The Rice Bacterial Pathogen *Xanthomonas oryzae* pv. *oryzae* Produces 3-Hydroxybenzoic Acid and 4-Hydroxybenzoic Acid via XanB2 for Use in Xanthomonadin, Ubiquinone, and Exopolysaccharide Biosynthesis

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Submitted 22 April 2013. Accepted 22 May 2013.

Xanthomonas oryzae pv. *oryzae*, the causal agent of rice bacterial blight, produces membrane-bound yellow pigments, referred to as xanthomonadins. Xanthomonadins protect the pathogen from photodamage and host-induced peroxidation damage. They are also required for epiphytic survival and successful host plant infection. Here, we show that XanB2 encoded by PXO_3739 plays a key role in xanthomonadin and coenzyme Q8 biosynthesis in *X. oryzae* pv. *oryzae* PXO99A. A *xanB2* deletion mutant exhibits a pleiotropic phenotype, including xanthomonadin deficiency, producing less exopolysaccharide (EPS), lower viability and H₂O₂ resistance, and lower virulence. We further demonstrate that *X. oryzae* pv. *oryzae* produces 3-hydroxybenzoic acid (3-HBA) and 4-hydroxybenzoic acid (4-HBA) via XanB2. 3-HBA is associated with xanthomonadin biosynthesis while 4-HBA is mainly used as a precursor for coenzyme Q (CoQ)8 biosynthesis. XanB2 is the alternative source of 4-HBA for CoQ8 biosynthesis in PXO99A. These findings suggest that the roles of XanB2 in PXO99A are generally consistent with those in *X. campestris* pv. *campestris*. The present study also demonstrated that *X. oryzae* pv. *oryzae* PXO99A has evolved several specific features in 3-HBA and 4-HBA signaling. First, our results showed that PXO99A produces less 3-HBA and 4-HBA than *X. campestris* pv. *campestris* and this is partially due to a degenerated 4-HBA efflux pump. Second, PXO99A has evolved unique xanthomonadin induction patterns via 3-HBA and 4-HBA. Third, our results showed that 3-HBA or 4-HBA positively regulates the expression of *gum* cluster to promote EPS production in PXO99A. Taken together, the results of this study indicate that XanB2 is a key metabolic enzyme linking xan-

thomonadin, CoQ, and EPS biosynthesis, which are collectively essential for *X. oryzae* pv. *oryzae* pathogenesis.

Xanthomonas is a genus of gram-negative, polarly flagellated, phytopathogenic bacteria. These bacteria infect nearly 400 different plant hosts, including economically important crops such as rice, cotton, soybean, oil-rape, citrus, and banana (Leyns et al. 1984). A characteristic feature of this genus is the production of yellow, membrane-bound pigments called xanthomonadins (Starr 1981). Xanthomonadins are mixtures of unusual brominated, aryl-polyene esters (Andrewes et al. 1976; Stephens and Starr 1963), and xanthomonadins from different *Xanthomonas* spp. differ in bromination and methylation patterns (Starr et al. 1977). In addition to being a diagnostic characteristic of the genus (Starr 1981), xanthomonadins may protect *Xanthomonas* spp. from photobiological and peroxidation damage induced by host defense mechanisms (He et al. 2011; Jenkins and Starr 1982; Poplawsky et al. 2000; Rajagopal et al. 1997) and are required for epiphytic survival and host infection (Goel et al. 2001; He et al. 2011; Park et al. 2009; Poplawsky et al. 2000). These findings have established the role of xanthomonadins in maintaining the ecological fitness and virulence of *Xanthomonas* spp.

A diffusible factor (DF) has been implicated in the regulation of xanthomonadin production and extracellular polysaccharide (EPS) in *Xanthomonas campestris* pv. *campestris* B-24 (Poplawsky and Chun 1997). Subsequent investigation showed that XCC4014 (renamed as *xanB2*) in *X. campestris* pv. *campestris* ATCC33913 is required for DF biosynthesis (Poplawsky et al. 2005). Moreover, disruption of *xanB2* abolishes xanthomonadins and DF production in *X. campestris* pv. *campestris* strains B-24, 8004, and XC1 (He et al. 2011; Poplawsky and Chun 1997). The DF produced by *X. campestris* pv. *campestris* strains 8004 and XC1 has been purified and chemically characterized as 3-hydroxybenzoic acid (3-HBA) (He et al. 2011). Genetic and biochemical analysis showed that 3-HBA is associated with bacterial survival, cell viability, H₂O₂ resistance, and systemic invasion (He et al. 2011).

Numerous bacterial species within the genera *Xanthomonas*, *Xylella*, *Xylophilus*, and *Burkholderia* also display DF-like

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*The e-Xtra logo stands for “electronic extra” and indicates that eleven supplementary figures are published online and that Figures 1 and 8 appear in color online.

activity. An even wider range of bacterial genomes contain homologs of *xanB2* (He et al. 2011). These findings suggest that the biosynthesis and biological functions of DF are widely conserved in bacteria (He et al. 2011). Previously, XanB2 was predicted to be a putative pteridine-dependent deoxygenase (da Silva et al. 2002). Recent results describe XanB2 as a novel bifunctional chorismatase that hydrolyzes chorismate to produce 3-HBA and 4-hydroxybenzoic acid (4-HBA). 3-HBA is associated with the yellow xanthomonadin biosynthesis, whereas the XanB2-derived 4-HBA is a precursor for coenzyme Q (CoQ)8 biosynthesis (Zhou et al. 2013). Furthermore, along with XanB2, the shikimate pathway, xanthomonadin biosynthetic cluster, and ubiquinone biosynthetic pathway are found in all *Xanthomonas* spp., *Xylella fastidiosa*, and *Xylophilus ampelinus*, suggesting that XanB2 may act as a widely conserved metabolic link in these bacterial pathogens (Zhou et al. 2013).

The shikimate pathway is widely present in plants, bacteria, and fungi (Herrmann and Weaver 1999). The end product of the shikimate pathway is chorismate, which is the common precursor for the biosynthesis of aromatic amino acids and other aromatic compounds such as folate cofactors, phenazines, siderophores, salicylic acid, and hydroxybenzate (Andexer et al. 2011; Dosselaere and Vanderleyden 2001; Herrmann and Weaver 1999; Van Lanen et al. 2008; Zhou et al. 2013). CoQ is a naturally occurring coenzyme formed by the conjugation of a benzoquinone ring with a hydrophobic isoprenoid chain of varying length (Cluis et al. 2007). It is an obligatory cofactor in the aerobic respiratory electron transfer for energy generation. It also acts as an antioxidant that protects membrane phospholipids and proteins from lipid peroxidation by scavenging free radicals directly or by regenerating levels of tocopherol (Cluis et al. 2007; Martin et al. 2007). The CoQ biosynthetic pathway in *Escherichia coli* consists of at least nine enzymes, including UbiA, UbiB, UbiC, UbiD, UbiE, UbiF, UbiG, UbiH, and IspB (Bentinger et al. 2010; Meganathan 2001). The formation of 4-HBA from chorismate is the first committed step, which is catalyzed by chorismate-pyruvate lyase encoded by *ubiC* in *E. coli* or by Rv2949c in *Mycobacteria* (Siebert et al. 1994; Søballe and Poole, 1999; Stadthagen et al. 2005). Our previous results showed that *Xanthomonas campestris* pv. *campestris* lacks the genes encoding chorismate-pyruvate lyase and XanB2 is a novel chorismatase to provide an alternative source of 4-HBA for CoQ8 production (Zhou et al. 2013).

X. oryzae pv. *oryzae* is a rod-shaped, gram-negative bacterium (Nino-Liu et al. 2006). It causes one of the most serious diseases in rice, bacterial blight. *X. oryzae* pv. *oryzae* colonies are round, convex, and mucoid on solid media that contains glucose. The colonies present yellow in color due to the production of the pigment xanthomonadin (Bradbury 1984). Goel and associates (2001) characterized a 21.0-kb genomic region involved in xanthomonadin biosynthesis in the *X. oryzae* pv. *oryzae* BXO1, and further analysis showed that xanthomonadin might be synthesized through a novel type II polyketide pathway. The detailed chemical structures of xanthomonadins in *X. oryzae* pv. *oryzae* have yet to be elucidated. Studies suggest that xanthomonadins may be some of the most important virulence factors in *X. oryzae* pv. *oryzae*. Disruption of xanthomonadin biosynthesis led to a virulence deficiency in a previous study (Goel et al. 2001; Park et al. 2009; Song et al. 2011). In addition, xanthomonadin appears to protect *X. oryzae* pv. *oryzae* against photooxidative damage (Rajagopal et al. 1997).

DF-like activity has previously been detected in *X. oryzae* pv. *oryzae* strains PXO99A, HB21, and KACC10331 (Chun et al. 1997; He et al. 2011). A homolog of *xanB2* has also been found in the genomes of *X. oryzae* pv. *oryzae* strains PXO99A,

KACC10331, and MAFF 311018 (He et al. 2011). Moreover, mutations in genes involved in the shikimate pathway lead to deficiency in pigments (Goel et al. 2001; Park et al. 2009; Song et al. 2011). These findings suggest that *X. oryzae* pv. *oryzae* might use a similar mechanism in DF biosynthesis and DF-dependent xanthomonadin and ubiquinone biosynthesis. To this end, the biological functions of *xanB2* and DF in the rice pathogen *X. oryzae* pv. *oryzae* PXO99A were investigated. Our results indicate the biological functions of *xanB2* and DF in *X. oryzae* pv. *oryzae* are similar to those in *X. campestris* pv. *campestris*. In addition, results of the present study suggest that *X. oryzae* pv. *oryzae* has evolved several unique features in DF signaling.

RESULTS

A *xanB2* deletion mutant confers pleiotropic phenotypes in *X. oryzae* pv. *oryzae* PXO99A.

Previously, xanthomonadins were shown to be synthesized via the products of the *pig* cluster in *X. campestris* pv. *campestris* (Supplementary Fig. S1). Among the *pig* cluster, XCC4014 (*xanB2*) was responsible for 3-HBA and 4-HBA biosynthesis (He et al. 2011; Poplawsky et al. 2005; Zhou et al. 2013). The *X. oryzae* pv. *oryzae* wild-type strain PXO99A contained two loci with 20 open reading frames (PXO_2878 to PXO_2885 and PXO_3729 to PXO_3741, respectively). They are highly homologous to the *pig* cluster in *X. campestris* pv. *campestris*, suggesting that *X. oryzae* pv. *oryzae* might use a similar mechanism for xanthomonadin biosynthesis. To investigate this hypothesis, a PXO_3739 (a *xanB2* homolog) deletion mutation strain was created in PXO99A. The resulting mutation strain failed to produce xanthomonadins. However, the *in trans* overexpression of PXO_3739 restored xanthomonadin production (Fig. 1A and B). These findings confirm that PXO_3739, similar to its homolog *xanB2*, is involved in xanthomonadin biosynthesis. Therefore, PXO_3739 was also renamed as *xanB2* in this study.

Strain Δ xanB2 was further analyzed for other phenotypes. Disruption of *xanB2* did not affect *in vitro* growth in sucrose-containing peptone-yeast-sucrose (NAS) liquid medium or glycerol-containing peptone-yeast-glycerol (NYG) medium (Supplementary Fig. S2). EPS levels found in the supernatant of the mutant strain Δ xanB2 were 2.3 mg/ml at late stationary phase (optical density at 600 nm [OD₆₀₀] = 2.3). This level was significantly lower than EPS at 3.2 mg/ml produced by the wild-type strain PXO99A (Fig. 2A). Moreover, *in trans* overexpression of *xanB2* in Δ xanB2 strain restored EPS production to above wild-type levels (Fig. 2A).

The cell viability of PXO99A and derivatives were compared by counting CFU at different growth stages during a time course. No significant differences in viability were observed between the wild type and *xanB2* mutants during the exponential phase of bacterial growth (OD₆₀₀ = 1.2) (Fig. 3A). However, the viability of the *xanB2* mutant at early and late stationary phases (OD₆₀₀ = 2.0 and 2.3, respectively) was significantly lower than that of wild-type PXO99A (Fig. 3A). *In trans* overexpression of *xanB2* in the Δ xanB2 strain restored CFU viability to that of wild-type strains in stationary phase (Fig. 3A).

To assay oxidative stress resistance, *X. oryzae* pv. *oryzae* strains were grown in NAS medium until they reached the early stationary phase (OD₆₀₀ = 2.0). Next, the bacterial cells were precipitated and the pellet was resuspended in fresh NAS medium and put to the same density level. Hydrogen peroxide (H₂O₂) was then added to the cultures at final concentrations of 440 and 880 μ M, respectively. After 30 min of H₂O₂ treatment, the CFU values of each strain were compared. The results showed that deletion of *xanB2* substantially decreased bacte-

rial resistance to oxidative stress, as indicated by lower CFU values for the $\Delta xanB2$ strain with H_2O_2 treatment. Furthermore, overexpression of *xanB2* restored H_2O_2 resistance in the $\Delta xanB2$ strain to close to wild-type levels (Fig. 4).

Fig. 1. Reduced xanthomonadin production of the *Xanthomonas oryzae* pv. *oryzae* PXO99A $\Delta xanB2$ strain and restoration by *xanB2*, 3-hydroxybenzoic acid (3-HBA), and 4-hydroxybenzoic acid (4-HBA). **A**, Yellow pigmentation of *X. oryzae* pv. *oryzae* strains. **B**, Quantitative analysis of xanthomonadin production in *X. oryzae* pv. *oryzae* strains. **C**, Effect of increasing concentrations of 3-HBA on the xanthomonadin production of the $\Delta xanB2$ strain. **D**, Effect of increasing concentrations of 4-HBA on the xanthomonadin production of the $\Delta xanB2$ strain. (*xanB2*) = complemented with a plasmid pBBRMCS1 harboring the gene *xanB2*. (3-HBA) = addition of 3-HBA at a final concentration of 4.8 μM . (4-HBA) = addition of 4-HBA at a final concentration of 38.4 μM . WT indicates *X. oryzae* pv. *oryzae* PXO99A grown in a medium without 3-HBA and 4-HBA. Values shown are the means $\pm$ standard deviation from three independent experiments. Different letters indicate significant difference between treatments according to least significant difference at $P = 0.05$.

Finally, the virulence of wild-type strain PXO99A, the $\Delta xanB2$ strain, and the complemented strain was evaluated by measuring lesion lengths on rice plant leaves at 14 days post inoculation. The mean lesion length observed with the $\Delta xanB2$ strain was significantly reduced by approximately 3.5 cm compared with that observed with wild-type strain PXO99A (Fig. 5). These results demonstrate that *xanB2* is required for full virulence of *X. oryzae* pv. *oryzae* in rice. Consistently, the populations of strain $\Delta xanB2$ on adult rice leaves were significantly lower than those of PXO99A at weeks 1 and 2 (Fig. 5B). Overexpression of *xanB2* restored the populations to wild-type levels (Fig. 5B).

Fig. 2. Effects of *xanB2*, 3-hydroxybenzoic acid (3-HBA), and 4-hydroxybenzoic acid (4-HBA) on extracellular polysaccharide (EPS) production in *Xanthomonas oryzae* pv. *oryzae* PXO99A $\Delta xanB2$ strain. **A**, Overexpression of *xanB2*, or the exogenous addition of 3-HBA (19.2 μM) or 4-HBA (19.2 μM) restores EPS production to the $\Delta xanB2$ strain. **B**, Reverse-transcription polymerase chain reaction analysis of *gumB* and *gumK* expression. **C**, β -Glucuronidase (GUS) activity of PXO99A strains in the presence of 3-HBA or 4-HBA. (*xanB2*) = complemented with a plasmid pBBRMCS1 harboring the gene *xanB2*. (3-HBA) = addition of 3-HBA at a final concentration of 19.2 μM . (4-HBA) = addition of 4-HBA at a final concentration of 19.2 μM . Values shown are the means $\pm$ standard deviation from three independent experiments. Different letters indicate significant difference between treatments according to least significant difference at $P = 0.05$.

PXO99A produces 3-HBA and 4-HBA via XanB2.

Using the previously described method for 3-HBA and 4-HBA extraction and purification in *X. campestris* pv. *campestris* (He et al. 2011; Zhou et al. 2013), two compounds were identified from the supernatants of *X. oryzae* pv. *oryzae* PXO99A. These two compounds were chemically characterized as 3-HBA and 4-HBA based on UV absorbance, elution time in high-performance liquid chromatography (HPLC) analysis, mass spectrometry (MS) analysis, and in vitro pigment-induction assay (Figs. 1 and 6A and B; Supplementary Fig. S3). Furthermore, our results demonstrated that 3-HBA and 4-HBA production was cell-density dependent, with the maximum levels in the supernatant of PXO99A cell cultures being 1.2 and 1.1 μM , respectively (Fig. 6C). Deletion of *xanB2* completely abolished 3-HBA and 4-HBA production while overexpression of *xanB2* in strain ΔxanB2 restored 3-HBA and 4-HBA production (Fig. 6B). These findings show that XanB2 is required for 3-HBA and 4-HBA production in *X. oryzae* pv. *oryzae* PXO99A.

The differential effects of 3-HBA and 4-HBA on xanthomonadin induction in PXO99A.

Exogenous addition of 3-HBA to the ΔxanB2 strain fully restored xanthomonadin production (Fig. 1C). The color of 3-HBA-induced pigment was similar to that of the wild-type

strain (Fig. 1A). In addition, xanthomonadin production increased as the exogenous concentration of 3-HBA increased, ranging from 99.0% of the wild type at 4.8 μM to 175.5% at 38.4 μM (Fig. 1C). In contrast, the exogenous addition of 4-HBA induced the ΔxanB2 strain to produce atypical pale-yellow pigments (Fig. 1A). The minimum concentration of 4-HBA for full wild-type level of pigment induction was 38.4 μM (Fig. 1D). Further increasing the concentration of 4-HBA above 38.4 μM did not significantly increase pigment production (data not shown). Finally, when 3-HBA (4.8 μM) and 4-HBA (38.4 μM) were both added to the ΔxanB2 strain, xanthomonadin induction was similar to that of the wild-type strain (Fig. 1A). These findings suggest that 3-HBA is of greater biological importance for xanthomonadin biosynthesis in *X. oryzae* pv. *oryzae*.

3-HBA or 4-HBA is required for full EPS induction.

The above findings suggest that 3-HBA or 4-HBA is required for full EPS production. To explore these possibilities, 3-HBA or 4-HBA was added to the ΔxanB2 strain at early growth phase ($\text{OD}_{600} = 0.4$). EPS was extracted from the supernatant of cell cultures at $\text{OD}_{600} = 2.3$. Our results showed that exogenous addition of 3-HBA (4.8 μM) could fully restore EPS production in the ΔxanB2 strain to wild-type levels (3.1 mg/ml); higher concentrations of 3-HBA induced the ΔxanB2 strain to produce more EPS. For example, EPS at 3.8 or 4.7 mg/ml was detected in the supernatant of ΔxanB2 cell cultures supplemented with 9.6 or 19.2 μM 3-HBA, respectively (Fig. 2A; Supplementary Fig. S4A). The addition of 4-HBA had effects similar to those of 3-HBA on EPS production (Fig. 2A). Interestingly, the addition of both 3-HBA (4.8 μM) and 4-HBA (4.8 μM) did not show an additive effect on EPS production in cell cultures of the ΔxanB2 strain (Fig. 2A).

EPS in xanthomonads is a polymer of repeating pentasaccharide units (Sutherland 1993). The assembly of the pentasaccharide repeating unit, polymerization, and the export of EPS are directed by a gene cluster comprising 13 genes, *gumB* (PXO_01391) to *gumN* (PXO_01405) (Salzberg et al. 2008;

Fig. 3. Effects of *xanB2*, 3-hydroxybenzoic acid (3-HBA), and 4-hydroxybenzoic acid (4-HBA) on cell viability of *Xanthomonas oryzae* pv. *oryzae* PXO99A ΔxanB2 strain. **A**, CFU of *Xanthomonas oryzae* pv. *oryzae* strains at different growth stages. **B**, Effect of exogenous addition of 3-HBA or 4-HBA on the viability of the ΔxanB2 strain. (*xanB2*) = complemented with a plasmid pBBRMCS1 harboring the gene *xanB2*. (3-HBA) = addition of 3-HBA at a final concentration of 4.8 μM . (4-HBA) = addition of 4-HBA at a final concentration of 38.4 μM . Values shown are the means $\pm$ standard deviation from three independent experiments. Different letters indicate significant difference between treatments according to least significant difference at $P = 0.05$.

Fig. 4. H₂O₂ resistance of the *Xanthomonas oryzae* pv. *oryzae* PXO99A ΔxanB2 strain in the presence of 3-hydroxybenzoic acid (3-HBA) or 4-hydroxybenzoic acid (4-HBA). Bacterial cells at an optical density at 600 nm of 2.0 were collected for H₂O₂ treatment. After 30 min of H₂O₂ treatment, the CFU value of each strain was determined. (*xanB2*) = complemented with a plasmid pBBRMCS1 harboring the gene *xanB2*. (3-HBA) = addition of 3-HBA at a final concentration of 4.8 μM . (4-HBA) = addition of 4-HBA at a final concentration of 38.4 μM . Values shown are the means $\pm$ standard deviation from three independent experiments. Different letters indicate significant difference between treatments according to least significant difference at $P = 0.05$.

Sutherland 1993). Transcriptional analysis has revealed that the *gum* region is expressed mainly as an operon from a promoter located upstream of *gumB* (Katzen et al. 1996). In this study, by using reverse-transcription polymerase chain reaction (RT-PCR), we showed that the transcriptional levels of *gumB* and *gumK* in *xanB2* deletion mutant were significantly lower than those in the wild-type strain (Fig. 2B). Overexpression of *xanB2* or exogenous addition of 3-HBA (19.2 μ M) or 4-HBA (19.2 μ M) restored the expression levels of *gumB* and *gumK* (Fig. 2B). To further investigate the effects of 3-HBA or 4-HBA on the expression of *gum* cluster in PXO99A, a reporter construct was created by fusing the promoter region of the *gum* cluster with the coding sequence for β -glucuronidase (GUS) of *E. coli* (*gusA*). The GUS activity analysis results confirmed that addition of 3-HBA (19.2 μ M) or 4-HBA (19.2 μ M) can stimulate expression of the *gumgusA* reporter (Fig. 2C).

3-HBA and 4-HBA have a cumulative effect on cell viability and H₂O₂ resistance in *X. oryzae* pv. *oryzae*.

The exogenous addition of 3-HBA (4.8 μ g/ml) or 4-HBA (38.4 μ g/ml) to the Δ *xanB2* strain partially restored viability (Fig. 3B; Supplementary Fig. S5). In contrast, the addition of 3-HBA (4.8 μ M) and 4-HBA (4.8 μ M) together fully restored

the viability of the Δ *xanB2* strain to wild-type levels (Fig. 3B), suggesting a cumulative effect of 3-HBA and 4-HBA on cell viability. Similarly, the addition of 3-HBA or 4-HBA only partially restored H₂O₂ resistance in the Δ *xanB2* strain. However, the addition of 3-HBA and 4-HBA together fully restored H₂O₂ resistance in the Δ *xanB2* strain to wild-type levels (Fig. 4; Supplementary Fig. S6).

XanB2-derived 4-HBA is required for ubiquinone biosynthesis in *X. oryzae* pv. *oryzae* PXO99A.

Ubiquinones have been found to play multiple roles in respiration, gene regulation, and oxidative stress management

Fig. 5. Deletion of *xanB2* impairs **A**, virulence and **B**, epiphytic survival of *Xanthomonas oryzae* pv. *oryzae* PXO99A. Adult rice plants ('Shanyou 63', 2 months old) were used in virulence assays. (*xanB2*) = complemented with a plasmid pBBRMCS1 harboring the gene *xanB2*. For virulence assay, the values shown are the mean $\pm$ standard deviation (SD) from 10 measurements of the length of the lesions induced by the bacterium on the leaves of infected rice plants. For epiphytic assay, the significant differences could also be observed for the H₂O₂ treatment at 440 μ M. Values shown are the mean $\pm$ SD from three independent experiments. Different letters indicate significant difference between treatments according to least significant difference at $P = 0.05$.

Fig. 6. *Xanthomonas oryzae* pv. *oryzae* PXO99A produces 3-hydroxybenzoic acid (3-HBA) and 4-hydroxybenzoic acid (4-HBA) via XanB2. **A**, Chemical structure of 3-HBA and 4-HBA. **B**, High-performance liquid chromatography analysis of 3-HBA and 4-HBA production in the supernatant of *Xanthomonas oryzae* pv. *oryzae* strains. **C**, 3-HBA and 4-HBA production during the growth cycle. (*xanB2*) = complemented with a plasmid pBBRMCS1 harboring the gene *xanB2*. OD₆₀₀ = optical density at 600 nm.

(Sóballé and Poole 1999). Previous findings showed that XanB2 is an alternative source of 4-HBA, and that XanB2-derived 4-HBA is an essential precursor for CoQ8 biosynthesis (Zhou et al. 2013). In this study, ubiquinone was extracted and purified from *X. oryzae* pv. *oryzae* wild-type strain PXO99A using the method described by Zhou and associates (2013). HPLC and subsequent MS analysis revealed that the ubiquinone produced by PXO99A was CoQ8 (Fig. 7A; Supplementary Fig. S7A). The PXO99A genome contains most of the genes for ubiquinone biosynthesis—*ubiA* (PXO_02871), *ubiB* (PXO_02769), *ubiE* (PXO_04274), *ubiF* (PXO_04637), *ubiG* (PXO_00379), *ubiH* (PXO_04638), and *ispB* (PXO_02016)—but not the homolog of *ubiC* or Rv2949c encoding chorismate-pyruvate lyase (Siebert et al. 1994; Stadthagen et al. 2005). Deletion of *xanB2* significantly decreased CoQ8 production (Fig. 7B). Overexpression of *xanB2* or exogenous addition of 4-HBA (4.8 μ M) to the Δ xanB2 strain restored CoQ8 production to wild-type levels (Fig. 7B). Exogenous addition of 3-HBA (4.8 μ M) had no effect on CoQ8 production (Fig. 7B).

Fig. 7. *xanB2* and 4-hydroxybenzoic acid (4-HBA) are required for coenzyme Q (CoQ)8 biosynthesis in *Xanthomonas oryzae* pv. *oryzae* PXO99A. **A**, Chemical structure of CoQ8. **B**, High-performance liquid chromatography analysis of CoQ8 production in *X. oryzae* pv. *oryzae* strains. (*xanB2*) = complemented with a plasmid pBBRMCS1 harboring the gene *xanB2*. (3-HBA) = addition of 3-hydroxybenzoic acid at a final concentration of 4.8 μ M. (4-HBA) = addition of 4-HBA at a final concentration of 38.4 μ M.

PXO99A strain contains a degenerated 4-HBA efflux pump.

The *pig* clusters between *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* are highly conserved; however, the extracellular levels of 3-HBA and 4-HBA of *X. campestris* pv. *campestris* XC1 and *X. oryzae* pv. *oryzae* PXO99A are apparently different (Fig. 6C). To explore the underlying causes of the difference, we first did amino acid sequence alignment analysis and found that XanB2 in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* are highly conserved, with only a few amino acids being divergent (Supplementary Fig. S8). Furthermore, we found that *X. campestris* pv. *campestris* Δ xanB2 strains overexpressing *xanB2*^{Xcc} produce significantly more 4-HBA than *X. oryzae* pv. *oryzae* Δ xanB2 strains overexpressing *xanB2*^{Xcc} (Supplementary Fig. S9). These findings suggest that the differential extracellular production of 3-HBA and 4-HBA in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* is not due to XanB2.

A 4-HBA efflux pump AaeXAB was characterized in *E. coli* MG1655 (Van Dyk et al. 2004) (Fig. 8A). *X. campestris* pv. *campestris* ATCC33913 contains homologs of *aaeX* (XCC4168), *aaeA* (XCC4169), and *aaeB* (XCC4171), as well as an additional XCC4170 encoding an outer membrane efflux protein (Fig. 8A). Deletion of the gene cluster of XCC4168 to XCC4171 in *X. campestris* pv. *campestris* XC1 resulted in decreased production of exogenous 3-HBA and 4-HBA (J.-Y. Wang, unpublished data). The PXO99A genome contains two genes, PXO_03532 and PXO_03533, in the same genomic locus (Fig. 8A). PXO_03532 and PXO_03533 are highly homologous to XCC4170 and XCC4171, respectively (Supplementary Figs. S10 and S11); however, the products of PXO_03532 and PXO_03533 (188 and 145 amino acids) are much smaller than those of XCC4170 and XCC4171 (766 and 483 amino acids) (Fig. 8A). Deletion or overexpression of both PXO_03532 and PXO_03533 had no effects on the extracellular production of 3-HBA and 4-HBA in *X. oryzae* pv. *oryzae* PXO99A (Fig. 8B and C). Overexpression of the gene cluster of XCC4168 to XCC4171 in PXO99A significantly increased the extracellular level of 3-HBA and 4-HBA (Fig. 8B and C). Collectively, these data suggest that PXO_03532 and PXO_03533 encode a degenerated 4-HBA efflux pump in PXO99A.

DISCUSSION

In this study, we showed that XanB2, a recently functionally characterized chorismatase in *X. campestris* pv. *campestris*, plays important roles in adaptation and virulence in the *X. oryzae* pv. *oryzae* PXO99A. PXO99A produces 3-HBA and 4-HBA via XanB2. 3-HBA is exclusively associated with xanthomonadin production while 4-HBA is mainly involved in CoQ8 biosynthesis. A high concentration of 4-HBA alone also induced atypical xanthomonadin biosynthesis. In addition, 3-HBA or 4-HBA is required for full EPS production, optimal CFU values at late stationary phase, H₂O₂ resistance, and virulence. Our findings also demonstrate the *X. oryzae* pv. *oryzae* PXO99A produces CoQ8 via a new mechanism dependent on XanB2. XanB2 has been shown to be a bifunctional chorismatase that uses chorismate, the end product of shikimate pathway, to synthesize 3-HBA and 4-HBA in *X. campestris* pv. *campestris* XC1 (Zhou et al. 2013). 3-HBA was proposed to be a biosynthetic intermediate for xanthomonadin biosynthesis (He et al. 2011). In *X. oryzae* pv. *oryzae*, disruption of genes encoding key components of the shikimate pathway led to deficiency in xanthomonadin biosynthesis and DF production (Goel et al. 2001; Park et al. 2009; Song et al. 2011). Therefore, the roles of XanB2 in PXO99A are generally consistent with those in *X. campestris* pv. *campestris* (He et al. 2011; Zhou et al. 2013).

However, the current study also provides evidence that *X. oryzae* pv. *oryzae* PXO99A, compared with *X. campestris* pv. *campestris*, has evolved several specific features in 3-HBA and 4-HBA signaling. First, PXO99A produces significantly less 3-HBA and 4-HBA than *X. campestris* pv. *campestris* does. The maximum levels of 3-HBA and 4-HBA in the supernatant of *X. campestris* pv. *campestris* were previously determined to be 6.2 and 32.0 μM , respectively (He et al. 2011; Zhou et al. 2013), whereas the maximum levels of 3-HBA and 4-HBA produced by *X. oryzae* pv. *oryzae* were 1.2 and 1.1 μM , respectively (Fig. 6C). Our results further showed that the differential extracellular production of 3-HBA and 4-HBA in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* is not due to XanB2 itself but is partially due to a degenerated 4-HBA efflux pump. Nearly all bacterial species have the metabolic capacity to synthesize various aromatic compounds (Gosset 2009) and they also evolve diverse transporting systems for aromatic compounds (Chaudhry et al. 2007). AaeAB is the first characterized 4-HBA efflux pump in bacteria. *X. campestris* pv. *campestris* that contains a functional AaeAB-like efflux pump (XCC4168-XCC4169) (Fig. 8A) whereas *X. oryzae* pv. *oryzae* contains a degenerated efflux pump (Fig. 8B and C). The biological significance of the degenerated 4-HBA efflux pump in PXO99A remains to be elucidated.

Second, PXO99A has evolved unique xanthomonadin induction patterns via 3-HBA and 4-HBA. In *X. campestris* pv. *campestris* XC1, it was reported that exogenous addition of 3-HBA (approximately 6 μM) fully restored xanthomonadin biosynthesis in ΔxanB2 strains to wild-type levels. The addition of more 3-HBA (12.5 to approximately 50 μM) to ΔxanB2 cultures, however, failed to induce more xanthomonadin production in *X. campestris* pv. *campestris* (He et al. 2011). In contrast, in the *X. oryzae* pv. *oryzae* PXO99A, addition of 3-HBA (4.8 μM) fully induced xanthomonadin production. Higher concentrations of 3-HBA (9.6 to 38.4 μM) significantly increased xanthomonadin production (Fig. 1C). Furthermore, it was previously shown that exogenous addition of higher concentrations of 4-HBA (100 to 1,000 μM) induced darker yellow xanthomonadins in *X. campestris* pv. *campestris* (He et al. 2011). Conversely, this study demonstrates that the xanthomonadins induced by addition of higher concentrations of 4-HBA (100 to 1,000 μM) are pale yellow in PXO99A (Fig. 1A).

Third, previous results by Poplawsky and Chun (1997) showed that null mutation of *pigB* in *X. campestris* pv. *campestris* B-24 abolished the production of xanthomonadins and EPS and resulted in impaired production of EPS. In *X. campestris* pv. *campestris* strains 8004 and XC1, it was found that deletion of *xanB2* only slightly affected EPS production and the addition of 3-HBA had no significant effect on EPS production (He et al. 2011). The present study showed that deletion of *xanB2* in *X. oryzae* pv. *oryzae* led to a significant decrease in EPS levels in the supernatant of PXO99A. The exogenous addition of 3-HBA (4.8 μM) or 4-HBA (4.8 μM) fully restored EPS production to wild-type levels (Fig. 2). EPS production increased with the extracellular addition of 3-HBA or 4-HBA. Our results further demonstrated that XanB2, 3-HBA, or 4-HBA induces EPS production through promoting the transcriptional expression of *gum* cluster (Fig. 2). The mechanism behind the strain-specific EPS induction by 3-HBA or 4-HBA in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* remains to be elucidated.

Taken together, our findings with *X. oryzae* pv. *oryzae* PXO99A further support a growing body of evidence that XanB2 is a key metabolic link between the shikimate pathway, xanthomonadin, and CoQ biosynthesis. The mechanisms underlying *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* differences in response to 3-HBA or 4-HBA remain undefined.

The yellow xanthomonadin pigments are a mixture in *Xanthomonas* spp.; only xanthomonadin I from *X. juglandis* has been chemically characterized (Andrewes et al. 1976). The detailed chemical structures of *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris* xanthomonadins remain to be investigated. In *X. campestris* pv. *campestris*, the *pig* cluster, which is composed of 20 genes (XCC3995 to XCC4015), is responsible for xanthomonadin biosynthesis. In *X. oryzae* pv. *oryzae*, xan-

Fig. 8. *Xanthomonas oryzae* pv. *oryzae* PXO99A encodes a degenerated 4-hydroxybenzoic acid (4-HBA) efflux pump. **A**, Genes encoding 4-HBA efflux pump in *Escherichia coli*, *X. campestris* pv. *campestris*, and *X. oryzae* pv. *oryzae*. **B** and **C**, Overexpression of XCC4168 to XCC4171 significantly increased exogenous 3-hydroxybenzoic acid (3-HBA) and 4-HBA production in *X. oryzae* pv. *oryzae* PXO99A. $\Delta 3233$ = the double deletion mutant of PXO_03532 and PXO_03533. (3233) = complemented with a plasmid pBBRMCS1 harboring the genes PXO_03532 and PXO_03533. (6871) = complemented with a plasmid pBBRMCS1 harboring the gene cluster XCC4168 to XCC4171. Values shown are the mean $\pm$ standard deviation from three independent experiments. Different letters indicate significant difference between treatments according to least significant difference at $P = 0.05$.

thomonadin biosynthetic genes are clustered at two separated loci (PXO_02877 to PXO_02885 and PXO_03730 to PXO_03741). The *X. oryzae* pv. *oryzae* pig clusters contain two additional genes encoding conserved hypothetical proteins PXO_02879 and PXO_03740 and lack two genes (XCC4002 and XCC4006) identified in *X. campestris* pv. *campestris*. The relevance of how these genetic differences in the pig clusters contribute to the differential effects of 3-HBA and 4-HBA remains to be determined. Further characterization of the chemical structure of xanthomonadins in *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris* will contribute to a better understanding of how 3-HBA and 4-HBA are involved in xanthomonadin biosynthesis.

The results of this study indicate that, in PXO99A, both xanthomonadin and CoQ8 biosynthesis are linked together by a single metabolic enzyme, XanB2. The membrane-bound yellow-pigment xanthomonadins have been shown to play essential roles in protecting bacteria from photobiological and peroxidative damage and are important for epiphytic survival and systemic infection in *Xanthomonas* spp. (He et al. 2011; Jenkins and Starr 1982; Poplawsky et al. 2000; Rajagopal et al. 1997). Aerobic respiration and active plant defenses can result in increasing production of reactive oxygen species, including superoxide, H₂O₂, and OH⁻ (Bestwick et al. 1997; Bindschedler et al. 2006; Sutherland 1991). Therefore, the ability of PXO99A to survive oxidative stress is of critical importance for successful colonization in the rice plant. Microbial ubiquinones also play roles in oxidative stress management (Sóballé and Poole 1999). Hence, understanding the role of XanB2 in 3-HBA and 4-HBA production for use in xanthomonadins and ubiquinone biosynthesis provides new insight into factors that allow *X. oryzae* pv. *oryzae* to survive oxidative stress.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

X. oryzae pv. *oryzae* wild-type strain PXO99A and its derivatives were grown at 28°C in NYG medium (5 g of peptone, 3 g of yeast extract, and 20 g of glycerol, pH 7.0) or NAS medium (5 g of peptone, 1 g of yeast extract, 3 g of beef extract, and 50 g of sucrose, pH 7.0) in the dark. *E. coli* strains were grown at 37°C in Luria-Bertani (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl) medium. Antibiotics were added at the following concentrations when required: kanamycin, 50 µg/ml; cephalixin, 20 µg/ml; and ampicillin, 100 µg/ml.

Gene deletion and functional complementation analysis.

PXO_03739 in-frame deletion mutants of *X. oryzae* pv. *oryzae* PXO99A were generated according to He and associates (2006) using the following primers: 3739D-FOR1: cgggatccaggccgcgctggtgatg, 3739D-REV1: gtgcgtggtctggcctgggaag, 3739D-FOR2: ccagggccagaccacgcacgcgatgagctggcgtggag, and 3739D-REV2: CCAAGCTTcggggggacgaatcagaat. For complementation analysis, the coding region of PXO_03739 was amplified by PCR using 3739-FOR: gctctagatcagccatgcaccccatgatc and 3739-REV: ggaattcgtggcggccaccgcccgc and was cloned under the control of the *lac* promoter in pBBR-1-MCS (Kovach et al. 1995). The resulting construct was transferred into PXO99A strain via electroporation. The PXO_3739 deletion mutant was further verified by PCR and sequencing analysis. The DNA fragment consisting of XCC4168 to XCC4171 was amplified by PCR using 416871-FOR: cccaagcttatgtggcgtatgacggagaattcag and 416871-REV: gctcagactgaggtgcgtggtgctgttag and was cloned under the control of the *lac* promoter in pBBR-1-MCS. The resulting construct was transferred into strain PXO99A via electroporation. The DNA fragment consisting of PXO_03532 to PXO_03533 was amplified by PCR

using 3233FOR: ggaattcGTGGCCGGCAGTGATCTGGAGA and 3233REV: cctctagaTTATCGCCCTGACGGCAGAGGGG and was cloned in pBBR-1-MCS for overexpression.

Isolation of total RNA and RT-PCR analysis.

RNA extraction and RT-PCR analysis were conducted as described previously (Zhou et al. 2011). Briefly, cells from bacterial culture grown in NAS medium at OD₆₀₀ = 1.6 were collected by centrifugation at 4°C for 5 min at 10,000 rpm. Total RNA samples were prepared by using RNeasy midi columns following the manufacturer's instructions (Qiagen, Valencia, CA, U.S.A.). RNA integrity was confirmed by electrophoresis using a 1.3% formaldehyde agarose gel. RT-PCR analysis was conducted using a One-Step RT-PCR Kit (Qiagen) following the manufacturer's instructions. A total of 200 ng of total RNA was used for reverse transcription.

Construction of pL3gumGUS and assay of *gumGUS* expression in culture.

The reporter plasmid pL3gumGUS was constructed and assayed following the methods previously described (Vojnov et al. 2001; Wang et al. 2004). Briefly, the promoter region of the gum operon (located upstream of *gumB*) was amplified by PCR with primers XOOgum-FOR: GGATCCGGGCAGTAATCGC GAGCTAC and XOOgum-REV: cccaagcttccgatcagtttcttcagctgtg. The PCR product was cleaved and ligated into the plasmid pGEM-4-GUS to generate the intermediate construct pGegumGUS. The *gumGUS* cassette was then cloned as a *HindIII*-*EcoRI* fragment into the broad-host-range vector pLAFR3 to create pL3gumGUS. The plasmid was transferred to wild-type and *xanB2* mutant strains of PXO99A by triparental mating. For GUS activity assay, flasks (250 ml) containing 50 ml of media were inoculated with 0.5 ml of an overnight culture of PXO99A strains diluted to an OD₆₀₀ of 0.5. The cells were collected from the culture at OD₆₀₀ = 2.0 and the GUS activity was measured as described previously (Jefferson et al. 1987) and defined as picomoles of methyl umbelliferone produced per minute per microgram of total soluble protein.

Extraction and quantification of xanthomonadins.

The previously described procedure for the extraction of pigments from *X. juglandis* (Irey and Stall 1982) was used in this study, with minor modifications. Briefly, PXO99A cultures were grown until stationary phase (OD₆₀₀ = 2.1) was reached, and the cells from 5 ml of culture were recovered by centrifugation (10,000 rpm for 10 min). Pigments were extracted with 1 ml of methanol by shaking for 5 min at room temperature. The amount of pigment produced was expressed as the absorbance (OD at 445 nm) of crude pigment extracts (Poplawsky and Chun 1997).

Extraction, purification, and bioassay of 3-HBA and 4-HBA.

X. oryzae pv. *oryzae* PXO99A was cultured in NAS medium for 72 h. Bacterial supernatants (30 liters) were collected by centrifugation (3,800 rpm for 30 min at 4°C). 3-HBA and 4-HBA were extracted and purified following a previously described method (He et al. 2011; Zhou et al. 2013).

Bacterial H₂O₂ resistance test and CFU assay.

X. oryzae pv. *oryzae* strains were grown in NAS medium with or without 3-HBA or 4-HBA. The H₂O₂ resistance assay and the bacterial CFU assay were conducted as previously described by He and associates (2011).

EPS production assay.

To analyze the production of EPS, the supernatants of bacteria cultured in NAS medium (10 ml, OD₆₀₀ of approximately 2.3)

were collected by centrifugation at 10,000 rpm for 10 min. KCl solution (400 µl, 3.4 M) was added to the supernatants followed by 20 ml of absolute ethanol and incubated at -20°C for a half-hour. The precipitated EPS were spun down (10,000 rpm for 10 min) and dried at 55°C overnight before determination of dry weights. Each experiment was conducted three times.

Epiphytic survival and virulence test.

All rice plants ('Shanyou 63', 2 months old) were grown and maintained in a greenhouse with 12-h day-and-night cycle illuminations by fluorescent lamps with a light intensity of approximately 50,000 to 60,000 lx. A constant temperature of 25°C with relative humidity at 75% was maintained. For epiphytic survival assay, the bacterial pellets from the culture at OD₆₀₀ = 1.6 were resuspended in sterile phosphate-buffered saline (PBS) buffer to an OD of 0.1 and sprayed on adult rice leaves. Each week thereafter, the leaves were sampled for populations of *X. oryzae* pv. *oryzae* by plating PBS extracts on NAS agar plate with rifampicin (25 µg/ml), as previously described (Poplawsky and Chun 1998). The data were log transformed, and the means and the standard errors of the mean were then calculated and plotted. For the virulence assay, the bacterial pellets from the culture at OD₆₀₀ = 1.6 were resuspended in sterile PBS buffer to an OD of 0.05 and inoculated into adult rice plants by leaf-needling for lesion length measurement. Lesion lengths were measured 14 days after inoculation. Ten leaves were inoculated for each independent experiment and each treatment was repeated three times.

Ubiquinone extraction, purification, and HPLC analysis.

Ubiquinone was extracted as previously described (Zhou et al. 2013), with slight modifications. Briefly, bacteria were grown in NAS medium until an OD of 1.6 and the cells from 50 ml of cultures were collected by centrifugation. Cell pellets were resuspended in 2 ml of 0.2 M acetate buffer (pH 5.6) after washing twice by 1× PBS. Cryo-impacted cell homogenate was subjected to sonication for a total of 90 s. Hexane-acetone (5 ml, 1:1, vol/vol) reaction mixture was added to the cell homogenate followed by sonication and vortexing. The hexane fractions were collected and the solvent was removed by rotary evaporation at 40°C to dryness. The residue was then dissolved in 1.5 ml of chloroform-methanol (1:1, vol/vol), followed by washing with 0.75 ml of 0.7% NaCl. The chloroform fraction was collected and the solvent was removed by rotary evaporation at 40°C to dryness. The residue was dissolved in 100 µl of methanol. The crude extract was further analyzed by a liquid chromatographic system (Agilent 1260 Infinity; Agilent Technologies, Palo Alto, CA, U.S.A.). The eluate containing the purified ubiquinone was subjected to electrospray ionization-MS analysis on an Agilent 6230 TOF MS equipped with a Jet Stream ESI source (Agilent Technologies), using 100% methanol containing 1.5% formic acid as mobile phase at a flow rate of 0.2 ml/min. Data were acquired under the control of Mass Hunter workstation software.

Statistical analysis.

Analysis of variance for experimental datasets was performed using JMP software (version 5.0; SAS Institute Inc., Cary, NC, U.S.A.). Significant effects of treatment were determined by the *F* value (*P* = 0.05). When a significant *F* test was obtained, separation of means was accomplished by Fisher's protected least significant difference at *P* = 0.05.

ACKNOWLEDGMENTS

We thank B. Zhu for technical help with MS analysis. This work was supported by a grant from the National Natural Science Foundation of

China (number 31272005) and a China Postdoctoral Science Foundation Grant (number 2011M500770).

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