

AsnB, regulated by diffusible signal factor and global regulator Clp, is involved in aspartate metabolism, resistance to oxidative stress and virulence in *Xanthomonas oryzae* pv. *oryzicola*

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SUMMARY

Xanthomonas oryzae pv. *oryzicola* (*Xoc*) causes bacterial leaf streak in rice, which is a destructive disease worldwide. *Xoc* virulence factors are regulated by diffusible signal factor (DSF) and the global regulator Clp. In this study, we have demonstrated that *asnB* (*XOC_3054*), encoding an asparagine synthetase, is a novel virulence-related gene regulated by both DSF and Clp in *Xoc*. A sequence analysis revealed that AsnB is highly conserved in *Xanthomonas*. An *asnB* mutation in *Xoc* dramatically impaired pathogen virulence and growth rate in host rice, but did not affect the ability to trigger the hypersensitive response in nonhost (plant) tobacco. Compared with the wild-type strain, the *asnB* deletion mutant was unable to grow in basic MMX (–) medium (a minimal medium without ammonium sulphate as the nitrogen source) with or without 10 tested nitrogen sources, except asparagine. The disruption of *asnB* impaired pathogen resistance to oxidative stress and reduced the transcriptional expression of *oxyR*, *katA* and *katG*, which encode three important proteins responsible for hydrogen peroxide (H₂O₂) sensing and detoxification in *Xanthomonas* in the presence of H₂O₂, and nine important known *Xoc* virulence-related genes in plant cell-mimicking medium. Furthermore, the *asnB* mutation did not affect extracellular protease activity, extracellular polysaccharide production, motility or chemotaxis. Taken together, our results demonstrate the role of *asnB* in *Xanthomonas* for the first time.

INTRODUCTION

Xanthomonas is a large genus of Gram-negative bacteria with the typical characteristic of yellow pigment. This genus consists of a number of phytopathogenic bacteria that infect more than

400 plant hosts, including a wide variety of economically important plants, such as rice, citrus, banana, cabbage and bean (Ryan *et al.*, 2011; Watt *et al.*, 2005). One member of this genus, *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), is the causal agent of bacterial leaf streak (BLS), which is a serious bacterial disease of rice in Asia and parts of Africa (Niño-Liu *et al.*, 2006; Zhao *et al.*, 2011). *Xoc* penetrates the leaf mainly through stomata, multiplies in the substomatal cavity and colonizes the parenchymal apoplast, causing interveinal lesions (Wang *et al.*, 2007). *Xoc* can also gain access through wounds, but, in this case, it remains restricted to the mesophyll tissue apoplast and does not invade the xylem (Niño-Liu *et al.*, 2006). To date, the virulence mechanisms of *Xoc* are not entirely clear, and control measures for BLS are poorly developed.

Quorum sensing (QS) is a sophisticated cell–cell communication mechanism that relies on small signal molecules to control bacterial behaviour and coordinate gene expression in a cell density-dependent manner (Mole *et al.*, 2007). In Gram-negative bacteria, the *N*-acyl homoserine lactones (AHLs) are a major class of signal molecules. AHLs are composed of a homoserine lactone ring carrying C₄–C₁₈ acyl chains and are thought to function as interspecific signalling molecules (Danhorn and Fuqua, 2007; Miller and Bassler, 2001). Bacterial behaviours or phenotypes generally controlled by AHL signals include virulence, symbiosis, biofilm formation, motility, conjugation, competence, antibiotic production and sporulation (Danhorn and Fuqua, 2007; Goo *et al.*, 2010; Miller and Bassler, 2001).

In contrast with other Gram-negative bacteria, *Xanthomonas* does not produce AHL, but has evolved to produce a different type of QS signal, called the diffusible signal factor (DSF). DSF is significantly different from AHL in chemical structure (Barber *et al.*, 1997; Chatterjee and Sonti, 2002; Tang *et al.*, 1991). The DSF structure has been identified as *cis*-11-methyl-2-dodecenoic acid in *X. campestris* pv. *campestris* (causal agent of crucifer black rot) (Wang *et al.*, 2004). The QS signals have been identified as BDSF (*cis*-dodecenoic acid) and CDSF (a novel unsaturated fatty acid, which is otherwise identical to DSF, except for the double bond

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between C5 and C6) in *X. oryzae* pv. *oryzae* (*Xoo*). A recent study has suggested that BDSF is the major *in vivo* signal when *Xoo* infects rice (Professor Yawen He, Shanghai Jiao Tong University, Shanghai, personal communication). Furthermore, DSF signals have also been identified in *Xylella fastidiosa* (causal agent of citrus variegated chlorosis) and the *Burkholderia cepacia* complex (a pathogen that particularly affects cystic fibrosis patients) (Boon *et al.*, 2008; Chatterjee *et al.*, 2008). Similar to AHL, DSF has been linked to the regulation of virulence, motility, toxin production, aerobic respiration, biofilm dispersal, extracellular enzymes and extracellular polysaccharide (EPS) production in DSF-producing bacteria (Deng *et al.*, 2011; He and Zhang, 2008).

Studies have shown that the regulation of pathogenicity factor (*rpf*) gene cluster (core genes including *rpfF*, *rpfC* and *rpfG*) is responsible for DSF production and signal transduction (Chatterjee and Sonti, 2002; He *et al.*, 2006; Siciliano *et al.*, 2006; Tang *et al.*, 1991). *rpfF* encodes a protein similar to enoyl-CoA hydratase, which catalyses DSF synthesis. RpfC (sensor protein) and RpfG (response regulator), consisting of a unique two-component regulatory system, are responsible for extracellular DSF sensing and intracellular signal transduction, which leads to the regulation of downstream gene expression.

A transcriptional factor, cyclic adenosine monophosphate (cAMP) receptor protein-like protein (Clp), is known as a master regulator in the *X. campestris* pv. *campestris* DSF signalling network. Clp regulates the expression of genes belonging to the DSF regulon, such as those encoding extracellular enzymes, components of type II and type III secretion systems, and genes involved in EPS synthesis (Büttner and Bonas, 2010; He *et al.*, 2006, 2007). However, Clp is not involved in DSF-dependent regulation of biofilm formation (He *et al.*, 2007).

In a previous study, we reported that DSF-mediated QS is required for *Xoc* pathogenicity on host rice, and revealed 48 differentially expressed intracellular proteins between the wild-type strain and QS-defective mutants using the two-dimensional method (Zhao *et al.*, 2011). Among the 48 proteins, 18 were identified using mass spectrometry and predicted to be involved in nitrogen transfer, protein folding, resistance to oxidative stress and flagellar synthesis (Zhao *et al.*, 2011). We in-frame deleted each of the 18 genes to investigate the role of these 18 DSF-regulated proteins in *Xoc* virulence. Here, we report the identification and functional characterization of one of the 18 genes, *asnB* (*XOC_3054*), which encodes the enzyme that converts aspartic acid to asparagine in *Xoc*. Our data show that *asnB* is involved in aspartate metabolism, resistance to oxidative stress, virulence and the regulation of the expression of nine known important virulence-related genes, and is regulated by both DSF-mediated QS and the global regulator Clp in *Xoc*. Our results provide a foundation for further molecular understanding of DSF-mediated proteins and their functions in the *Xoc*–rice interaction. This is the first report of a functional analysis of *asnB* in *Xanthomonas*.

RESULTS

asnB organization in the genome, promoter prediction and mutant confirmation

Figure 1A shows *asnB* organization in the available genome of strain BLS256. The *asnB* nucleotide size was 1692 bp. *Xoryp_15010* (2124 bp) and *Xoryp_15015* (1008 bp), which encode the receptor and a hypothetical protein, respectively, were located upstream of *asnB*, whereas *Xoryp_15025* and *Xoryp_15030*, which encode succinyl-diaminopimelate desuccinylase and a hypothetical protein, respectively, were located downstream of *asnB*. Furthermore, the intergenic regions between *Xoryp_15010* and *Xoryp_15015*, *Xoryp_15015* and *asnB*, *asnB* and *Xoryp_15025*, and *Xoryp_15025* and *Xoryp_15030* were 476, 70, 294 and 6 bp, respectively.

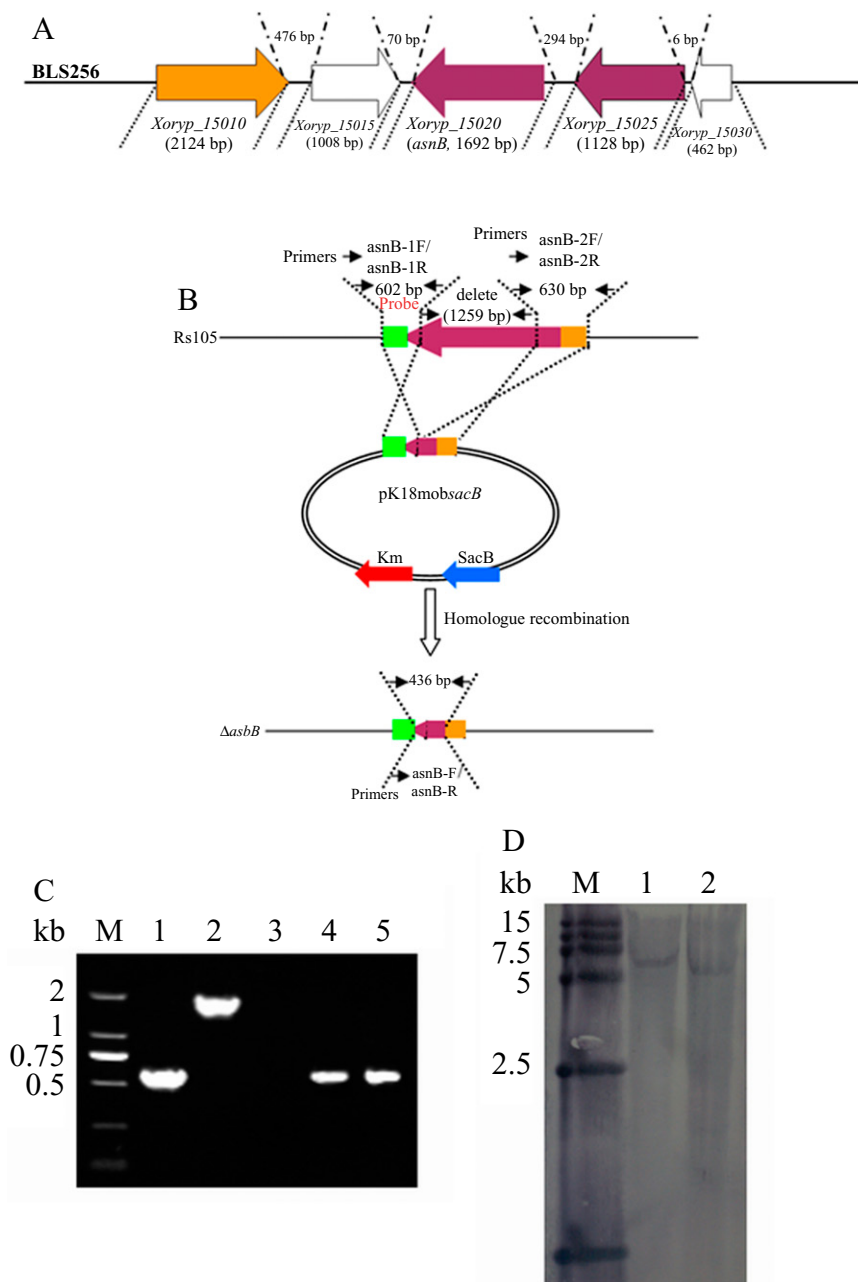
A 335-bp upstream fragment of the *asnB* 5' sequence was selected for promoter prediction at the Neural Network Promoter Prediction website (http://www.fruitfly.org/seq_tools/promoter.html). The results showed that two promoter sequences from –263 to –213 (with a score of 0.51) and –211 to –161 (with a score of 0.54) were predicted.

The scheme for *asnB* mutant construction and confirmation is shown in Fig. 1B. As shown in Fig. 1C, a 1.7-kb DNA fragment was amplified from the wild-type strain, whereas only a 0.4-kb DNA fragment was amplified in the *asnB* deletion mutant (lanes 4 and 5) because of the in-frame deletion of the 1.3-kb fragment from the *asnB* gene (Fig. 1B). This result shows correction of the *asnB* in-frame deletion. To confirm this result, we used the 602-bp DNA fragment amplified by *asnB*-1F/*asnB*-1R as a probe (Fig. 1B) for Southern blot analysis. As shown in Fig. 1D, a 6.5-kb DNA fragment was detected in the Rs105 wild-type strain, whereas only a 5.0-kb fragment was obtained from the *asnB* deletion mutant owing to in-frame deletion of the 1.3-kb fragment. This result was consistent with that of polymerase chain reaction (PCR) amplification. Taken together, these results show the correct construction of the *asnB* deletion mutant in *Xoc*.

asnB is required for *Xoc* pathogenicity in host rice, but is not necessary for the triggering of the hypersensitive response (HR) in nonhost (plant) tobacco

To investigate the role of *asnB* in *Xoc* virulence, we generated the *asnB* in-frame deletion mutant Δ *asnB* in a wild-type background. As shown in Fig. 2A,B,D, Δ *asnB* exhibited a significant decrease in virulence in rice seedlings and adult rice leaves, whereas the complemented strain Δ *asnB*(*asnB*) (Fig. 2A,B,D) displayed the same virulence as the wild-type. This result indicates that *asnB* is required for *Xoc* pathogenicity on host rice. In the following experiment, we aimed to understand whether *asnB* is also

Fig. 1 Physical map of *asnB* organization (A), mutant construction (B) and molecular confirmation (C, D) in *Xanthomonas oryzae* pv. *oryzicola*. (A) *asnB* organization in the available genome of strain BLS256. The nucleotide size of *asnB* is 1692 bp. *Xoryp_15010* (2124 bp) and *Xoryp_15015* (1008 bp), which encode an iron receptor and a hypothetical protein, respectively, were located upstream of *asnB*, whereas *Xoryp_15025* and *Xoryp_15030*, which encode succinyl-diaminopimelate desuccinylase and a hypothetical protein, respectively, were located downstream of *asnB*. (B) The gene deletion scheme. The 602-bp (amplified by *asnB*-1F/*asnB*-1R) (Table 1) and 630-bp (amplified by *asnB*-2F/2R) (Table 1) DNA fragments were used as the 5' and 3' fragments for homologous recombination, respectively. The internal 1259-bp DNA fragment was deleted in the *asnB* mutant. The *asnB*-F/R primer (Table 1) was used for molecular confirmation of the *asnB* mutant. If the 1259-bp internal fragment of *asnB* was successfully deleted, a 436-bp DNA fragment would be amplified from the *asnB* mutant. (C) Polymerase chain reaction (PCR) confirmation of the *asnB* mutant. Owing to deletion of the *asnB* 1259-bp internal fragment, only an approximately 0.5-kb DNA fragment was amplified from the *asnB* deletion mutant (lanes 4 and 5). M, mark; 1, pKS-A, positive control; 2, Rs105 wild-type strain; 3, H₂O, negative control; 4 and 5, Δ *asnB*. (D) Southern blotting analysis of the *asnB* deletion mutant. The 602-bp fragment was used as the probe for Southern blotting. A 6.5-kb DNA fragment was detected in the Rs105 wild-type strain (lane 1), whereas only an approximately 5.0-kb fragment was obtained in the *asnB* deletion mutant (lane 2) owing to in-frame deletion of the 1259-bp fragment.



required for the triggering of HR (programmed cell death) in nonhost (plant) tobacco. The *asnB* mutation did not affect the ability of the wild-type to trigger HR (Fig. 2C), indicating that *asnB* is not involved in the triggering of HR in nonhost (plant) tobacco. This result also suggests the diverse functions of *asnB*.

The *AsnB* mutation lowers the growth rate of the wild-type rice strain

The growth rate of Δ *asnB* in nutrient-rich broth (NB), nutrient-limiting broth (MMX) and rice tissue was determined to

understand the relationship between the growth rate of Δ *asnB* and its virulence deficiency. The *asnB* mutant exhibited the wild-type growth rate in NB medium (data not shown). However, the ability to grow in MMX medium was abolished by the mutation (Fig. 3A). The complemented strain restored the wild-type growth rate under the same conditions (Fig. 3A). Interestingly, the growth rate of Δ *asnB* in infected rice tissue decreased, but was not abolished, when compared with the complemented and wild-type strains (Fig. 3B). This result indicates that the virulence deficiency of the *asnB* deletion mutant is, at least partially, caused by the growth decrease in rice tissue.

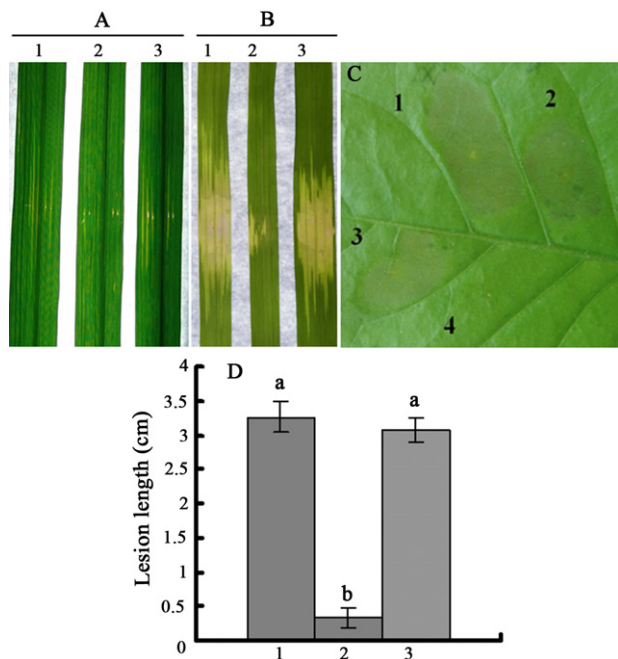


Fig. 2 Pathogenicity test and hypersensitive response (HR) assay of the *Xanthomonas oryzae* pv. *oryzaicola* (*Xoc*) strains in rice and tobacco, respectively. (A) Lesion lengths on the leaves of adult susceptible rice (cv. Shanyou63, 2 months old) inoculated with the *Xoc* strains. (B) Lesion lengths on the rice seedling leaves (cv. Shanyou63, 2 weeks old) by infiltration with the *Xoc* strains. (C) Phenotypes of HR in nonhost (plant) tobacco by inoculation with *Xoc* strains. (D) Calculated lesion lengths on the leaves of adult susceptible rice (cv. Shanyou63, 2 months old) inoculated with the *Xoc* strains. 1, Rs105 wild-type strain; 2, *asnB* deletion mutant Δ *asnB*; 3, Rs105 *asnB* complemented strain Δ *asnB*(*asnB*); 4, negative control (double-distilled H₂O). Three replicates were used for each treatment, and the experiment was repeated three times. Vertical bars represent standard errors. Different letters above the data bars indicate a significant difference between the wild-type strain and *asnB* deletion mutant or *asnB* complemented strain ($P < 0.05$; *t*-test).

The growth deficiency of the *asnB* mutant in MMX is restored by supplementation with the rice leaf extract

The results in Fig. 3 show that the *asnB* mutation abolished the growth ability of the wild-type in MMX minimal medium, but only decreased the growth rate in rice leaves compared with that in the wild-type strain. To confirm this result, we investigated whether the growth deficiency of the *asnB* mutant in MMX medium could be restored by supplementation with the rice leaf extract. As shown in Fig. 4, the *asnB* deletion mutant in MMX medium was restored to growth in the presence of rice tissue. As the concentration of rice tissues supplemented with MMX increased, growth restoration of the *asnB* deletion mutant also increased. In MMX medium supplemented with a 3% final concentration of rice tissue, growth of the *asnB* deletion mutant was significantly higher than that of the control (MMX broth). These findings

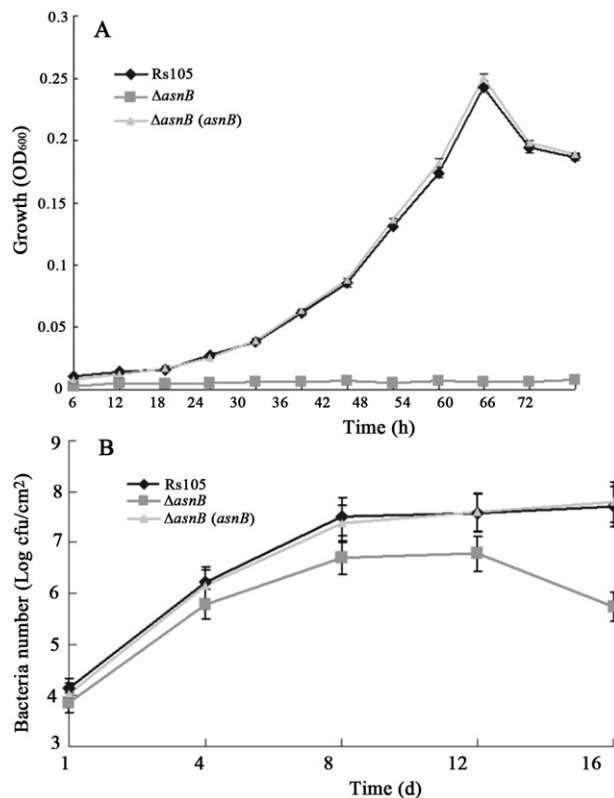


Fig. 3 Growth rate of *Xanthomonas oryzae* pv. *oryzaicola* (*Xoc*) strains in MMX basic medium and rice leaves. (A) Growth rate of *Xoc* wild-type strain Rs105, the *asnB* deletion mutant Δ *asnB* and its complemented strain Δ *asnB*(*asnB*) in MMX basic medium. OD_{600 nm}, optical density at 600 nm. (B) Growth rate of *Xoc* wild-type strain Rs105, the *asnB* deletion mutant Δ *asnB* and its complemented strain Δ *asnB*(*asnB*) in adult-susceptible rice leaves (cv. Shanyou63) during infection. cfu, colony-forming unit. Three replicates for each treatment were used, and the experiment was repeated three times. Vertical bars represent standard errors.

provide support for the growth determination results of the *asnB* deletion mutant with rice leaves (Fig. 3B). Taken together, these results suggest that a component(s) from rice leaves might restore the asparagine metabolism defect in the *asnB* mutant.

AsnB is involved in aspartate metabolism in *Xoc*

asnB encodes the enzyme that converts aspartic acid into asparagine in bacteria. To confirm that the *AsnB* protein in *Xoc* is involved in aspartate metabolism, we tested the growth rate of Δ *asnB* in MMX with or without aspartic acid or asparagine. We clearly observed that Δ *asnB* was unable to grow in minimal medium (Fig. 3A). However, Δ *asnB* displayed the wild-type growth rate when asparagine, but not aspartic acid, was added to the MMX medium, indicating that *asnB* is involved in aspartate metabolism. To further investigate whether *asnB* is involved in the metabolism of other nitrogen sources, the growth rate of Δ *asnB* in

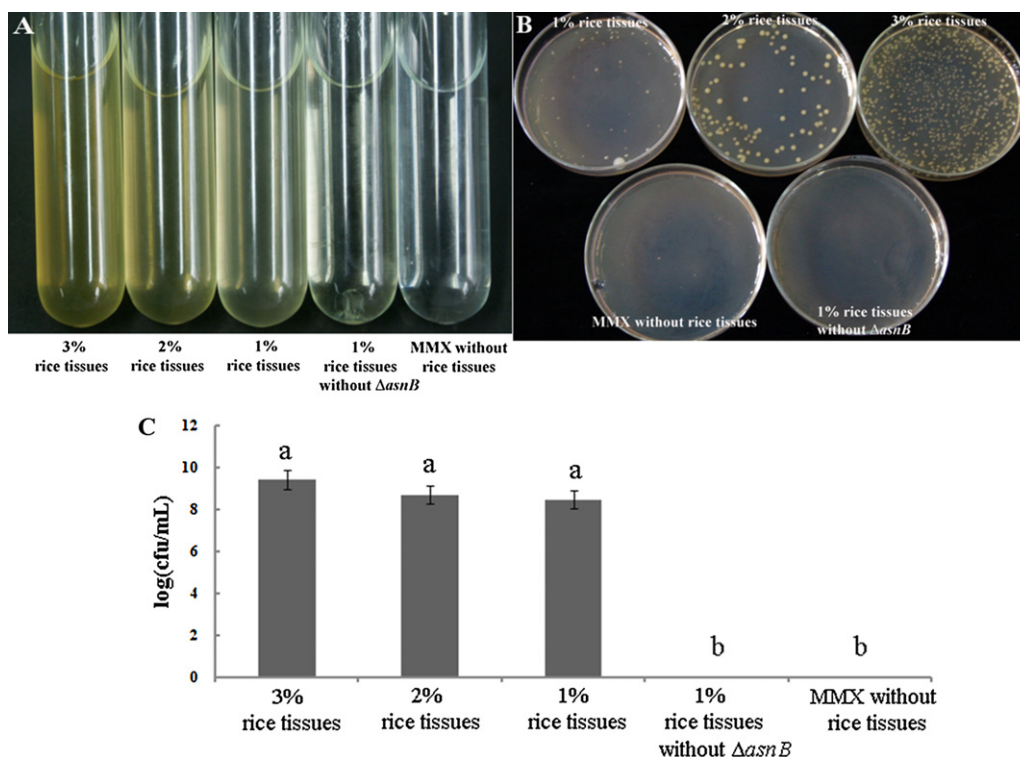


Fig. 4 Growth of the *asnB* mutant in MMX minimal medium was restored in the presence of rice tissue. (A) Representative photographs of growth of the *asnB* mutant in liquid MMX with or without different final concentrations of rice tissue (1%, 2% and 3%). (B) Representative photographs of growth of the *asnB* mutant on MMX plates with or without different final concentrations of rice tissue (1%, 2% and 3%). (C) Calculated data for growth of the *asnB* mutant on MMX plates with or without different final concentrations of rice tissues (1%, 2% and 3%). The 1% rice tissue without the *asnB* mutant, and $\Delta asnB$ and MMX without rice tissue, but containing $\Delta asnB$, were used as negative controls in all experiments. Three replicates per treatment were used, and the experiment was repeated three times. Vertical bars represent standard errors. Different letters above the bars indicate a significant difference between the rice tissue treatments and the control ($P < 0.05$; *t*-test). cfu, colony-forming unit.

MMX (–) medium (a minimal medium without ammonium sulphate as the nitrogen source) was assessed when the medium was amended with other nitrogen sources. As shown in Fig. 5, the ability of $\Delta asnB$ to grow in MMX (–) medium containing peptone and yeast extract was partially restored. Other nitrogen sources (glutamine, glutamate, urea, potassium nitrate and alanine) did not restore the growth rate of $\Delta asnB$ under the same conditions. Taken together, these results indicate that *asnB* is involved in aspartate metabolism.

The *asnB* mutation impairs bacterial resistance to hydrogen peroxide (H_2O_2) and reduces the expression of three genes responsible for H_2O_2 sensing and detoxification in *Xoc*

Sensing, detoxification and adaptation to oxidative stress play critical roles during successful pathogen infection and pathogenesis in *Xanthomonas* (Charoenlap *et al.*, 2011). We conducted the following experiments to understand the role of *asnB* in resistance to H_2O_2 . As shown in Fig. 6, the *asnB* mutant clearly displayed a greater sensitivity than the Rs105 wild-type strain and

$\Delta asnB$ (*asnB*) complemented strain to H_2O_2 . Furthermore, the expression of three important genes (*kagA*, *katG* and *oxyR*), which are responsible for H_2O_2 sensing and detoxification, clearly decreased in the *asnB* mutant compared with that in the Rs105 wild-type strain in the presence of 0.1 M H_2O_2 (Fig. 7A). However, we observed that the expression of only one gene (*katG*) decreased remarkably in the *asnB* mutant compared with that in the wild-type strain in NB without H_2O_2 (Fig. 7B)

asnB is regulated by QS and the Clp global regulator

In a previous proteomic study, it was evident that AsnB is regulated by DSF-mediated QS in *Xoc* (Zhao *et al.*, 2011). To understand whether *asnB* is regulated by DSF at the mRNA level, the *asnB* transcriptional levels in the Rs105 wild-type strain and $\Delta rpfF$ DSF-deficient strain were measured. As shown in Fig. 8, the transcriptional level of the *asnB rpfF* mutant decreased relative to that in the Rs105 wild-type strain. Furthermore, the *asnB* transcriptional level decreased in the *clp* mutant (Fig. 8). These results indicate that *asnB* is positively regulated by both DSF and the Clp global regulator.

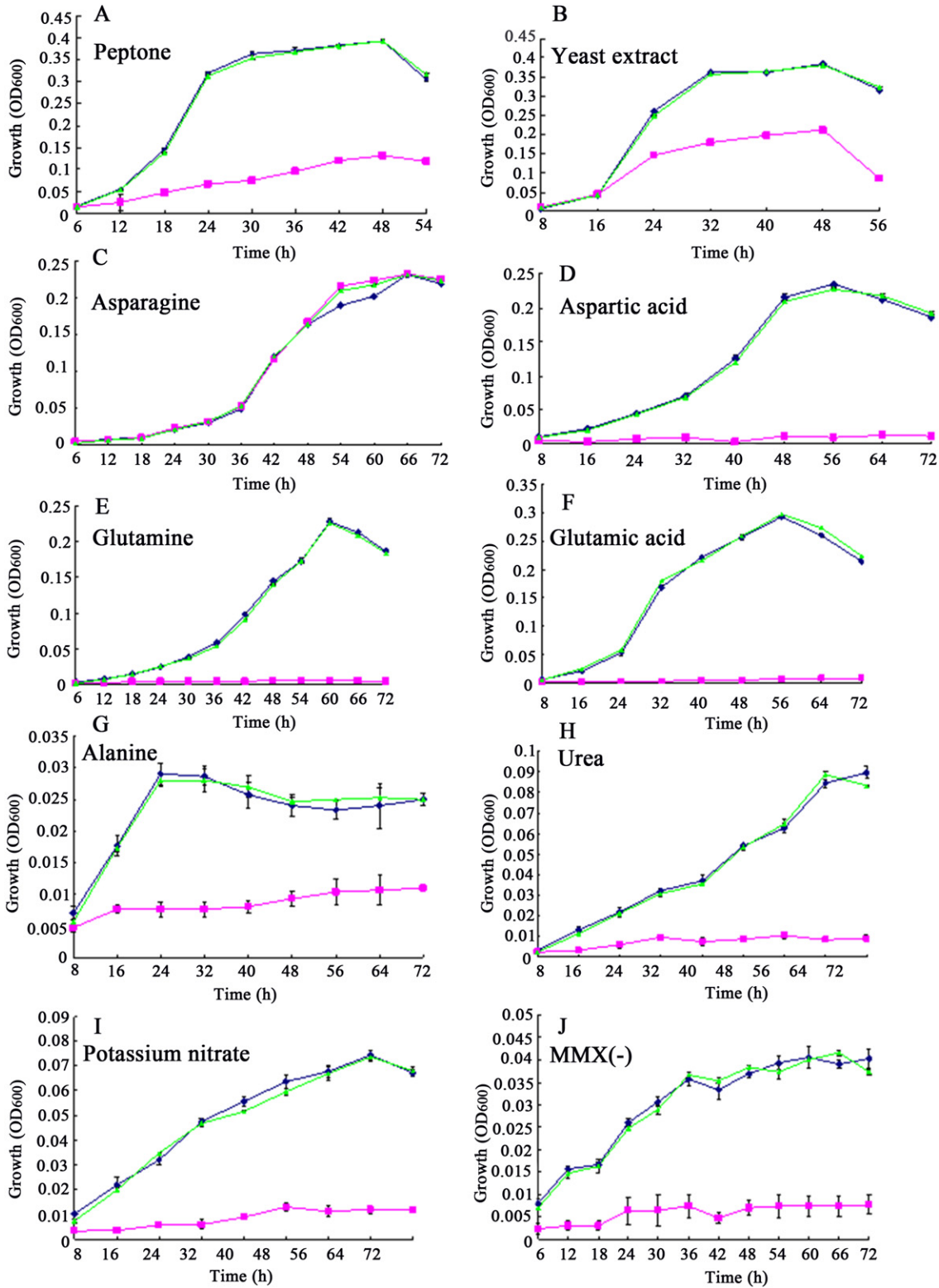


Fig. 5 Growth rate of *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) strains in MMX (-) basic medium supplemented with different nitrogen sources. The Rs105 wild-type strain (blue), *asnB* deletion mutant (pink) and corresponding complemented strain (green) were tested with 10 nitrogen sources to evaluate the role of *asnB* in *Xoc* nitrogen utilization. MMX (-), MMX medium without ammonium sulphate. Three replicates for each treatment were used, and the experiment was repeated three times. Vertical bars represent standard errors.

Fig. 6 *asnB* mutations impair resistance to H_2O_2 in *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*). *Xoc* strains, including the wild-type strain Rs105, the *asnB* deletion mutant Δ *asnB* and its complemented strain Δ *asnB*(*asnB*), were grown on nutrient broth (NB) agar plates with 0 mM H_2O_2 (A), 0.1 mM H_2O_2 (B) or 0.25 mM H_2O_2 (C). Three replicates for each treatment were used, and the experiment was repeated three times.

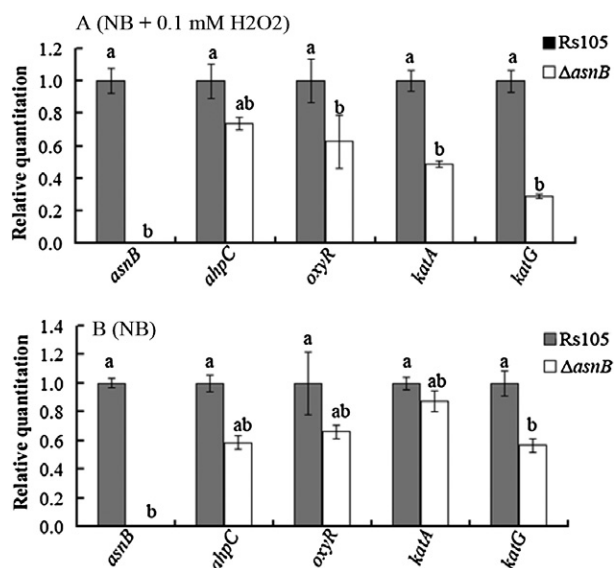
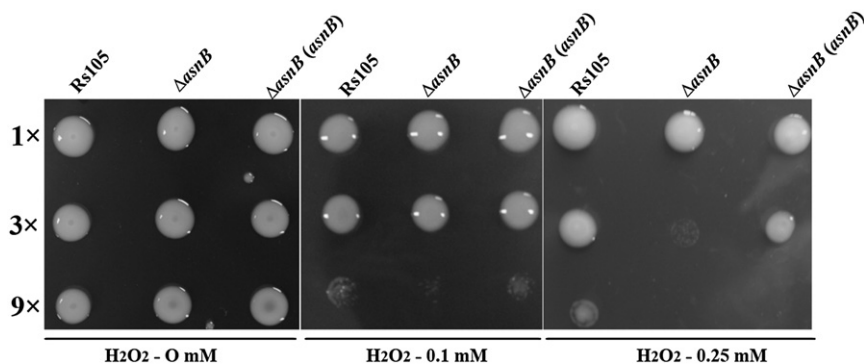


Fig. 7 *asnB* mutation reduces *KatA*, *katG* and *oxyR* transcriptional expression in *Xanthomonas oryzae* pv. *oryzicola* in the presence of hydrogen peroxide (H_2O_2). Total RNA was extracted from strains Rs105 and Δ *asnB* and cultured on nutrient broth (NB) plus 0.1 mM H_2O_2 (A) and NB (B). Three replicates for each treatment were used, and the experiment was repeated three times. Vertical bars represent standard errors. Different letters above the bars indicate a significant difference between the wild-type strain and *asnB* deletion mutant ($P < 0.05$; *t*-test).

Transcriptional expression of nine known virulence-associated genes is reduced in the *asnB* deletion mutant in plant cell-mimicking XOM3 medium, but not in NB medium

To date, a series of important virulence-associated genes, including *purF* (encodes amidophosphoribosyltransferase), *wxolB* (encodes putative glycosyltransferase), *pgk* (encodes phosphoglycerate kinase), *thrC* (encodes threonine synthase), *gapA* (encodes glyceraldehyde-3-phosphate dehydrogenase, type I), *tal-C10c-like* (encodes a transcriptional activator-like TAL effector), *trpA* (encodes tryptophan synthase α subunit), *dsbC* (encodes disulphide isomerase), *rfaA* (encodes glucose-1-phosphate thymidyltransferase) and *atmR* (encodes aminotransferase), has been

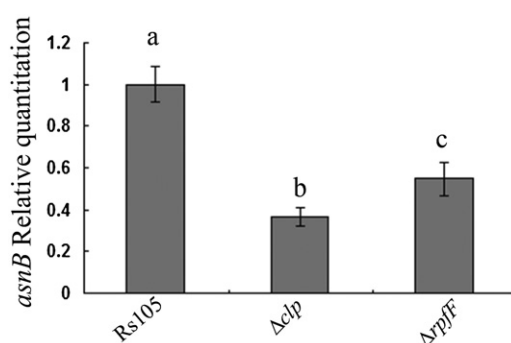


Fig. 8 *asnB* transcription is positively regulated by diffusible signal factor (DSF) and the Clp global regulator. Differential *asnB* expression was confirmed by relative quantitative real-time polymerase chain reaction (PCR). Total RNA was extracted from *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) strains Rs105, Δ *prpF* (DSF-deficient strain) (Zhao *et al.*, 2011) and Δ *clp* (Zhao *et al.*, 2012) at the mid-stage of growth [optical density at 600 nm ($OD_{600\text{ nm}}$) = 1.6]. Three replicates for each treatment were used, and the experiment was repeated three times. Vertical bars represent standard errors. Different letters above the bars indicate a significant difference between the wild-type strain and Δ *prpF* or Δ *clp* ($P < 0.05$; *t*-test).

identified and analysed in *Xoc* (Guo *et al.*, 2012; Wang *et al.*, 2007). A corresponding quantitative real-time reverse transcription-PCR (qRT-PCR) experiment was performed between the wild-type and *asnB* deletion mutant when both were cultivated in NB and plant cell-mimicking medium (XOM3) to investigate whether *asnB* has a regulatory role for transcriptional expression of these known virulence-associated genes in *Xoc*. We observed that deletion of *asnB* reduced significantly the transcriptional expression of the nine known virulence-associated genes (*purF*, *wxocB*, *pgk*, *gapA*, *thrC*, *tal-C10c-like*, *trpA*, *rfaA* and *atmR*) in plant cell-mimicking XOM3 medium (Fig. 9B), whereas all selected genes exhibited wild-type expression levels in NB medium (Fig. 9A).

AsnB is not involved in motility, chemotaxis, EPS or protease production in *Xoc*

Motility, chemotaxis, EPS and proteases are associated with virulence in *Xanthomonas* (Chen *et al.*, 2009; He *et al.*, 2006; Tang

et al., 1991; Yin *et al.*, 2011). Because the *asnB* mutation in *Xoc* led to lost virulence ability in the wild-type strain in host rice, it was reasonable to investigate whether or not the *asnB* mutation affected these virulence-associated functions that result in virulence deficiency of the *asnB* mutant in rice (detailed methods were provided in part B, C and D of Supplementary File 1). Interestingly, the *asnB* mutation did not affect EPS production (Fig. S1-2A, see Supporting Information), protease activity (Fig. S1-2B), chemotaxis (Fig. S1-3) or motility (Fig. S2-7, see Supporting Information) in *Xoc*.

DISCUSSION

In this study, we demonstrated, for the first time, that *asnB*, encoding an asparagine synthetase, is involved in aspartate metabolism, resistance to H₂O₂ and virulence in *Xoc*. Two families of asparagine synthetases have been identified in bacteria (Yoshida *et al.*, 1999). One is the AsnA family, such as the AsnA of *Escherichia coli*, whose members are found in prokaryotes, such as *E. coli* and *Klebsiella aerogenes* (Humbert and Simoni, 1980; Reitzer and Magasanik, 1982). Members of the AsnA family are only able to use ammonia as the amino group donor (Yoshida *et al.*, 1999). The other is the AsnB family, such as the AsnB protein in *E. coli* (Hughes *et al.*, 1997; Ren and Liu, 2006). Compared with the AsnA family, AsnB family members are found in both prokaryotes and eukaryotes, and are able to use both glutamine and ammonia as nitrogen donors, but glutamine is preferred (Hughes *et al.*, 1997; Ren and Liu, 2006). *Escherichia coli* and *K. aerogenes* have two asparagine synthetase genes, *asnA* and *asnB*, and the presence of either ensures sufficient asparagine biosynthesis, whereas disruption of both causes asparagine auxotrophy (Humbert and Simoni, 1980; Reitzer and Magasanik, 1982; Yoshida *et al.*, 1999). A bioinformatic analysis revealed that no AsnA homologue was detected in the *Xoc* genome. Further analysis indicated that the AsnA protein is absent in all published *Xanthomonas* species genomes, unlike for *E. coli*. However, a BLASTP search revealed that a number of *Xanthomonas* species, including *Xoo*, *X. axonopodis* pv. *citri*, *X. campestris* pv. *vesicatoria*, *X. fuscans*, *X. vesicatoria*, *X. gardneri* and *X. campestris* pv. *campestris*, contain a protein with >95% amino acid identity and similar size to AsnB found in *Xoc*. *Xanthomonas*-related species, such as *Xylella fastidiosa* and *Stenotrophomonas maltophilia*, also contain a protein with 80%–90% amino acid identity and a similar size to AsnB of *Xoc*. Taken together, these results indicate that AsnB is highly conserved in *Xanthomonas* and the related genera *Xylella* and *Stenotrophomonas*. However, the role(s) of the AsnB homologue in these bacterial species remains to be investigated.

Another defined asparagine synthetase (*Xoc_2999*) is found in the *Xoc* strain BLS256 genome, named AsnB2. A domain analysis of AsnB2 (564 amino acids) using the Pfam database (<http://pfam.sanger.ac.uk/>) showed that AsnB2 contains two domains

(Fig. S2-1): one is the glutamine amidotransferase_7 domain, at positions 50–170, and the other is the asparagine synthase domain, at positions 214–458. These two domains were also detected in the AsnB protein. An alignment analysis showed only 33% similarity between AsnB and AsnB2 at the amino acid level.

Furthermore, similar to AsnB, AsnB2 was also highly conserved, with 95%–99% similarity at the amino acid level to its counterparts in several available *Xanthomonas* genomes, including *Xoo* strain KACC 10331 (AE013598.1), *X. campestris* pv. *vesicatoria* strain 85-10 (NC_007508.1), *X. campestris* pv. *campestris* strain B100 (NC_010688.1) and *X. perforans* strain 91–118 (NZ_AEQW01000228.1).

The *asnB* mutation in *Mycobacterium smegmatis* leads to sensitivity of the wild-type strains to multiple antibiotics, including rifampicin, erythromycin, novobiocin and fusidic acid (Ren and Liu, 2006). However, our results showed that the *asnB* and *asnB2* mutations (Fig. S2-2) in *Xoc* did not impair bacterial resistance to rifampicin, gentamicin and kanamycin (Figs S1-1 and S2-3; detailed method was provided in part A of Supplementary File 1). These results indicate that the role of *asnB* and *asnB2* in bacterial resistance to antibiotics might be diverse in different bacterial species. Exogenous supplementation of asparagine into minimal medium as the sole nitrogen source facilitated the growth rate of the *Xoc* *asnB* and *asnB2* mutants (Figs 3 and S2-4), which was significantly different from the observation in *M. smegmatis* (Ren and Liu, 2006). These results also indicate that both *asnB* and *asnB2* are involved in aspartate metabolism in *Xoc*. Furthermore, the addition of other nitrogen sources did not restore the growth rate of the *asnB* mutant to the wild-type level in the corresponding medium (Fig. 5), indicating that AsnB might not be related to the metabolism of other nitrogen sources in *Xoc* under *in vitro* conditions.

The *asnB* mutant of the Gram-positive bacterium *Bacillus subtilis* shows poorer growth than that of the wild-type strain (Yoshida *et al.*, 1999), which was also observed in *Xoc* when the *asnB* mutant was grown in MMX basic medium. These results indicate that the contribution of *asnB* to bacterial growth might be conserved in *Bacillus* and *Xanthomonas*. Interestingly, the growth rate of the *asnB* mutant in rice leaves decreased, but was not deficient (Fig. 3B), which is clearly different from the *asnB* mutant in MMX basic medium. This result indicates that another mechanism(s) might be involved in aspartate metabolism when *Xoc* is grown in rice leaves. Interestingly, supplementation with the rice leaf extracts restored the growth deficiency of the *asnB* mutant in MMX basic medium (Fig. 4), indicating that a component(s) from rice leaves might restore the *asnB* mutant defect in aspartate metabolism, resulting in restored growth ability in the host rice. Whether this component(s) was identical to asparagine should be investigated further. More interestingly, supplementation with complex nitrogen sources (peptone and yeast extract) partially restored the growth ability of the *asnB* mutant. This indicates that *Xoc* might possess other *asnB*-independent mechanism(s)

involved, at least partially, in aspartate metabolism (Fig. 5), which requires further investigation.

Oxidative stress plays a crucial role in plant defence against invasion and colonization by pathogens (Baker and Orlandi, 1995). To survive and proliferate, pathogenic bacteria must overcome the plant defence response (Charoenlap *et al.*, 2011). Four proteins in *Xanthomonas*, including KatA (encoding a monofunctional catalase), KatG (encoding a catalase-peroxidase), AhpC (alkyl hydroperoxide reductase) and OxyR (encoding an H₂O₂ sensor and a LysR-type transcriptional regulator) are involved in the bacterial H₂O₂ sensing and protective system (Charoenlap *et al.*, 2011; Jitawuttipoka *et al.*, 2009). Furthermore, the *X. campestris* pv. *campestris* *katA*, *oxyR*, and *ahpC* mutations impair pathogen virulence in Chinese radish (*Raphanus sativus*) (Charoenlap *et al.*, 2011). Similarly, the disruption of *oxyR* impairs bacterial virulence in *Xoc* (Guo *et al.*, 2012). In the present study, the *Xoc asnB* mutation impaired bacterial resistance to H₂O₂ and virulence (Figs 2 and 6), whereas the *asnB2* mutation did not impair wild-type antioxidative ability or virulence (Fig. S2-5 and S2-6), indicating that *asnB*, not *asnB2*, plays a critical role in antioxidative ability and virulence in *Xoc*. Furthermore, *katA*, *katG* and *oxyR* expression in the presence of H₂O₂, which mimics the *Xoc* initial infection condition in rice cells, was positively regulated by *asnB* (Fig. 7). We assumed that the reduction in *katA*, *katG* and *oxyR* expression in the *asnB* mutant might contribute to the sensitivity of the *asnB* mutant to an increase in H₂O₂ (Fig. 6) and virulence deficiency (Fig. 2) in *Xoc*. However, further investigation is needed to clarify how *asnB* affects the expression of these genes.

Current evidence indicates that the global transcriptional regulator Clp is located downstream of the RpfC/RpfG two-component system in the DSF signalling pathway (He *et al.*, 2007; Mole *et al.*, 2007), which is involved in the regulation of virulence, resistance to oxidative stress, extracellular enzymes and EPS biosynthesis (He *et al.*, 2007; Zhao *et al.*, 2011). In the present study, the *asnB* mutant displayed similar virulence and resistance phenotypes to those of oxidative stress in the *Xoc clp* mutant reported by Zhao *et al.* (2012). These overlapping functions of *clp* and *asnB* indicate that a hierarchical relationship may exist between *clp* and *asnB* in the DSF signalling pathway in *Xoc*. qRT-PCR showed that *asnB* transcriptional expression was regulated by *clp* in *Xoc*. Taken together, these results indicate that *asnB* might be located downstream of *clp*. Furthermore, Crp regulates the expression of downstream genes in *E. coli* by binding directly to the motif (TGTTGANNNNNTCACCA) of the target gene promoter (Zheng *et al.*, 2004). Interestingly, an imperfect Clp (Crp-like protein) binding motif (TGCGGGTTTTTTATG), spaced 68 bp away from the 'ATG' *asnB* start codon, was found in the *asnB* promoter region, indicating that Clp might regulate *asnB* expression by binding directly to its promoter in *Xoc*.

In addition, two transcriptional regulators in *X. campestris* pv. *campestris*, named Zur and FhrR (a TetR-type transcription factor),

are located downstream of Clp and are involved in the regulation of a wide range of Clp regulons (He *et al.*, 2007). Whether Clp regulates *asnB* expression via Zur or in a FhrR-dependent manner should be investigated further in *Xoc*.

Because the *asnB* mutant simply showed a decrease in growth in rice leaves, but was not deficient, the contribution of *asnB* to virulence was only partially dependent on growth. This also indicates that other unknown mechanism(s) might contribute to virulence in the *asnB* mutant. We observed that the expression of nine important known virulence-related genes was reduced in the *asnB* mutant in XOM3 plant cell-mimicking medium (Fig. 9), indicating

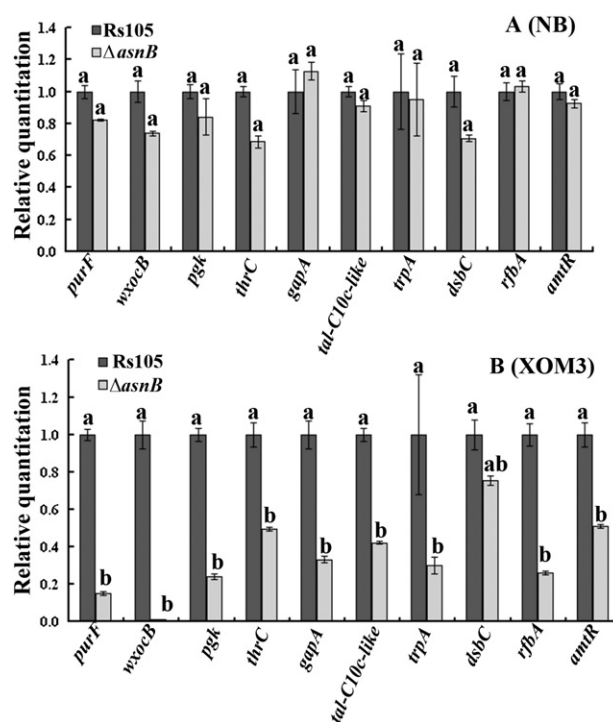


Fig. 9 Transcriptional expression of 10 known *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) virulence-associated genes in the *asnB* deletion mutant and wild-type strain. (A) Comparison of transcriptional expression of 10 known *Xoc*-associated genes cultivated in nutrient-rich broth (NB) between the *asnB* deletion mutant and the wild-type strain. Total RNA was extracted from the Rs105 wild-type strain of *Xoc* and *ΔasnB* (*asnB* deletion mutant) at the mid-stage of growth [optical density at 600 nm ($OD_{600\text{ nm}}$) = 1.6]. (B) Comparison of the transcriptional expression of 10 known *Xoc*-associated genes cultivated in plant cell-mimicking broth (XOM3) between the *epv* deletion mutant and the Rs105 wild-type strain. The *Xoc* strains were preincubated in NB medium overnight, resuspended at $OD_{600\text{ nm}} = 2.0$ in XOM3 medium and washed twice. Then, 2 mL of the bacterial suspension was inoculated into 50 mL of XOM3 plant cell-mimicking broth (pH 6.5) at 28 °C for 16 h (Li *et al.*, 2011). Total RNA was extracted from the Rs105 wild-type strain of *Xoc* and *ΔasnB* (*asnB* deletion mutant). Three replicates for each treatment were used, and the experiment was repeated three times. Vertical bars represent standard errors. Different letters above the bars indicate a significant difference between the wild-type strain and *asnB* deletion mutant ($P < 0.05$; *t*-test).

that *asnB* has a wide impact on virulence-related gene expression. More importantly, seven of these nine genes, including *pgk*, *thrC*, *gapA*, *tal-C10c-like*, *trpA*, *rfbA* and *atmR*, have been described as being responsible for *Xoc* virulence, but not as being involved in the induction of HR in nonhost (plant) tobacco, which was consistent with the result of *asnB* during HR induction.

HR, EPS production, motility, protease activity and chemotaxis are important virulence-associated functions in *Xoc* (Chen *et al.*, 2009; Dharmapuri and Sonti, 1999; Zou *et al.*, 2006). However, we found that the *asnB* mutation did not impair these virulence-associated functions (Figs 2, S1-2, S1-3 and S2-7), indicating that the contribution of *asnB* to virulence was independent of HR, EPS, motility, protease activity and chemotaxis in *Xoc*.

In summary, we hypothesize that *asnB* is regulated by a DSF-dependent QS located downstream of the Clp global regulator. The ability of this protein to contribute to pathogen virulence was dependent, at least partially, on the regulation of bacterial anti-oxidative ability and the expression of several critical virulence-related genes in *Xoc*.

EXPERIMENTAL PROCEDURES

Strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultivated at 37 °C in Luria–Bertani (LB) medium or on LB agar plates. Unless specified otherwise, *Xanthomonas* strains were grown at 28 °C in NB medium (beef extract, 3 g/L; yeast

extract, 1 g/L; polypeptone, 5 g/L; sucrose, 10 g/L) or on nutrient agar (NA). MMX [glucose, 5 g/L; (NH₄)₂SO₄, 2 g/L; MgSO₄·7H₂O, 0.2 g/L; K₂HPO₄, 4 g/L; KH₂PO₄, 6 g/L; trisodium citrate, 1 g/L; pH 7.0] was used as minimal medium. Antibiotics were added to the medium at the following final concentrations when required: 100 µg/mL ampicillin and 50 µg/mL kanamycin (Km) for *E. coli* and 100 µg/mL rifampicin (Rif) and 50 µg/mL Km for *Xanthomonas campestris* 8523 (pKLN55) and *Xoc*.

Generation of the *asnB* deletion mutant in *Xoc*

The *Xoc* Rs105 wild-type strain was used as the parental strain to generate the in-frame deletion mutant via allelic homologous recombination. In-frame deletion of *asnB* was performed as described previously (Zhao *et al.*, 2011). Briefly, two *asnB* flanking regions were generated by PCR using the primer pairs *asnB*-1F/*asnB*-1R and *asnB*-2F/*asnB*-2R (Table 2). The *asnB*-1 fragment (digested with *Bam*HI and *Kpn*I) and the *asnB*-2 fragment (digested with *Kpn*I and *Xba*I) were ligated into pK18mobsacB and digested with *Bam*HI and *Xba*I. This recombinant vector, designated pK18SA, was transformed into the Rs105 wild-type strain by electroporation. Transconjugants were selected on NA plates without sucrose, but with Rif (100 µg/mL) and Km (50 µg/mL). Positive colonies were plated onto NA plates containing 10% (w/v) sucrose and Rif (100 µg/mL) to select for resolution of the construct by a second cross-over event. The resulting mutant, containing the *asnB* in-frame deletion, was confirmed by PCR and Southern blotting (data not shown). One of the confirmed mutants, named Δ *asnB*, was selected for further study.

Complementation of the *asnB* mutant

The *asnB* mutation was complemented as described previously (Chen *et al.*, 2009; Zhao *et al.*, 2011, 2012). Briefly, a 2110-bp DNA fragment

Table 1 Strains, plasmids and primers used in this study.

Strains, plasmids and primers	Characteristics	Source
Strains		
<i>Escherichia coli</i> DH5 α	Φ 80 <i>lacZ</i> Δ M15, Δ (<i>lacZYA-argF</i>)U169. <i>recA</i> 1	Laboratory collection
Rs105	Rif ^R , wild-type strain of <i>Xanthomonas oryzae</i> pv. <i>oryzicola</i>	Laboratory collection
Δ <i>rpfF</i>	Rif ^R , deletion of <i>rpfF</i> in wild-type strain Rs105	Zhao <i>et al.</i> (2011)
Δ <i>clp</i>	Rif ^R , deletion of <i>clp</i> in wild-type strain Rs105	Zhao <i>et al.</i> (2011)
Δ <i>asnB</i>	Rif ^R , deletion of <i>asnB</i> in wild-type strain Rs105	This study
Δ <i>asnB2</i>	Rif ^R , deletion of <i>asnB2</i> in wild-type strain Rs105	This study
Δ <i>asnB</i> (<i>asnB</i>)	Rif ^R , Km ^R , complemented strain of Δ <i>asnB</i>	This study
Plasmids		
pK18mobsacB	Km ^R , allelic exchange suicide vector, <i>sacB</i> oriT(RP4)	Zou <i>et al.</i> (2011)
pK18SA	Km ^R , a 1238-bp fusion fragment of <i>asnB</i> -1, <i>asnB</i> -2 gene ligated in pK18mobsacB	This study
pHMI	Sp ^R , broad-host-range cosmid vector, pSa <i>ori</i>	Laboratory collection
pHMIA5	Sp ^R , <i>asnB-H</i> gene ligated in pHMI	This study
Primers		
<i>asnB</i> -1F	5'-CGGGATCCCCAAGGCATCTATCTCTCGG-3'	This study
<i>asnB</i> -1R	5'-GGGGTACCGCAGACCAAGGAAGCGTATT-3'	This study
<i>asnB</i> -2F	5'-GGGGTACCATCTGCCATTGACCGCCAG-3'	This study
<i>asnB</i> -2R	5'-GCTCTAGACAACGCCAGCATTATCAGG-3'	This study
<i>asnBH</i> -F	5'-CCAAGCTTCGCTGTATCGCAGCTGATC-3'	This study
<i>asnBH</i> -R	5'-CGGAATCCACGAGCAATAAGACACGCG-3'	This study
<i>asnB2</i> -1F	5'-CGGGATCCTGTTAGATACGCCAGCACG-3'	This study
<i>asnB2</i> -1R	5'-GGGGTACCTGTGGACGGTCTGATGTT-3'	This study
<i>asnB2</i> -2F	5'-GGGGTACCGCAAGCGAGCAACAGTCT-3'	This study
<i>asnB2</i> -2R	5'-GCTCTAGACCAAGAATCCCATCGCAAG-3'	This study

Table 2 Primers for quantitative real-time polymerase chain reaction (PCR).

Gene fragment	Primer sequence for 5' sequence	Primer sequence for 3' sequence	Primer source	Gene source
16S rRNA	5'-AATGGGCGCAAGCCTGATC-3'	5'-AACCACCACCTACGCACGC-3'	Zhao <i>et al.</i> (2011)	Zhao <i>et al.</i> (2011)
<i>katA</i> (<i>Xoryp_4325</i>)	5'-ATATCCGGAGGCAGCAAAGT-3'	5'-AATTCCTTGAACGGCGTATG-3'	This study	Charoenlap <i>et al.</i> (2011)
<i>katG</i> (<i>Xoryp_3410</i>)	5'-AGTGGTCCGTGCTGATCTTC-3'	5'-ATAGTTGTCGGCAGCGTCTT-3'	This study	Charoenlap <i>et al.</i> (2011)
<i>oxyR</i> (<i>Xoryp_0957</i>)	5'-CAGATGGCAGACATCCAGTG-3'	5'-TTCGAAGAACCTTTCGTGCT-3'	This study	Charoenlap <i>et al.</i> (2011)
<i>ahpC</i> (<i>Xoryp_0959</i>)	5'-CAGACCGATGGACTCCTT-3'	5'-CGACCAGTACGGTCAATTCC-3'	This study	Jittawuttiopoka <i>et al.</i> (2009)
<i>asnB</i> (<i>Xoryp_3054</i>)	5'-CTTCCGTCTTATCCACAGC-3'	5'-CTACAACCATCGCGAACTCA-3'	This study	This study
<i>tal-C10c-like</i> (<i>Xoryp_4384</i>)	5'-ACCGTGATGTGGGAACAAGATG-3'	5'-GAGGCAATAGCTCCCTCAACC-3'	Laboratory collection	Guo <i>et al.</i> (2012)
<i>dsbC</i> (<i>Xoryp_3811</i>)	5'-GCATTCAAGGATTCGGTGCG-3'	5'-ATGGTGAGCGGGCGGTCTTC-3'	Laboratory collection	Guo <i>et al.</i> (2012)
<i>wxocB</i> (<i>Xoryp_3864</i>)	5'-CCGCGTACTGGACGATAAGT-3'	5'-TGGATAAAGAGCGTTGACAC-3'	Laboratory collection	Wang <i>et al.</i> (2007)
<i>pgk</i> (<i>Xoryp_3589</i>)	5'-CGCAGTGGACAAGTACGACA-3'	5'-CTTGCTCCAGAACTCCA-3'	Laboratory collection	Wang <i>et al.</i> (2007)
<i>gapA</i> (<i>Xoryp_3592</i>)	5'-GCTGGGGTATACCGAAGACA-3'	5'-TCGTTGTCGTACCAGGACAC-3'	Laboratory collection	Wang <i>et al.</i> (2007)
<i>rfbA</i> (<i>Xoryp_3845</i>)	5'-GGCGTTGTTCAATCCTTG-3'	5'-AGTCGCGACCGATCAAATAC-3'	Laboratory collection	Guo <i>et al.</i> (2012)
<i>atmR</i> (<i>Xoryp_2825</i>)	5'-GTCGGTGGGCAAGTGCTGTA-3'	5'-GACGATGCGGTGCGCTTT-3'	Laboratory collection	Guo <i>et al.</i> (2012)
<i>thrC</i> (<i>Xoryp_2208</i>)	5'-ATGCGTTGGCGTTGATTCTG-3'	5'-CGATGGCCTTCCACCCT-3'	Laboratory collection	Guo <i>et al.</i> (2012)
<i>purF</i> (<i>Xoryp_1105</i>)	5'-CAGGATCTGGAAGACCTGGA-3'	5'-TGATGTACTCGCGTTGAAG-3'	Laboratory collection	Guo <i>et al.</i> (2012)
<i>trpA</i> (<i>Xoryp_2950</i>)	5'-CTTGCGTACGTGTTGGAAGC-3'	5'-TCTCGATCGGGTTGAGGTAG-3'	Laboratory collection	Guo <i>et al.</i> (2012)

containing *asnB* and its predicted promoter region was amplified from the Rs105 wild-type strain with *asnBH-F/asnBH-R* primers. The amplified fragment was digested with the *HindIII/EcoRI* enzyme and cloned into *HindIII/EcoRI*-digested pHMI, resulting in the pHMIAS plasmid (Table 1) used for complementation. Plasmid pHMIAS was transformed into Δ *asnB* competent cells by electroporation. Finally, one positive complemented strain, named Δ *asnB(asnB)*, was selected on NA plates with Rif (100 μ g/mL) and Km (50 μ g/mL) for further study.

Pathogenicity testing and determination of bacterial load in plants

Pathogenicity assays were conducted in a glasshouse at 22–30 °C as described previously (Zhao *et al.*, 2011, 2012; Zou *et al.*, 2006). Briefly, *Xoc* strains were cultivated in NB medium with appropriate antibiotics at 28 °C. The strains were adjusted to an optical density at 600 nm ($OD_{600\text{ nm}}$) ~ 0.1 and inoculated into leaves of 6-week-old rice plants (Shanyou-63, susceptible to the pathogen) using leaf needling. Lesion lengths were measured 14 days after inoculation. Twenty-five leaves were inoculated for each *Xoc* strain in each treatment. The same experiment was repeated three times.

The growth of each *Xoc* strain in rice leaf tissue was detected by homogenizing five inoculated leaves in 9 mL of sterile water. The leaves were cut into 6-mm sections around the inoculation spots on days 0, 4, 8, 12 and 16 after inoculation (Lee *et al.*, 2008). Diluted homogenates were plated onto NA plates supplemented with Rif (for the wild-type and mutants) or Rif plus Km (for the complemented strains). The number of bacterial colonies on these plates was counted after a 2-day incubation at 28 °C (Feng *et al.*, 2009). Each diluted homogenate was plated onto three plates. Three replicates for each treatment were used, and the experiment was repeated three times.

H₂O₂ resistance assay

H₂O₂ resistance assays were performed as described previously (Lan *et al.*, 2010), with some modifications. NB agar plates were prepared containing H₂O₂ concentrations of 0, 0.1 and 0.25 mM. *Xoc* strains were cultured to

the mid-logarithmic phase ($OD_{600} = 1.0$) in NB medium, and three-fold and nine-fold dilutions were made. A 5- μ L aliquot of the initial culture and diluted cultures for each strain were spotted onto NB agar plates (in triplicate) and cultured for 36 h at 28 °C (Lan *et al.*, 2010). The same experiment was repeated at least three times.

HR assay

Xoc strains were grown in NB medium at 28 °C with shaking at 200 rpm. Cells were pelleted at the early logarithmic phase by centrifugation at 6000 rpm. Cell pellets were suspended in water for HR or pathogenicity tests. The HR test was conducted as described previously (Zou *et al.*, 2006). Briefly, suspensions ($OD_{600\text{ nm}} = 0.1$) of *Xoc* strains were infiltrated into leaves of glasshouse-grown tobacco (*Nicotiana tabacum* L. cv. *Samsun*), and the results were observed after 48 h of infiltration. If the strain had the ability to trigger HR, the phenomenon of programmed cell death would be observed around the inoculation sites on tobacco leaves.

Growth determination of Δ *asnB* in MMX minimal medium supplemented with different nitrogen sources

asnB in bacteria encodes the enzyme that converts aspartic acid to asparagine. To confirm that the AsnB protein in *Xoc* is involved in aspartate metabolism, we tested the growth rate of Δ *asnB* in basic medium with or without aspartic acid, asparagine or other nitrogen sources. In brief, *Xoc* strains were grown in NB broth at 28 °C with shaking at 200 rpm. Cells were pelleted at the early logarithmic phase ($OD_{600\text{ nm}} = 0.5$) by centrifugation at 6000 rpm. Cell pellets were suspended in an equal volume of water. Then, 0.5 mL of cell suspension was inoculated into 100 mL of basic MMX medium broth with or without different nitrogen sources, including peptone, yeast extract, asparagine, aspartic acid, glutamine, glutamic acid, ammonium sulphate, urea, potassium nitrate and alanine. All inoculation broths were grown at 28 °C with shaking at 200 rpm, and the $OD_{600\text{ nm}}$ value was determined every 6 or 8 h until bacterial growth reached the stationary stage.

Determination of the growth status of the *asnB* mutant in MMX minimal medium broth supplemented with different concentrations of rice leaf extract

Rice leaves were extracted as described previously (Angela and Yoko, 2001), with some modifications. Briefly, susceptible IR24 rice leaves (500 g) at the tillering stage were selected, followed by sterilization with 75% (v/v) ethanol. After carefully milling and the addition of sterilized double-distilled H₂O, the constant volume of rice leaves was 1 L. The supernatant was collected after centrifugation (7000 rpm, 4 °C, 30 min) and was concentrated to 10 mg/mL using a vacuum freeze drier. In the following assay, the concentrated rice leaf extracts were added to MMX minimal medium at final concentrations of 1%, 2% and 3%. The *asnB* deletion mutant was inoculated into the prepared MMX broth with the rice leaf extracts to determine growth. The colony formation unit-based method was used to determine bacterial growth (Zhao *et al.*, 2012).

qRT-PCR

qRT-PCR was carried out using a SYBR Premix EX Tag™ II kit (TaKaRa Bio, Shiga, Japan) in an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). As the endogenous control, 16S rRNA was used. The primer sequences are listed in Table 2. RNA was extracted from *Xoc* wild-type strain Rs105, $\Delta rpfF$, Δclp and $\Delta asnB$ at different growth stages using the RNAiso Plus Reagent (TaKaRa Bio) following the manufacturer's instructions. To remove genomic DNA, the eluted RNA samples were treated with RNase inhibitors and DNaseI (TaKaRa Bio). RNA integrity was confirmed by electrophoresis on 1.2% agarose gels. Then, 2 µg of each RNA sample was used to synthesize cDNA with a cDNA Synthesis kit (TaKaRa Bio). The same experiment was repeated three times. The *asnB* transcriptional levels in the wild-type strain, $\Delta rpfF$ and Δclp were assessed and compared. The transcriptional levels of *katA* (*Xoryp_4325*), *katG* (*Xoryp_3410*), *oxyR* (*Xoryp_0957*), *aphC* (*Xoryp_0959*), *wxolB* (*Xoryp_3864*), *pgk* (*Xoryp_3589*), *gapA* (*Xoryp_3592*), *tal-C10c-like* (*Xoryp_4384*), *dsbC* (*Xoryp_3811*), *rfaA* (*Xoryp_3845*), *atmR* (*Xoryp_2825*), *thrC* (*Xoryp_2208*), *purF* (*Xoryp_1105*), *trpA* (*Xoryp_2950*) and *gumbB* (*Xoryp_1867*) in the wild-type strain and $\Delta asnB$ were also assessed and compared.

Data analysis

All analyses were conducted using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). The hypothesis test of percentages (*t*-test, *P* = 0.05) was used to determine significant differences in the pathogenicity test, gene expression and bacterial growth.

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REFERENCES

- Angela, M. and Yoko, B.R. (2001) Differentially expressed proteins in the interaction of *Xanthomonas axonopodis* pv. *citri* with leaf extract of the host plant. *Proteomics*, **1**, 1111–1118.
- Baker, C.J. and Orlandi, E.W. (1995) Active oxygen in plant pathogenesis. *Annu. Rev. Phytopathol.* **33**, 99–321.
- Barber, C.E., Tang, J.L., Feng, J.X., Pan, M.Q., Wilson, T.J., Slater, H., Dow, J.M., Williams, P. and Daniels, M.J. (1997) A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Mol. Microbiol.* **24**, 555–566.
- Boon, C., Deng, Y., Wang, L.H., He, Y.W., Xu, J.L., Fan, Y., Pan, S.Q. and Zhang, L.H. (2008) A novel DSF-like signal from *Burkholderia cenocepacia* interferes with *Candida albicans* morphological transition. *J. Int. Soc. Microb. Ecol.* **2**, 27–36.
- Büttner, D. and Bonas, U. (2010) Regulation and secretion of *Xanthomonas* virulence factors. *FEMS Microbiol. Rev.* **34**, 107–133.
- Charoenlap, N., Buranajitpakorn, S., Duang-Nkern, J., Namchaiw, P., Vattanaviboon, P. and Mongkolsuk, S. (2011) Evaluation of the virulence of *Xanthomonas campestris* pv. *campestris* mutant strains lacking functional genes in the OxyR regulon. *Curr. Microbiol.* **63**, 232–237.
- Chatterjee, S. and Sonti, R.V. (2002) *rpfF* mutants of *Xanthomonas oryzae* pv. *oryzae* are deficient for virulence and growth under low iron conditions. *Mol. Plant-Microbe Interact.* **15**, 463–471.
- Chatterjee, S., Newman, K.L. and Lindow, S.E. (2008) Cell-to-cell signaling in *Xylella fastidiosa* suppresses movement and xylem vessel colonization in grape. *Mol. Plant-Microbe Interact.* **21**, 1309–1315.
- Chen, L., Hu, B.S., Qian, G.L., Wang, C., Yang, W.F., Han, Z.C. and Liu, F.Q. (2009) Identification and molecular characterization of twin-arginine translocation system (Tat) in *Xanthomonas oryzae* pv. *oryzae* strain PXO99. *Arch. Microbiol.* **191**, 163–170.
- Danhorn, T. and Fuqua, C. (2007) Biofilm formation by plant-associated bacteria. *Annu. Rev. Microbiol.* **61**, 401–422.
- Deng, Y., Wu, J.E., Tao, F. and Zhang, L.H. (2011) Listening to a new language: DSF-based quorum sensing in Gram-negative bacteria. *Chem. Rev.* **111**, 160–173.
- Dharmapuri, S. and Sonti, R.V. (1999) A transposon insertion in the *gumG* homologue of *Xanthomonas oryzae* pv. *oryzae* causes loss of extracellular polysaccharide production and virulence. *FEMS Microbiol. Lett.* **179**, 53–59.
- Feng, J.X., Song, Z.Z., Duan, C.J., Zhao, S., Wu, Y.Q., Wang, C., Dow, J.M. and Tang, J.L. (2009) The *xrvA* gene of *Xanthomonas oryzae* pv. *oryzae*, encoding an H-NS-like protein, regulates virulence in rice. *Microbiology*, **155**, 3033–3044.
- Goo, E., Kang, Y.S., Kim, H.S. and Hwang, I. (2010) Proteomics analysis of quorum-sensing-dependent proteins in *Burkholderia glumae*. *J. Proteome Res.* **9**, 3184–3199.
- Guo, W., Cui, Y.P., Li, Y.R., Che, Y.Z., Yuan, L., Zou, L.F., Zou, H.S. and Chen, G.Y. (2012) Identification of seven genes of *Xanthomonas oryzae* pv. *oryzicola* potentially involved in pathogenesis in rice. *Microbiology*, **158**, 505–518.
- He, Y.W. and Zhang, L.H. (2008) Quorum sensing and virulence regulation in *Xanthomonas campestris*. *FEMS Microbiol. Rev.* **32**, 842–857.
- He, Y.W., Xu, M., Lin, K., Ng, Y.J., Wen, C.M., Wang, L.H., Liu, Z.D., Zhang, H.B., Dong, Y.H., Dow, J.M. and Zhang, L.H. (2006) Genome scale analysis of diffusible signal factor regulon in *Xanthomonas campestris* pv. *campestris*: identification of novel cell-cell communication-dependent genes and functions. *Mol. Microbiol.* **59**, 610–622.
- He, Y.W., Ng, A.Y., Xu, M., Lin, K., Wang, L.H., Dong, Y.H. and Zhang, L.H. (2007) *Xanthomonas campestris* cell-cell communication involves a putative nucleotide receptor protein Clp and a hierarchical signalling network. *Mol. Microbiol.* **64**, 281–292.
- Hughes, C.A., Beard, H.S. and Matthews, B.F. (1997) Molecular cloning and expression of two cDNAs encoding asparagine synthetase in soybean. *Plant Mol. Biol.* **33**, 301–311.
- Humbert, R. and Simoni, R.D. (1980) Genetic and biochemical studies demonstrating a second gene coding for asparagine synthetase in *Escherichia coli*. *J. Bacteriol.* **142**, 212–220.

- Jittawuttipoka, T., Buranajitpakorn, S., Vattanaviboon, P. and Mongkolsuk, S. (2009) The catalase-peroxidase KatG is required for virulence of *Xanthomonas campestris* pv. *campestris* in a host plant by providing protection against low levels of H₂O₂. *J. Bacteriol.* **191**, 7372–7377.
- Lan, L.F., Murray, T.S., Kazmierczak, B.I. and He, C. (2010) *Pseudomonas aeruginosa* OspR is an oxidative sensing regulator that affects pigment production, antibiotic resistance and dissemination during infection. *Mol. Microbiol.* **75**, 76–91.
- Lee, S.W., Jeong, K.S., Han, S.W., Lee, S.E., Phee, B.K., Hahn, T.R. and Ronald, P. (2008) The *Xanthomonas oryzae* pv. *oryzae* PhoPQ two-component system is required for AvrXA21 activity, *hrpG* expression, and virulence. *J. Bacteriol.* **190**, 2183–2197.
- Li, Y.R., Zou, H.S., Che, Y.Z., Cui, Y.P., Guo, W., Zou, L.F., Chatterjee, S., Biddle, E.M., Yang, C.H. and Chen, G.Y. (2011) A novel regulatory role of HrpD6 in regulating *hrp-hrc-hpa* genes in *Xanthomonas oryzae* pv. *oryzicola*. *Mol. Plant-Microbe Interact.* **24**, 1086–1101.
- Miller, M.B. and Bassler, B.L. (2001) Quorum sensing in bacteria. *Annu. Rev. Microbiol.* **55**, 165–199.
- Mole, B.M., Baltrus, D.A., Dangi, J.L. and Grant, S.R. (2007) Global virulence regulation networks in phytopathogenic bacteria. *Trends Microbiol.* **15**, 363–371.
- Niño-Liú, D.O., Ronald, P.C. and Bogdanove, A.J. (2006) *Xanthomonas oryzae* pathogens: model pathogens of a model crop. *Mol. Plant Pathol.* **7**, 303–324.
- Reitzer, L.J. and Magasanik, B. (1982) Asparagine synthetase of *Klebsiella aerogenes*: properties and regulation of synthesis. *J. Bacteriol.* **151**, 1299–1313.
- Ren, H.P. and Liu, J. (2006) AsnB is involved in natural resistance of *Mycobacterium smegmatis* to multiple drugs. *Antimicrob. Agents Chemother.* **50**, 250–255.
- Ryan, R.P., Vorhölter, F.J., Potnis, N., Jones, J.B., Van Sluys, M.A., Bogdanove, A.J. and Dow, J.M. (2011) Pathogenomics of *Xanthomonas* understanding bacterium-plant interactions. *Nat. Rev.* **9**, 344–355.
- Siciliano, F., Torres, P., Sendin, L., Bermejo, C., Filippone, P., Vellice, G., Ramallo, J., Castagnaro, A., Vojnov, A. and Marano, M.R. (2006) Analysis of the molecular basis of *Xanthomonas axonopodis* pv. *citri* pathogenesis in *Citrus limon*. *Electron. J. Biotechnol.* **9**, 200–204.
- Tang, J.L., Liu, Y.N., Barber, C.E., Dow, J.M., Wootton, J.C. and Daniels, M.J. (1991) Genetic and molecular analysis of a cluster of *rpf* genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris* pathovar *campestris*. *Mol. Gen. Genet.* **226**, 409–417.
- Wang, L., Makino, S., Subedee, A. and Bogdanove, A.J. (2007) Novel candidate virulence factors in rice pathogen *Xanthomonas oryzae* pv. *oryzicola* as revealed by mutational analysis. *Appl. Environ. Microbiol.* **73**, 8023–8027.
- Wang, L.H., He, Y.W., Gao, Y.F., Wu, J.E., Dong, Y.H., He, C.Z., Wang, S.X., Weng, L.X., Xu, J.L., Tay, L., Fang, R.X. and Zhang, L.H. (2004) A bacterial cell-cell communication signal with cross-kingdom structural analogues. *Mol. Microbiol.* **51**, 903–912.
- Watt, S.A., Wilke, A., Patschkowski, T. and Niehaus, K. (2005) Comprehensive analysis of the extracellular proteins from *Xanthomonas campestris* pv. *campestris* B100. *Proteomics*, **5**, 153–167.
- Yin, F.Q., Zhao, Y.C., Liu, C.H., Qian, G.L., Fan, J.Q., Hu, B.S. and Liu, F.Q. (2011) Analysis of the *flgD* and *flgE* genes regulated by diffusible signal factor in *Xanthomonas oryzae* pv. *oryzicola*. *Acta Microbiol. Sin.* **51**, 891–897.
- Yoshida, K.I., Fujita, Y. and Ehrlich, S.D. (1999) Three asparagine synthetase genes of *Bacillus subtilis*. *J. Bacteriol.* **181**, 6081–6091.
- Zhao, Y.C., Qian, G.L., Yin, F.Q., Fan, J.Q., Zhai, Z.W., Liu, C.H., Hu, B.S. and Liu, F.Q. (2011) Proteomic analysis of the regulatory function of DSF-dependent quorum sensing in *Xanthomonas oryzae* pv. *oryzicola*. *Microb. Pathog.* **50**, 48–55.
- Zhao, Y.C., Qian, G.L., Fan, J.Q., Liu, C.H., Yin, F.Q., Zhou, Y.J., Shen, Q., Hu, B.S. and Liu, F.Q. (2012) Identification and characterization of a novel gene hshB in *Xanthomonas oryzae* pv. *oryzicola* co-regulated by quorum sensing and *clp*. *Phytopathology*, **102**, 252–259.
- Zheng, D., Constantinidou, C., Hobman, J.L. and Minchin, S.D. (2004) Identification of the CRP regulon using *in vitro* and *in vivo* transcriptional profiling. *Nucleic Acids Res.* **32**, 5874–5893.
- Zou, L.F., Wang, X.P., Xiang, Y., Zhang, B., Li, Y.R., Xiao, Y.L., Wang, J.S., Walmsley, A.R. and Chen, G.Y. (2006) Elucidation of the *hrp* clusters of *Xanthomonas oryzae* pv. *oryzicola* that control the hypersensitive response in nonhost tobacco and pathogenicity in susceptible host rice. *Appl. Environ. Microbiol.* **72**, 6216–6224.
- Zou, L.F., Li, Y.R. and Chen, G.Y. (2011) A non-marker mutagenesis strategy to generate poly-*hrp* gene mutants in the rice pathogen *Xanthomonas oryzae* pv. *oryzicola*. *Agric. Sci. China*, **10**, 1139–1150.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

- Supplementary File 1** Outlines, Results, Figures S1-1 to S1-3.
Supplementary File 2 Figures S2-1 to S2-7.