUS activity, hrpB1 mRNA levels, and HrpB1 protein expression levels was observed in PΔminD and PΔminCDE compared with that in the wild-type PXO99\textsuperscript{A} (Figures 1E–G and S1B). These results indicate that the Min system participates in the negative regulation of T3SS expression.

3.2. Min System Inhibits T3SS Expression through the HrpG–HrpX Regulatory Pathway

To further determine whether the Min system regulates hrpB1 and hrpF expression through the key hrp regulator HrpG and HrpX, we analyzed the hrpG and hrpX expression in minC and minD mutants and the triple mutant PΔminCDE. We measured the GUS activity of the wild-type PXO99\textsuperscript{A}, PΔminC, PΔminD, and PΔminCDE carrying the reporters pHG2-hrpG and pHG2-hrpX, which contain transcriptional fusions of hrpG and hrpX promoters with the uidA gene, respectively. A significant increase in GUS activity of hrpG and hrpX promoter was observed in PΔminC, PΔminD, and PΔminCDE in comparison to that in wild-type (Figure 2A,B). The enhanced hrpG and hrpX promoter-driven GUS activity of PΔminC could be restored to the wild-type levels in CPAminC. In addition, the GUS activity of PΔminC, PΔminD, and PΔminCDE carrying the reporter pHG3-hrpG-post that contains a post-transcriptional fusion of hrpG with the uidA gene, was measured. Similarly, the mutants PΔminC, PΔminD, and PΔminCDE exhibited a dramatic increase in hrpG expression-driven GUS activity (Figure 2C). We next investigated the HrpG and HrpX protein expression in the mutants. The Western blotting assays showed that the HrpG and HrpX expression levels were significantly enhanced in minC mutants 8–24 and PΔminC, minD mutants 24–46 and PΔminD, as well as the triple mutant PΔminCDE compared with that in the wild-type PXO99\textsuperscript{A} (Figure 2D,E). These results suggest that the Min system negatively regulates T3SS expression through the HrpG–HrpX regulatory pathway.

3.3. Min System Is Involved in Positive Regulation of Two Key Virulence Regulators RpfG and Clp

To further explore whether the known key virulence regulators, including the quorum-sensing system RpfF/RpfC/RpfG, two hrpG positive regulators Trh and XrvA, the transcriptional regulator Clp, and the hrpX rather than hrpG positive regulator Zur, were involved in the MinCDE-T3SS regulatory pathway, we first analyzed the hrpG promoter activity in the mutants defective in the genes encoding the regulator mentioned above. The quantitative GUS assays indicated that the hrpG expression was significantly enhanced in the quorum-sensing mutants PΔrpfF, PΔrpfC, and PΔrpfG, and the clp mutant PΔclp than that in the wild-type PXO99\textsuperscript{A} (Figure 3A). However, the hrpG promoter-driven GUS activity was lower in the trh and xrvA mutants PΔtrh and PΔxrvA, and no differences in the hrpG promoter-driven GUS activity were observed between the zur mutant PΔzur and the wild-type PXO99\textsuperscript{A} (Figure 3A), which is in agreement with the previous studies [14,42]. Similar results were obtained by the Western blotting assays in which the HrpG expression levels were dramatically higher in PΔrpfF, PΔrpfC, PΔrpfG, and PΔclp than that in the wild-type PXO99\textsuperscript{A}, suggesting that RpfF/RpfC/RpfG and Clp functions as a hrpG negative regulator. We next investigated the mRNA levels of rpfF/rpfC/rpfG, clp, trh, and xrvA in the Min mutants by qRT-PCR. The results showed that the mRNA levels of rpfG were significantly reduced in PΔminC, PΔminD, and PΔminCDE, but the mRNA levels of clp were lower in PΔminC and PΔminD, and not in PΔminCDE, compared with the wild-type (Figure 3C). However, the rpfF, rpfC, and trh mRNA levels in the Min mutants were almost
the same as that in the wild-type. From these results, we speculate that RpfG and Clp might be involved in T3SS regulation by the Min system in Xoo.

Figure 2. The Min system negatively regulates the expression of hrpG and hrpX. (A) The hrpG promoter-driven GUS activity of Xoo wild-type PXO99A, P∆minC, CP∆minC, P∆minD, and P∆minCDE in XOM3 at 3 hr post-induction. (B) The hrpX promoter-driven GUS activity of PXO99A, P∆minC, CP∆minC, P∆minD, and P∆minCDE in XOM3 at 3 hr post-induction. (C) The hrpG expression-driven GUS activity of PXO99A, P∆minC, P∆minD, and P∆minCDE harboring a post-transcription hrpG::uidA fusions in XOM3 at 12 hr post-induction. (D) The abundance of HrpG proteins in PXO99A, 8–24, P∆minC, 24–46, P∆minD, and P∆minCDE by Western blotting. The data revealed that mutation of Min system increased the HrpG protein levels by more than 3.16-fold. (E) The abundance of HrpX proteins in PXO99A, 8–24, P∆minC, 24–46, P∆minD, and P∆minCDE by Western blotting. P∆hrpG was a negative control of HrpX protein expression. The data revealed that mutation of Min system increased the HrpX protein levels by more than 2.68-fold. The total protein extracts were analyzed by Western blotting using anti-FLAG antibodies. RNAP, RNA polymerase subunit alpha from E. coli was used as a loading control. Relative protein abundance was calculated by ImageJ software. Similar results were observed in two independent experiments. As assessed by Duncan’s test, different letters indicate statistically significant differences, and the same letter displays no significant differences (p < 0.05) between Xoo strains.

3.4. Deficiency of the Min System Causes Aberrant Cell Morphology and Division

It has been reported that cells with Min system deficiency fail to prevent the Z-ring from localizing to the cell poles and have aberrant cell division resulting in forming filamentous cells, minicells, or branching [27,30]. We investigated the cell size and shape of the Min mutants defective in minC, minD, and minCDE by transmission electron microscopy (TEM), scanning electron microscope (SEM), and fluorescent microscope (FM). The TEM observation showed that the cell elongation and asymmetric division of 8–24, P∆minC, P∆minD, and P∆minCDE were evident when compared to the wild-type PXO99A, which are normal rod-shaped cells (Figure 4A). P∆minC exhibited the classic Min-defective cell phenotypes: minicells and short filamentation observed in other bacteria such as Xcc with the minC deletion [30], whereas the complementary strain CP∆minC looked normal, like the wild-type (Figure 4B). Although the short filaments were observed in P∆minD and P∆minCDE, the occurrence frequency of short filamentations in the minC mutants 8–24 and P∆minC was higher than that in P∆minD and P∆minCDE. Similar phenotypes of minicells
and short filaments were obtained in 8–24, PΔminC, PΔminD, and PΔminCDE carrying a highly expressed green fluorescent protein (GFP) by FM (Figure 4A), indicating that the alterations (short filaments and minicells) are typical in Min mutants.

To define the length distribution of the Min mutants, we divided the cell populations into four categories based on their cell body lengths: <0.5 \( \mu \text{m} \), minicells; 0.5–1 \( \mu \text{m} \); 1–2 \( \mu \text{m} \); and >2 \( \mu \text{m} \). The standard deviation for each population was obtained after averaging under SEM conditions. The wild-type PXO99\(^A\) cells had a mean length of 1.43 ± 0.28 \( \mu \text{m} \) (n = 265) and did not form minicells. The minC insertion mutant 8–24 had a mean length of 1.79 ± 1.26 \( \mu \text{m} \) (n = 259). The shortest minicell of 8–24 was 0.176 \( \mu \text{m} \), and the frequency of minicells was about 7.72%, whereas the most extended cell was 9.135 \( \mu \text{m} \), and the proportion of filamentous cells was about 33.59% (Figure S4 and Table 1). The minD deletion mutant PΔminD possesses a mean length of 1.32 ± 0.633 \( \mu \text{m} \) (n = 268), with filamentous cells recording for 11.94% and minicells accounting for 2.99% (Figure S4 and Table 1). The recovered strain CPΔminC possesses a mean length of 1.58 ± 0.50 \( \mu \text{m} \) (n = 264) without the minicells, indicating similar morphology (cell shape and cell length variation) to the wild-type PXO99\(^A\). Minicells were almost absent in PΔminD (n = 257) and PΔminCDE (n = 258), while the ratios of cells length than 2 \( \mu \text{m} \) (18.29% and 13.18%) in PΔminD (n = 257) and PΔminCDE (n = 258) were significantly longer than that in the PXO99\(^A\) (Figure S4 and

**Figure 3.** HrpG expression levels in the mutants of Xoo virulence regulator genes and the rpfG and clp expression levels in the Min mutants. (A) The abundance of HrpG proteins in PXO99\(^A\), PΔthr, PΔxvrA, PΔtur, PΔrpfC, PΔrpfG, PΔrpfF, and PΔclp by Western blotting. The total protein extracts were analyzed by Western blotting using anti-FLAG antibodies. RNAP, RNA polymerase subunit alpha from E. coli was used as a loading control. Relative protein abundance was calculated by ImageJ software. Similar results were observed in two independent experiments. (B) Expression ratios of rpfG in PΔminC, PΔminD, and PΔminCDE compared to that in Xoo wild-type PXO99\(^A\) by qRT-PCR. (C) Expression ratios of clp in PΔminC, PΔminD, and PΔminCDE compared to that in PXO99\(^A\) by qRT-PCR. Similar results were observed in more than three independent experiments. As assessed by Duncan’s test, different letters indicate statistically significant differences, and the same letter displays no significant differences (p < 0.05) between Xoo strains.
Table 1). These results indicated that mutation of min genes, especially the minC gene, causes aberrant morphology and asymmetric division of Xoo cells.

| Strain     | Minicells Percentage | Filamentous Cells Percentage | Mean Cell Length (µm) | Minimum (µm) | Maximum (µm) |
|------------|----------------------|-------------------------------|-----------------------|--------------|--------------|
| PXO99A     | 0%                   | 4.15%                         | 1.432 ± 0.281         | 0.791        | 2.717        |
| 8–24       | 7.72%                | 33.59%                        | 1.793 ± 1.260         | 0.176        | 9.135        |
| PΔminC     | 2.99%                | 11.94%                        | 1.318 ± 0.628         | 0.233        | 4.630        |
| CPΔminC    | 0%                   | 17.05%                        | 1.579 ± 0.496         | 0.582        | 3.820        |
| PΔminD     | 0.40%                | 16.80%                        | 1.521 ± 0.462         | 0.500        | 4.155        |
| PΔminCDE   | 0.80%                | 13.18%                        | 1.426 ± 0.402         | 0.405        | 3.672        |

3.5. Effect of Min System on Bacterial Virulence, Motility, and Biofilm Formation

In Xoo, the T3SS is essential for bacterial pathogenicity on susceptible host rice and triggering HR on nonhost. To verify whether the deletion of min genes affects Xoo virulence, we inoculated the Min mutants and relative complementary strains on IR24, a susceptible rice variety, by the leaf-clipping method. The result showed that all mutants could cause the water-soaked lesions on IR24 (Figure S5A), whereas the minC insertion mutant 8–24 exhibited a significant decrease in lesion length on IR24 when compared to the wild-type PXO99A, and the deletion mutants PΔminC, PΔminD, and PΔminCDE displayed a weaker reduction in virulence on IR24 (Figure 5A). The corresponding complementary strains CPΔminD and CPΔminCDE, in which the minD and minCDE genes were expressed...
in trans, could be retained the wild-type ability to cause lesion length on IR24, indicating that the Min system is required for *Xoo* full virulence on host rice. The inoculation assays on tobacco indicated that the absence of the Min system did not affect the capacity of *Xoo* to trigger HR on nonhost tobacco (Figure S5B).

![Figure 5](image)

**Figure 5.** MinC affects *Xoo* virulence, biofilm formation, and swimming motility. (A) Lesion lengths of the leaves of IR24 caused by *Xoo* wild-type PXO99<sup>A</sup>, 8–24, PΔminC, PΔminD, CPΔminD, PΔminCDE, CPΔminCDE, and PΔhrpG at 14 days post-inoculation by leaf-clipping. Bacterial suspensions (OD<sub>600</sub> = 0.6) were inoculated in the leaves of susceptible rice IR24. PΔhrpG as a negative control strain without pathogenicity on rice. Similar results were observed in two independent experiments. (B) Swimming motility of PXO99<sup>A</sup>, 8–24, PΔminC, 24–46, PΔminD, and CPΔminD on NA medium with 0.15% agar. Swimming zones were measured and evaluated after bacterial growth on the NA plates for 3 days. As assessed by Duncan’s test, different letters indicate statistically significant differences (p < 0.05) between *Xoo* strains. (C) Biofilm formation of PXO99<sup>A</sup>, 8–24, PΔminC, 24–46, PΔminD, and PΔminCDE on glass test tube surfaces after 3 days of incubation. The biofilm formation was visualized by crystal violet staining, then was quantified by measuring the absorbance at 590 nm. The tests were repeated three times. As assessed by Duncan’s test, different letters indicate statistically significant differences (p < 0.05) between *Xoo* strains.

It has been shown that Min system proteins prevent the septa formation at the cell pole by inhibiting the Z-ring [19,30]. Taking into account that the swimming motility of *Xoo* is dependent on a polar flagellum, we explored the role of the Min system in *Xoo* swimming motility, and conducted the swimming motility assays in which the Min mutants were inoculated on the semi-solid NA medium with 0.15% agar. Similar to the transposon mutant 8–24, the minC deletion mutant PΔminC did not exhibit any significant swimming motility, but the minD mutants 24–46 and PΔminD, as well as the triple mutant PΔminCDE showed slightly reduced swimming motility compared to the wild-type PXO99<sup>A</sup> (Figure 5B). The complementary strain CPΔminD nearly reverted swimming motility to wild-type levels.
we screened 198 and 106 DEGs in P (∆minC) and transferase activity involved in the alkyl or aryl transfer (GO: 0016765). 

\[ \text{copB} \] (GO:0042592), including three up-regulated DEGs, (Figures 6D and S6B). All DEGs were classified into three main categories based on their function. The findings revealed that the majority number of DEGs enriched in biological processes in strains 8–24, with significant enrichment in the homeostatic process. As with 8–24, P∆minCDE are highly enriched in functions associated with protein maturation (∆minC, and 8–24 strains (Figure 6A,B). Three genes (PXO_04154, PXO_04552, and PXO_04756) were significantly down-regulated in 8–24 (Figure 6A). PXO_04756 was reported to have a role related to cardiolipin synthesis. Furthermore, the expression of 99 genes, including two copies of clpA (PXO_06136 and PXO_01030), was significantly up-regulated. ClpA was annotated as an ATP-binding subunit of the Clp protease. In P∆minC, 14 and 184 genes were significantly down-regulated and up-regulated, respectively (Figure 6A). Six hrp genes, hrpD6 (PXO_03410), hpaA (PXO_03408), hpaB (PXO_03412), hrcL (PXO_03402), hrpD5 (PXO_03409) and hrpE (PXO_03411), and the TCS genes raxH (PXO_04467) and raxR (PXO_04469) were all up-regulated in the minC mutant P∆minC (Figure 6C). The expression levels of these genes were dramatically higher in 8–24 than in wild-type PXO99A. Furthermore, the cytokinesis-related gene zipA (PXO_00742) was also significantly enriched. In P∆minC and 8–24, Venn diagram analysis revealed that 78 up-regulated DEGs and 2 down-regulated DEGs were overlapped, demonstrating the precision of the RNA-seq.

We investigated DEGs for GO enrichment in P∆minC and 8–24, respectively (Figures 6D and S6B). All DEGs were classified into three main categories based on their putative function. The findings revealed that the majority number of DEGs enriched in biological processes in 8–24, with significant enrichment in the homeostatic process (GO:0042592), including three up-regulated DEGs, copB (PXO_03131), cutC (PXO_01619), and ferricyanide receptor (PXO_03287). These results suggest that MinC can regulate DEGs expression by altering the function of the homeostatic process. As with 8–24, P∆minC concentrated a significant proportion of the DEGs in biological processes. The DEGs up-regulated in P∆minC are highly enriched in functions associated with protein maturation (GO: 0051604) and transferase activity involved in the alkyl or aryl transfer (GO: 0016765). The interaction between MinC and transferase activity genes might occur in stress response modulation and differential stability. These genes are possibly associated with the virulence mediated by MinC.

The KEGG pathway would be used to categorize further and study the biological functions of these DEGs. Among these pathways, a p-value ≤ 0.05 was necessary for analysis. DEGs are implicated in various pathways. The DEGs influence six critical pathways in strains 8–24 (Figure S6C), including cell growth and death, immune disease, and infecting diseases. Similarly, DEGs in P∆minC were considerably more abundant in primary immunodeficiency (ko05340) of immunological disorders (Figure 6E). In P∆minC, the uracil-DNA glycosylase gene (UDG, PXO_03712) expression was increased significantly. UDGs exist in different bacteria and possess base activity to excise damaged bases in DNA. Furthermore, they can increase heat resistance. These results suggest the potential stress resist functions of MinC in Xoo.
Figure 6. Analysis of the differentially expressed genes (DEGs) in PXO99A versus in PΔminC and 8–24.
(A) The log2 Foldchange of DEGs in 8–24 and (B) PΔminC was plotted against the p-value. Statistically significant differentially expressed genes, with a log2 Foldchange ≥ 1 or ≤ −1, are depicted as the red and green dots, respectively, and insignificant as grey dots. For each organism, the shade of the color represents the level of gene expression. Dark blue dots represent T3SS-associated genes. Light blue dots represent cell division-associated genes. (C) Heatmap of gene expression of the differentially expressed genes (DEGs) in minC mutants 8–24 and PΔminC. The color gradient indicates the normalized base mean values of DEGs (high expression (red) and low expression (purple)). (D) GO analysis of DEGs in PΔminC mutant. The abscissa axis represents the GO category, and the ordinate axis represents the value of significance (p < 0.05). (E) KEGG analysis of DEGs in PΔminC mutant. The abscissa axis represents the KEGG pathway, and the ordinate axis represents the value of significance (p < 0.05).
4. Discussion

The Min system comprising three proteins, MinC, MinD, and MinE, is conserved among genera of rod-shaped bacteria, and its function in cell division has been well studied in *E. coli* and *B. subtilis*. However, most studies focused on understanding its role in interaction with other divisome proteins such as FtsZ, whereas other roles in cellular processes including virulence, bacterial motility, and colonization were not explored. In this study, we found that apart from involvement of the *Xoo* Min system in cell division, the Min system also participates in the regulation of T3SS expression, bacterial full virulence, swimming motility, and biofilm formation, suggesting that the function of Min proteins is not strictly confined to cytokinesis; more cellular functions must be elucidated.

Generally, the Min system mutation leads to abnormal morphology such as minicells, short filamentations, and branching in bacteria [17,30]. Our microscopy observations showed that the 8–24, *P∆minC*, *P∆minD*, and *P∆minCDE* mutants exhibited obvious cell elongation and asymmetric division. The aberrant cell division phenotypes including minicells and short filamentations were observed in 8–24, *P∆minC*, *P∆minD* and *P∆minCDE*, especially in the *minC* 8–24 and *P∆minC* mutants, which are classic Min-defective cell phenotypes, and also similar to cell shapes of the *minC*-defective mutants in *Xcc* 306 and *Helicobacter pylori* [27,30], indicating that the *Xoo* Min system indeed plays a key role in cell division. However, some branching cells comprising less than 20% of total cells were observed in the *minC* mutant of *Xcc* 306 [30], which were not observed in our microscopy assays. Branched cells impair the divisome formation, the nucleoid organization, and the incorporation of peptidoglycans. The phenotype of branching cells reported in the *E. coli* Min mutants was dependent on the growth medium used in the experiments [43,44]. Almost no branching cells were observed in the *minC* mutant of *Xcc* when the rich NYG/CB media were used [30]. We speculated that the absence of branching cells in the Min mutants of *Xoo* might be the reason for the nutrient-rich NB medium employed in our assays.

Some studies in pathogenic bacteria have shown that Min proteins are essential for full virulence. The *minD* mutant of the pathogenic enterohemorrhagic *E. coli* (EHEC) reduced its adherence to the human epithelial tissues [45]. Both mutations of *minC* and *minD* in *Neisseria gonorrhoea*, a sexually-transmitted bacterium, reduced its ability to adhere to and invade urethral epithelial cells, but did not alter its potential to produce other virulence factors [46]. Our results showed that the *minC* insertion mutant 8–24 exhibited an attenuated virulence in rice, whereas the deletion mutants *P∆minC*, *P∆minD*, and *P∆minCDE* displayed a weaker reduction in virulence. We speculate that the different phenotypic effects on virulence between 8–24 and *P∆minC* could be related to the mutation sites in the *minC* gene in these two mutants. We deleted the middle open reading fragment of *minC* in *P∆minC*, but the Tn5 transposon was inserted in the 3’-terminal of *minC* in 8–24. This suggests that the C-terminal domain of MinC is important for the function of MinC in bacterial virulence. Similarly, a current study in *Xoo* PXO99Δ has shown that MinD was significantly downregulated during its interaction with host rice IR24, and the average lesion lengths caused by the *minD* mutant were significantly shorter than those caused by the wild-type PXO99Δ [31]. Taken together, these results indicate that MinC and MinD are essential for *Xoo* full virulence in susceptible host rice.

Our swimming motility assays showed that the mutants with inactivation of *minC*, *minD*, or *minCDE* showed reduced swimming motility as compared to the wild-type PXO99Δ. However, the *minC* mutants 8–24 and *P∆minC* nearly lost swimming motility, indicating that MinC plays a critical role in swimming motility. This result is in agreement with some studies in *Proteus mirabilis* and *H. pylori* [26,27]. Both *minC* mutants in these two bacteria exhibited reduced swarming motility. It has been determined that alteration in cell morphology might affect motility. Although the mutations of *minC*, *minD*, and *minCDE* resulted in elongation in cell lengths, a *minC* mutation alone was found to lose swimming motility. Therefore, we speculate that asymmetric division may affect bacterial motility whereas, more to the point, some underlying mechanisms or connections between MinC and flagellar biosynthesis are essential for swimming motility. Current studies...
have indicated some relation between the Min system and flagella regulators, such as FlhG and FlhDC, a master regulator for flagellar synthesis [17,26,47]. These findings also indicate that Xoo is an ideal model bacterium to study the role of cell division proteins in motility function.

In this study, we discovered that the Min system is extensively conserved in seven species of the genus Xanthomonas, and the minCDE gene cluster co-transcribed with the flanking genes PXO_04462 and PXO_04466. A similar study has been observed in pathogenic N. gonorrhoea, in which the minCDE gene cluster is transcribed with oxyR, which encodes a redox-response transcriptional regulator (LysR-NodD family) that can directly bind the promoter regions of some catalase genes such as katA [28]. The mutation of N. gonorrhoea oxyR led to defective cell division and enhanced minD expression [28]. The Xoo PXO_04462 gene encodes a putative GNAT-family protein. The C-terminal domain of the GNAT family contains an acetyl-CoA binding fold that transfers the acetyl group from acetyl-CoA to a variety of N-terminal amino groups. The mutation of the GNAT gene in Dickeya zeae MS2 has been shown to decrease virulence in potatoes [48]. We have constructed the deletion mutants of PXO_04462 and PXO_04466. Whether these two genes are involved in cell division inhibition and expression of minCDE genes needs to be examined further. Moreover, we found that raxR-raxH, a pair of genes associated with a two-component system directly orthologous to Pseudomonas colS-colR [49,50], was located upstream of the minCDE operon in Xoo. Our RNA-seq data showed that the expression of raxH and raxR was significantly higher in the minC mutants than that in the wild-type. It has been determined that, in response to Zn\textsuperscript{2+} stress, RaxH-RaxR regulates the arnT-lpxT-eptA gene cluster to participate in lipid A remodeling enzyme synthesis [49,51]. Therefore, we hypothesized that the Xoo Min system might be involved in other cellular processes associated with stress response.

Our study demonstrated that the Min system inhibited the hrp genes (hrpB1 and hrpF) expression through HrpG and HrpX in XOM3. To our knowledge, this is the first report about the Min system participating in the regulation of T3SS expression in Xoo. This finding is further confirmed by the RNA-seq data, by which we found that hrpD6 (PXO_03410), hpaA (PXO_03408), hpaB (PXO_03412), hrcU (PXO_03402), hrd5 (PXO_03409), and hrpE (PXO_03411) were induced in the minC mutants 8–24 and PΔminC. These results are consistent with a current finding that MinD was significantly downregulated during early interaction of Xoo with host rice IR24 [31]. As the reduced MinD expression causes increased expression of hrp genes, it is logical for inducible expression of hrp genes in the early stage of interaction with host rice. Our results showed that the mutations of minC, minD, or minCDE caused an increase in hrp genes (hrpF and hrpB1), but the mutants PΔminC, PΔminD, and PΔminCDE displayed a weaker reduction in virulence. We speculate that high expression of hrp genes does not necessarily cause an increase in bacterial virulence on the host plant. For example, in our previous study, a metB mutant of Xoo PXO99\textsuperscript{A} exhibited the enhanced hrpG expression in XOM3, but showed impaired virulence in host rice, as the metB gene is the EPS and LPS synthesis-related gene [52]. Our results showed that rpfG and clp were down-regulated in the minC and minD mutants, whereas hrpG was up-regulated in the rpfG and clp mutants. RpfG is a response regulator of the two-component system RpfG/RpfC with the capacity of degrading c-di-GMP, and Clp is a homologue of cyclic AMP receptor protein (CRP) with the ability to bind c-di-GMP [53,54]. Therefore, DSF and c-di-GMP (or cAMP) signal pathways were speculated to participate in T3SS expression regulated by the Min system in Xoo. Our RNA-seq data also indicated that MinC regulates the expression of two copies of clpA. ClpA, a Clp protease, has been demonstrated to be a virulence factor in Xoo and protect the cytoplasm against the detrimental effects of stressful conditions imposed by host defense mechanisms and environmental events [55]. Taken together, we speculate that negative regulation of T3SS expression by the Min system in Xoo is complex, and that a combination is involved in multiple signaling pathways.
5. Conclusions

In this study, we identified the Xoo Min system (MinC, MinD, and MinE) functioning as a negative regulator for T3SS expression through the key hrp regulators HrpG and HrpX. The mutations of minC, minD, and minCDE resulted in cell elongation and asymmetric division; meanwhile, mutation of minC in Xoo resulted in significantly impaired virulence in host rice, swimming motility, and enhanced biofilm formation. Our transcriptome profiling also indicated that some virulence genes were differentially expressed in the minC mutants. To our knowledge, this is the first report about the Min system participating in the regulation of T3SS expression. It provides some evidence for the complex T3SS regulatory networks and sheds light on the understanding of Xoo virulence mechanisms.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/10.3390/microorganisms10081549/s1, Figure S1A: The hrpF promoter-driven GUS activity of PXO99A, 8–24, P∆minC, and the complementary strains C8–24 and CP∆minC measured in XOM3 at 3 h post-induction. Figure S1B: The hrpF promoter-driven GUS activity of PXO99A, 24–46, P∆minD, and t P∆minCDE measured in XOM3 at 3 h post-induction. Figure S2: Synteny analysis of the minCDE gene cluster among Xanthomonas strains. Figure S3A: Co-transcription of PXO_04462-minC-minD-minE-PXO_04466 by RT-PCR. Figure S3B: Southern blot analysis of the Xoo Min mutants P∆minC, P∆minD, P∆minCDE, 8–24 and 24–46. Figure S4: Cell length distribution of the Xoo Min mutants 8–24, P∆minC, P∆minD, P∆minCDE, and the complementary strain CP∆minC. Figure S5A: Water-soaked lesions on IR24 caused by PXO99A, P∆minC, P∆minD, and P∆minCDE at 3 days post-inoculation. Figure S5B: Hypersensitive response in nonhost tobacco caused by PXO99A, 8–24, P∆minC, P∆minD, and P∆minCDE at 1 day post-inoculation. Figure S5C: Swimming motility of PXO99A, P∆minD, and CP∆minD on NA medium with 0.15% agar. Figure S6A: Venn diagram analysis of the up-regulated and down-regulated genes in PXO99A versus P∆minC and 8–24. Figure S6B: GO analysis of DEGs in the minC mutant 8–24. Figure S6C: KEGG analysis of DEGs in the minC mutant 8–24. Table S1: Bacterial strains and plasmids used in this study. Table S2: Primer sequences used in this study.

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