Genetic Interference Analysis Reveals that Both 3-Hydroxybenzoic Acid and 4-Hydroxybenzoic Acid Are Involved in Xanthomonadin Biosynthesis in the Phytopathogen *Xanthomonas campestris* pv. *campestris*

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Accepted for publication 12 October 2019.

ABSTRACT

A characteristic feature of phytopathogenic *Xanthomonas* bacteria is the production of yellow membrane-bound pigments called xanthomonadins. Previous studies showed that 3-hydroxybenzoic acid (3-HBA) was a xanthomonadin biosynthetic intermediate and also, that it had a signaling role. The question of whether the structural isomers 4-HBA and 2-HBA (salicylic acid) have any role in xanthomonadin biosynthesis remained unclear. In this study, we have selectively eliminated 3-HBA, 4-HBA, or the production of both by expression of the *mhb*, *pobA*, and *pchAB* gene clusters in the *Xanthomonas campestris* pv. *campestris* strain XC1. The resulting strains were different in pigmentation, virulence factor production, and virulence. These results suggest that both 3-HBA and 4-HBA are involved in xanthomonadin biosynthesis. When both 3-HBA and 4-HBA are present, *X. campestris* pv. *campestris* prefers 3-HBA for Xanthomonadin-A biosynthesis; the 3-HBA-derived Xanthomonadin-

A was predominant over the 4-HBA-derived xanthomonadin in the wild-type strain XC1. If 3-HBA is not present, then 4-HBA is used for biosynthesis of a structurally uncharacterized Xanthomonadin-B. Salicylic acid had no effect on xanthomonadin biosynthesis. Interference with 3-HBA and 4-HBA biosynthesis also affected *X. campestris* pv. *campestris* virulence factor production and reduced virulence in cabbage and Chinese radish. These findings add to our understanding of xanthomonadin biosynthetic mechanisms and further help to elucidate the biological roles of xanthomonadins in *X. campestris* pv. *campestris* adaptation and virulence in host plants.

Keywords: bacteriology, genetics and resistance, salicylic acid, *Xanthomonas campestris*, xanthomonadin, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid

Members of the Gram-negative genus *Xanthomonas* cause disease in a large number of plants worldwide and are responsible for serious losses in agricultural production (Hayward 1993; Leyns et al. 1984). *Xanthomonas campestris* pv. *campestris*, the causal agent of black rot of crucifers, represents a model organism for the study of pathogenicity in phytopathogenic bacteria (Mansfield et al. 2012). A characteristic feature of *Xanthomonas* bacteria is the production of yellow membrane-bound pigments called xanthomonadins (Stephens and Starr 1963). Several studies have indicated that xanthomonadins protect bacterial cells from photooxidative damage (Jenkins and Starr 1982; Poplawsky et al. 2000; Rajagopal et al. 1997). In addition, xanthomonadin extracts from *Xanthomonas oryzae* pv. *oryzae* displayed antioxidant activity by inhibiting lipid peroxidation in liposomes (Rajagopal et al. 1997). Furthermore, Poplawsky and Chun (1997) and Poplawsky et al. (2000) found that xanthomonadin-deficient *X. campestris* pv. *campestris*

mutant was reduced in the bacterial epiphytic survival rate and host infection following exposure to high-intensity natural light. Our previous results showed that deletion of *xanB2*, which is essential for xanthomonadin biosynthesis, led to reduced virulence in radish plants (He et al. 2011). Collectively, these findings suggest that xanthomonadins play important roles in maintaining ecological fitness by protecting the *Xanthomonas* bacteria against photooxidative and peroxidative stress.

Xanthomonadins were found to be located on the bacterial outer membrane (Dianese and Schaad 1982; Stephens and Starr 1963). Structural analysis of xanthomonadin isobutyl esters by X-ray crystallographic and mass spectrum methods demonstrated that they represent a unique group of halogenated aryl-polyene pigments (Andrewes et al. 1973, 1976). One *Xanthomonas* species usually produces several xanthomonadins, which differ in methylation and bromination patterns at the aryl ring and also, polyene chain length (Starr et al. 1977). Our latest data showed that there is no methylation at the aryl ring in XC1 xanthomonadins (Cao et al. 2018). Arriati and Kester (1985) isolated two major xanthomonadin esters from *Xanthomonas juglandis* (*campestris*); additional analysis showed that both esters were phospholipid-like compounds. The phospholipid-like structure was further supported by the findings that xanthomonadins are always coextracted from *X. campestris* pv. *campestris* together with phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, and cardiolipin (Moser et al. 2014). Nevertheless, because of the difficulty in obtaining pure and stable pigments, the detailed chemical compositions and structures of xanthomonadins remain unclear.

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Funding: This work was supported by National Natural Science Foundation of China grants 31471743 (to Y.-W. He) and 31772121 (to Y.-W. He).

X.-Q. Cao and X.-Y. Ouyang contributed equally to this work.

*The e-Xtra logo stands for “electronic extra” and indicates that supplementary materials are published online.

The author(s) declare no conflict of interest.

A 25.4-kb *pig* genomic region containing seven transcriptional units (*pigA-G*) was first identified to be responsible for xanthomonadin biosynthesis in *X. campestris* pv. *campestris* (Poplawsky and Chun 1997; Poplawsky et al. 1993). *pig*-like gene clusters were also reported in *X. oryzae* pv. *oryzae* and *Lysobacter enzymogenes* (Goel et al. 2002; Wang et al. 2013), and disruption of the genes for enzymes in the shikimate pathway abolished *X. oryzae* pv. *oryzae* xanthomonadin biosynthesis (Goel et al. 2001; Park et al. 2009). In the genome of *X. campestris* pv. *campestris* strain ATCC33913, the putative *pig* cluster is composed of the 20 genes *Xcc3996* to *Xcc4015*. *Pseudomonas aeruginosa* strain M18 expressing this *X. campestris* pv. *campestris* *pig* gene cluster produced a darker yellow pigment that had structural changes, including the substitution of chlorine for bromine on the aryl ring (Cao et al. 2018). Single-gene deletion analysis showed that only 11 genes of the *pig* cluster (named *xanA2B2CDEFGHKLM*) are essential for xanthomonadin biosynthesis in *X. campestris* pv. *campestris* (Cao et al. 2018). Among them, *xanB2* (*Xcc4014*) was shown to encode a bifunctional chorismatase that hydrolyzes chorismate into 3-hydroxybenzoic acid (3-HBA) and 4-HBA in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* (Zhou et al. 2013a, b). The gene *xanA2* (*Xcc4015*) was found to encode an ATP-dependent 3-HBA:acyl carrier protein (ACP) ligase, whereas *xanC* (*Xcc4012*) encoded an ACP. Both XanA2 and XanC work together to activate 3-HBA to generate 3-HBA-S-ACP, which is needed for the initiation of xanthomonadin biosynthesis (Cao et al. 2018). The other proteins may be involved in xanthomonadin biosynthesis via an unusual type II polyketide synthase pathway (Cao et al. 2018). In our previous results, we showed that the proposed signal molecule DF, which was required for xanthomonadin production, was actually a mixture of 3-HBA and 4-HBA. Subsequently, it was proposed that 3-HBA and 4-HBA were intermediates in xanthomonadin and coenzyme Q (CoQ) biosynthesis, respectively, in both *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* (He et al. 2011; Zhou et al. 2013a). However, accumulating evidence suggests that 4-HBA might also be involved in xanthomonadin biosynthesis. First, exogenous addition of 4-HBA to the pigment-deficient mutant Δ *xanB2* resulted in the biosynthesis of darker yellow pigments (Zhou et al. 2013b). Second, our 2018 findings showed that XanA2, an ATP-dependent 3-HBA:ACP ligase, can also activate 4-HBA in vitro (Cao et al. 2018). To further elucidate the roles of 3-HBA and 4-HBA in xanthomonadin biosynthesis, we applied three strategies to selectively eliminate 3-HBA, 4-HBA, or the production of both in the *X. campestris* pv. *campestris* wild-type strain XC1 (Fig. 1). Our results clearly demonstrated that *X. campestris* pv. *campestris* uses both 3-HBA and 4-HBA for xanthomonadin biosynthesis.

MATERIALS AND METHODS

Bacteria strains, plasmids, and growth condition. The bacterial strains and plasmids used in this study are listed in Supplementary Table S1. *X. campestris* pv. *campestris* strains were grown in nitrogen yeast glycerol (NYG) medium (5 g/liter of peptone, 3 g/liter of yeast extract, and 20 g/liter of glycerol, pH 7.0), nutrient agar (NA) medium (5 g/liter of peptone, 3 g/liter of beef extract, 10 g/liter of sucrose, and 1 g/liter of yeast extract, pH 7.0), or nutritionally poor XOLN medium (0.7 g/liter of K_2HPO_4 , 0.2 g/liter of KH_2PO_4 , 1.0 g/liter of $(NH_4)_2SO_4$, 0.1 g/liter of $MgCl_2 \cdot 6H_2O$, 0.01 g/liter of $FeSO_4 \cdot 7H_2O$, 0.001 g/liter of $MnCl_2 \cdot 4H_2O$, 0.0625% yeast extract, and 0.0625% peptone, pH 7.0) at 28°C. *Escherichia coli* strains were cultured at 37°C in Luria-Bertani (LB) medium (10 g/liter of tryptone, 5 g/liter of yeast extract, and 10 g/liter of NaCl, pH 7.0), and *P. aeruginosa* M18 and *Klebsiella pneumoniae* M5a1 were grown in LB medium at 28°C. If necessary, antibiotics were added at the following concentrations: rifampicin (Rif), 25 µg/ml; kanamycin (Km), 50 µg/ml; spectinomycin, 50 µg/ml; gentamycin (Gm), 20 µg/ml for *E. coli* and 30 µg/ml for *X. campestris* pv. *campestris*; and ampicillin, 100 µg/ml.

Construction of gene or gene cluster overexpression in *X. campestris* pv. *campestris*. The coding regions of *pchAB*, *mhbDHIM*, and *pobA* were amplified by PCR using the primers in Supplementary Table S2. These genes were further cloned into the expression vector pBBR1MCS-2 (Km^R) (Kovach et al. 1995), and they were transferred to *X. campestris* pv. *campestris* strains by triparental mating. The positive transformants growing on NYG plate containing Rif (25 µg/ml) and Km (50 µg/ml) were further confirmed by colony PCR. For single-copy *pchAB* heterologous expression in *X. campestris* pv. *campestris*, the coding region of *pchAB* plus the promoter region from pBBR-*pchAB* was amplified and cloned into a versatile Mini-Tn7 delivery vector mini-Tn7T-Gm plasmid. The resultant construction was transformed into *X. campestris* pv. *campestris* by electroporation together with the helper plasmid pTNS2 (Choi and Schweizer 2006) (Supplementary Fig. S1). Chromosomal integration of mini-Tn7 was verified by PCR using three pairs of primers listed in Supplementary Figure S2.

Extraction and quantitative analyses of 3-HBA, 4-HBA, and salicylic acid. 3-HBA, 4-HBA, and salicylic acid (SA) extractions were performed as described (Zhou et al. 2013b) with minor modifications. Briefly, 2.5 ml of supernatants of *X. campestris* pv. *campestris* culture were collected and adjusted to pH 4.0 by addition of 1 M HCl prior to extraction with a twofold volume of ethyl acetate. The ethyl acetate fractions were then collected, and the solvent was removed by rotary evaporation to dryness at 40°C. The residues were then dissolved in 0.1 ml of methanol and submitted to high-performance liquid chromatography (HPLC) with a C18 reverse-phase column (Zorbax XDB; 5 µm, 4.6 × 150 mm), which was eluted with methanol and water containing 0.05% formic acid in each phase (25/75 [vol/vol]) at 1 ml/min. Commercially available 3-HBA, 4-HBA, and SA were used as standards.

For liquid chromatography-mass spectrum (LC-MS) analysis of SA, the extracted sample or SA standard was injected into the ultra-performance liquid chromatography (UPLC) coupled with MS apparatus (Agilent UPLC1290-TOF-MS6230) under the following conditions: Agilent Zorbax XDB C18 reverse phase (5 µm, 4.6 × 150 mm) eluted with methanol and water containing 0.05% formic acid (25/75 [vol/vol]) at 0.4 ml/min. The mass spectrum (MS) analysis was performed in the negative ion mode with an electrospray ionization source. Data were acquired in the centroid mode using Agilent MassHunter Workstation Data Acquisition Software (revision B.04).

Extraction and quantification of xanthomonadins. Pigments were extracted from *X. campestris* pv. *campestris* using previously described procedures (He et al. 2011). Briefly, *X. campestris* pv. *campestris* liquid cultures were grown until the stationary phase (optical density at 600 nm [OD₆₀₀] = 2.3) in NYG medium. The cell pellets from 10 ml of cultures were collected by centrifugation. Pigments were extracted with 1 ml methanol by

Fig. 1. The three strategies used to genetically interfere with xanthomonadin biosynthesis in this study. The solid lines indicate known pathways, and dotted red lines indicate postulated pathways in this study. CoQ, co-enzyme Q; E4P, erythrose-4-phosphate; HBA, hydroxybenzoic acid; PEP, phosphoenol-pyruvate; SA, salicylic acid; TCA cycle, tricarboxylic acid cycle.

Fig. 2. The effects of heterologous expression of the 3-hydroxybenzoic acid (3-HBA) degradation gene cluster in *Xanthomonas campestris* pv. *campestris*. **A**, The *mhb* gene cluster and putative 3-HBA degradation mechanism in *Klebsiella pneumoniae*. **B**, The time course of bacterial growth of *X. campestris* pv. *campestris* strains in nutritionally poor XOLN medium. **C**, The time course of 3-HBA degradation in XOLN medium. **D**, The 3-HBA levels of strains XC1(pBBR) and XC1(*mhb*) in nitrogen yeast glycerol (NYG) medium. **E**, The 4-HBA levels of strains XC1(pBBR) and XC1(*mhb*) in NYG medium. Shown are averages for three biological replicates with standard deviation. OD, optical density. *Statistically significant differences with respect to strain XC1(pBBR) ($P \leq 0.05$).

shaking for 5 min at room temperature. The amount of pigment produced was expressed as the absorbance (OD₄₄₁) of crude pigment extracts as described previously (Poplawsky and Chun 1997). The methyl esters of xanthomonadins were prepared following a method previously described by Cao et al. (2018).

Exogenous addition of 3-HBA or 4-HBA. Exogenous addition of 3-HBA or 4-HBA (from Sigma; purity >99%) was performed following previously described methods (He et al. 2011). In liquid NYG culture, 3-HBA or 4-HBA was added to the *X. campestris* pv. *campestris* cultures when the OD₆₀₀ reached 0.02. Xanthomonadins were then extracted using the above-mentioned methods. *X. campestris* pv. *campestris* cultures were grown overnight on NYG agarose plates, diluted to an OD₆₀₀ of 0.05, and spotted at the agarose bars. Subsequently, either 3-HBA or 4-HBA was added to the upper end of the agarose bars. The resulting plates were sealed and incubated at 28°C for 2 days. The presence of yellow-colored colonies indicated the biosynthesis of xanthomonadins.

Quantitative determination of extracellular polysaccharide and ubiquinone. Extracellular polysaccharide (EPS) was extracted and quantified following methods previously described (He et al. 2011). The extraction and quantitative analysis of ubiquinone was performed as previously described with slight modifications (Zhou et al. 2013b); 20 ml of *X. campestris* pv. *campestris* cultures were grown in NYG medium to an OD₆₀₀ of 1.6, and cells were then collected by centrifugation. After washing twice with 1× phosphate buffer saline (PBS), cell pellets were resuspended in 5 ml of 0.2 M acetate buffer (pH 5.6). Cryo-impacted cell homogenate was then subjected to sonication for a total of 90 s. Hexane-acetone (12.5 ml; 1:1 [vol/vol]) reaction mixture was added to the cell homogenate followed by further sonication and vortexing. The hexane fractions were collected, and the solvent was removed by rotary evaporation to dryness at 40°C. The resulting 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 then collected, and the solvent was removed by rotary evaporation to dryness at 40°C. The resulting residue was then dissolved in 100 μl of methanol, and it was subjected to HPLC with a C18 reverse-phase column (Zorbax XDB; 5 μm, 4.6 × 150 mm) eluting with methanol and isopropanol in each phase (50:50 [vol/vol]) for 20 min at a flow rate of 0.5 ml/min and ultraviolet detection at 275 nm. Commercially available CoQ₈ was used as a standard.

***X. campestris* pv. *campestris* virulence tests.** *X. campestris* pv. *campestris* virulence in cabbage and radish was estimated by the leaf clipping method. Fresh cell cultures at an OD₆₀₀ of 0.1 were used to inoculate clipped leaves of cabbage (cultivar Jingfeng no. 1) and Chinese radish (cultivar Manjianghong). The inoculated leaf was subsequently kept in an incubator at 28°C with 80% humidity, and lesion length was scored 10 days after inoculation. Fifteen leaves were inoculated with each tested strain. Each strain was tested in at least three separate experiments.

RESULTS

Heterologous expression of the *K. pneumoniae* 3-HBA degradation gene cluster in *X. campestris* pv. *campestris* results in the biosynthesis of a different xanthomonadin. Previous studies have proposed that the aryl ring moiety of xanthomonadin is derived from 3-HBA (He et al. 2011). To further confirm the role of 3-HBA in xanthomonadin biosynthesis, the *mhb* gene cluster (*mhbDHIM*), encoding the enzymes that cleave 3-HBA into fumarate and pyruvate in *K. pneumoniae* (Xu et al. 2012), was cloned and overexpressed in the *X. campestris* pv. *campestris* wild-type strain XC1 via a constitutive promoter in a broad host-range expression vector pBBR1MCS-2 (Fig. 2A). XC1 carrying the empty vector was used as a control. The resulting strains XC1(*mhb*) and XC1(pBBR) were grown in the following two media for functional analysis. First, when grown in the poor nutrient medium XOLN supplemented with 0.5 mM 3-HBA, no significant difference in bacterial growth was observed between the two strains

(Fig. 2B). The levels of 3-HBA in cultures of strain XC1(*mhb*) decreased over time, whereas 3-HBA levels remained relatively stable in the control strain XC1(pBBR) cultures (Fig. 2C). Thus, the *mhb* gene cluster from *K. pneumoniae* was functional in *X. campestris* pv. *campestris*. Second, although both strains produced 3-HBA, when cultured in glycerol-containing NYG medium in the absence of 3-HBA, the levels in the XC1(*mhb*) culture at 24, 36, and 48 h after inoculation were much lower than those in the XC1(pBBR) culture (Fig. 2D). Surprisingly, this overexpression of the *mhb* cluster in XC1 resulted in an increase of 4-HBA levels ranging from 14.2 to 18.8% depending on the growth stage (Fig. 2E).

We further investigated pigment biosynthesis in strains XC1(pBBR) and XC1(*mhb*). On NYG agar, colonies of XC1(*mhb*) appeared darker than those of XC1(pBBR) (Fig. 3A); however quantitative analysis (OD₄₄₁) revealed no significant difference in xanthomonadin yield in both strains (Fig. 3A). The absorption maximum for the XC1(pBBR)-derived xanthomonadins occurred at approximately 441 nm (Fig. 3B). In contrast, the absorption maximum for the XC1(*mhb*)-derived dark yellow pigment occurred at approximately 430 nm (Fig. 3B). Exogenous addition of 3-HBA or 4-HBA to the XC1(pBBR) cultures had no significant effect on xanthomonadin biosynthesis (Supplementary Fig. S1), whereas the addition of 3-HBA (25 μ M) to the XC1(*mhb*) cultures restored bright yellow pigment biosynthesis and significantly increased pigment yield by 24% (Fig. 3A). Exogenous addition of 4-HBA (25 μ M) to XC1(*mhb*) cultures resulted in dark yellow pigment biosynthesis and an increased pigment yield of about 27% (Fig. 3A). These findings suggest that, in absence of 3-HBA, 4-HBA is used for biosynthesis of the dark yellow pigment in the XC1(*mhb*) strain.

We have developed an ultrahigh performance liquid chromatography-time of flight mass spectrometry (UPLC-TOF-MS)-based method to characterize the methyl ester of the yellow pigments produced by strain XC1 (Cao et al. 2018). The main methyl ester of the XC1(pBBR)-derived xanthomonadins was dibrominated with a putative formula of $C_{24}H_{22}Br_2O_3$ denoted Xanthomonadin-A (Fig. 3C). However, the level of this pigment in strain XC1(*mhb*) was only 16% of that seen in the strain XC1(pBBR) (Fig. 3C), and the predominant pigment was a new methyl ester tentatively denoted Xanthomonadin-B (Fig. 3C). Xanthomonadin-B is structurally different from the previously characterized dibrominated Xanthomonadin-A.

Overexpression of the 4-HBA monooxygenase gene *pobA* significantly reduced xanthomonadin biosynthesis in *X. campestris* pv. *campestris*. A gene cluster required for 4-HBA degradation was previously identified in *X. campestris* pv. *campestris* (Wang et al. 2015). One of the genes, *pobA*, encodes a 3-monooxygenase, which catalyzes the degradation of 4-HBA into protocatechuate prior to further aromatic ring cleavage (Fig. 4A). To investigate whether 4-HBA is involved in xanthomonadin biosynthesis, we generated the *pobA* deletion and overexpression strains XC1(*pobA*), Δ *pobA*(pBBR), and Δ *pobA*(*pobA*) and measured their biosynthesis of 3-HBA, 4-HBA, and xanthomonadins. When grown in NYG liquid medium, all four strains showed similar growth rates (Fig. 4B). Deletion of *pobA* in strain XC1 had little effect on the levels of 3-HBA and 4-HBA (Fig. 4C and D). Overexpression of *pobA* in strains XC1 or Δ *pobA* resulted in a reduction of 4-HBA levels ranging from 88 to 98%; however, 3-HBA levels showed significant increases ranging from 18 to 23% at various times after inoculation (Fig. 4C and D).

When grown on NYG agar, the colonies of strains XC1(*pobA*) and Δ *pobA*(*pobA*) appeared lighter yellow in color than strains XC1(pBBR) and Δ *pobA*(pBBR) (Fig. 4E). Quantitative analysis showed that deletion of *pobA* had no significant effect on pigment yield; however, overexpression of *pobA* in XC1 or Δ *pobA* led to 23 and 25% decreases in pigment yield, respectively (Fig. 4E). The absorption maximum analysis and UPLC-TOF-MS analysis indicated that the predominant XC1(*pobA*)-derived xanthomonadin was Xanthomonadin-A (Fig. 3B and C). Quantitative analysis of

Fig. 3. The effect of heterologously expressing the *mhb* gene cluster on xanthomonadin biosynthesis in *Xanthomonas campestris* pv. *campestris*. **A**, Colony pigmentation and xanthomonadin biosynthesis in *X. campestris* pv. *campestris* strains. Shown are averages for three biological replicates with standard deviation. HBA, hydroxybenzoic acid; OD, optical density. *Statistically significant differences with respect to strain XC1(*mhb*) ($P \leq 0.05$). **B**, Absorption spectra of crude xanthomonadin extracts from XC1(pBBR), XC1(*mhb*), and *pobA* expression strain XC1(*pobA*). The absorption maxima are indicated with arrows. **C**, High-performance liquid chromatography analysis of the methyl esters in strains XC1(pBBR), XC1(*mhb*), and XC1(*pobA*). The dibrominated xanthomonadin methyl ester is indicated by a dashed line, and their relative ratios are indicated by percentages in parentheses.

Fig. 4. Overexpression of *pobA* significantly reduced xanthomonadin biosynthesis in *Xanthomonas campestris* pv. *campestris*. **A**, The gene cluster for 4-hydroxybenzoic acid (4-HBA) degradation and the proposed degradation pathway in *X. campestris* pv. *campestris*. TCA cycle, tricarboxylic acid cycle. **B**, Time course of bacterial growth in Nitrogen yeast glycerol medium. **C**, The 4-HBA biosynthesis in the *pobA* deletion and overexpression strains Δ *pobA*(pBBR), XC1(*pobA*), and Δ *pobA*(*pobA*). **D**, The 3-HBA biosynthesis in the *pobA* deletion and overexpression strains Δ *pobA*(pBBR), XC1(*pobA*), and Δ *pobA*(*pobA*). **E**, Colony pigmentation and xanthomonadin biosynthesis in *pobA* deletion and overexpression strains Δ *pobA*(pBBR), XC1(*pobA*), and Δ *pobA*(*pobA*). Shown are averages for three biological replicates with standard deviation. OD, optical density. *Statistically significant differences with respect to strain XC1(pBBR) ($P \leq 0.05$).

methyl esters showed that Xanthomonadin-A produced in the *pobA* overexpression strain XC1(*pobA*) was about 8% lower than that in the wild-type strain XC1(pBBR) (Fig. 3C).

The effect of the *pchAB* cluster on xanthomonadin biosynthesis depends on its copy number. The *pchA-G* cluster is involved in the biosynthesis of the siderophore pyochelin in *P. aeruginosa* (Gaille et al. 2002, 2003). *pchA* encodes an isochorismate synthase, and *pchB* encodes an isochorismate pyruvate-lyase, which hydrolyzes chorismate into SA via a two-step reaction (Supplementary Fig. S2A). Our previous results showed that chorismate is the substrate of a bifunctional chorismatase XanB2, which produces both 3-HBA and 4-HBA; 3-HBA is subsequently used as a precursor for xanthomonadin biosynthesis in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* (Zhou et al. 2013a, b). To further verify the roles of 3-HBA and 4-HBA in xanthomonadin biosynthesis, the *pchAB* gene cluster from *P. aeruginosa* strain M18 was expressed in strain XC1 in either of two ways: (i) via the multicopy expression vector pBBR1MCS-2 (Kan^R) [XC1(*pchAB*)] or (ii) via the vector mini-Tn7T-Gm, which integrates a single copy of the *pchAB* gene cluster into the chromosome (XC1::*pchAB*). We hypothesized that the introduced SA biosynthetic pathway would compete with the XanB2-dependent xanthomonadin biosynthetic pathway for the common precursor, chorismate.

Using UPLC-TOF-MS analysis as described by Wang et al. (2015), SA was detected in XC1(*pchAB*) but not XC1(pBBR) cultures (Supplementary Fig. S2B and C). The SA levels in XC1(*pchAB*) cultures increased over time, with the highest level of 292.3 μ M observed at 48 h after inoculation (Fig. 5A). This overexpression of *pchAB* in XC1 grown in NYG medium significantly reduced bacterial growth and the biosynthesis of 3-HBA, 4-HBA, and xanthomonadins (Fig. 5A to C). The subsequent exogenous addition of 3-HBA (25 μ M) or 4-HBA (25 μ M) to the XC1(*pchAB*) cultures resulted in bright or dark yellow pigment biosynthesis, respectively (Fig. 5C). Further analysis revealed two pigment absorption maxima at approximately 420 and approximately 450 nm with XC1(*pchAB*) in the presence of 3-HBA, and in the presence of 4-HBA, a pigment absorption maximum at approximately 430 nm was observed, similar to what was observed with strain XC1(*mhb*) (Fig. 5D).

Next, to avoid the depression of XC1 growth owing to the high copy number of plasmid-borne *pchAB*, a single copy of the *pchAB* cluster was integrated into the *X. campestris* pv. *campestris* chromosome at the *attTn7* site using the mini-Tn7T-Gm vector (Choi and Schweizer 2006) (Supplementary Fig. S3). The resulting strain XC1::*pchAB* showed a growth pattern similar to the wild-type strain XC1 in NYG medium (Fig. 6A). It produced less SA than the *pchAB* overexpression strain XC1(*pchAB*), with a maximum SA level of about 120 μ M observed at 48 h after inoculation (Fig. 6A). The levels of 3-HBA and 4-HBA in the XC1::*pchAB* cultures were 2.5 and 4.7 μ M, respectively, which were 35 and 41%, respectively, of those observed in the XC1 culture at 48 h after inoculation (Fig. 6B). When grown on NYG agar, the colonies of strains XC1 and XC1::*pchAB* showed no obvious difference. Exogenous addition of 3-HBA had no effect on colonies color of strain XC1::*pchAB*, whereas the addition of 4-HBA in XC1::*pchAB* resulted in biosynthesis of dark yellow pigmentation. Further quantitative analysis showed that xanthomonadin production in strain XC1::*pchAB* was 90.0% of that observed with strain XC1 as judged by OD₄₄₁ values of pigment extracts (Fig. 6C). The addition of 3-HBA or 4-HBA at a final concentration of 25 μ M to the XC1::*pchAB* culture restored pigment production to the level of XC1 (Fig. 6C).

To further rule out the possibility that the *pchAB*-dependent SA might be involved in or interfere with pigment biosynthesis, SA was exogenously added to XC1 cultures at final concentrations of 0.25 and 0.50 mM, respectively. SA had little effect on the biosynthesis of 3-HBA, 4-HBA, or xanthomonadins at 48 h after inoculation (Supplementary Fig. S4).

Fig. 5. The effect of *pchAB* overexpression on bacterial growth and 3-hydroxybenzoic acid (3-HBA), 4-HBA, and xanthomonadin biosynthesis in *Xanthomonas campestris* pv. *campestris*. **A**, The time course of bacterial growth (black curves) and salicylic acid (SA) biosynthesis (gray/red curve) in NYG (nitrogen yeast glycerol) medium. **B**, The 3-HBA and 4-HBA levels in XC1(pBBR) and XC1(*pchAB*) cultures 48 h after inoculation. **C**, Colony pigmentation and xanthomonadin biosynthesis in strains XC1(pBBR) and XC1(*pchAB*). OD, optical density. *Statistically significant differences with respect to the strain XC1(pBBR) ($P \leq 0.05$). **D**, Absorption spectra of crude xanthomonadin extracts from strains XC1(pBBR) and XC1(*pchAB*) in the presence of 3-HBA or 4-HBA. The absorption maxima are indicated with arrows. Combined with Figure 3B, the absorption maxima of crude xanthomonadin extracts from strains XC1(pBBR), XC1(*mhb*), and XC1(*pobA*) were 441, 430, and 441 nm, respectively, whereas the XC1(*pchAB*) strain had no obvious absorption maxima. Shown are averages for biological replicates repeats with standard deviation.

Interference with 3-HBA and/or 4-HBA biosynthesis also affected a range of other phenotypes in *X. campestris* pv. *campestris*. Previous findings showed an extensive cross-talk between the xanthomonadin biosynthetic pathway and those of other secondary metabolites, such as EPS and diffusible signal factor (DSF) family signals and CoQ8 (Cao et al. 2018; Zhou et al. 2013b). Thus, we further analyzed the biosynthesis of extracellular enzymes EPS and CoQ8 in the above-mentioned strains, which were compromised for the production of 3-HBA, 4-HBA, or both. HPLC analysis of CoQ8 extracts showed that overexpression of

Fig. 6. The effect of a single copy of the *pchAB* cluster on bacterial growth and 3-hydroxybenzoic acid (3-HBA), 4-HBA, and xanthomonadin biosynthesis in *Xanthomonas campestris* pv. *campestris*. **A**, Time course of bacterial growth (black curves) and salicylic acid (SA) biosynthesis (gray/red curve) in NYG (nitrogen yeast glycerol) medium. **B**, The 3-HBA and 4-HBA levels in XC1 and XC1::*pchAB* cultures 48 h after inoculation. **C**, Colony pigmentation and xanthomonadin biosynthesis in strains XC1 and XC1::*pchAB*. Shown are averages for three biological replicates with standard deviation. OD, optical density. *Statistically significant differences with respect to strain XC1 ($P \leq 0.05$). **Statistically significant differences with respect to strain XC1(pBBR) ($P \leq 0.01$).

pchAB or *pobA* in XC1 significantly reduced CoQ8 biosynthesis (Fig. 7A). CoQ8 levels in strains XC1(*pchAB*) and XC1(*pobA*) were 69 and 26%, respectively, of those observed in XC1(pBBR). Heterologous expression of the *mhb* gene cluster had no effect on CoQ8 biosynthesis (Fig. 7A). The overexpression of the *pchAB* gene cluster or *pobA* also significantly reduced EPS biosynthesis (Fig. 7B). The EPS levels (early stationary phase, OD₆₀₀ = 2.3) in cultures of XC1(*pchAB*) and XC1(*pobA*) were 0.6 and 1.2 mg/ml, respectively. These were much lower than the 2.0 mg/ml observed in the wild-type culture (Fig. 7B). As with CoQ8, the heterologous expression of the *mhb* gene cluster had no effect on EPS biosynthesis.

Interference with 3-HBA and/or 4-HBA biosynthesis in *X. campestris* pv. *campestris* led to reduced virulence. Xanthomonadins, EPS, CoQ8, and extracellular enzymes are important factors needed for *X. campestris* pv. *campestris* adaptation to different host plant ecological niches and virulence (Büttner and Bonas 2010). In this study, we further evaluated the virulence of four *X. campestris* pv. *campestris* strains, XC1(pBBR), XC1(*mhb*), XC1(*pobA*), and XC1(*pchAB*), by measuring lesion lengths on the leaves of cabbage and Chinese radish at 10 days postinoculation. Overexpression of the *mhb* gene cluster or *pobA* led to similar reductions in virulence on cabbage and radish, with average lesion length reduced by 18 to 23 and 22 to 30%,

respectively (Fig. 8). However, overexpression of the *pchAB* gene cluster led to a more substantial reduction of pathogenicity, with the average lesion length reduced by 71% on cabbage and 77% on Chinese radish (Fig. 8). This large reduction in pathogenicity may in part have been because of the compromised growth effect of the *pchAB* gene cluster as well as the production of SA and its effect on host resistance induction.

DISCUSSION

XanB2, a new bifunctional chorismatase responsible for 3-HBA and 4-HBA biosynthesis from the common precursor chorismite, is

Fig. 7. Overexpression of *mhb*, *pobA*, and *pchAB* affected pleiotropic phenotypes in *Xanthomonas campestris* pv. *campestris*. **A**, Coenzyme Q8 (CoQ8) biosynthesis. **B**, Extracellular polysaccharide biosynthesis. EPS, extracellular polysaccharide. *Statistically significant differences with respect to strain XC1(pBBR) ($P \leq 0.05$); **statistically significant differences with respect to strain XC1(pBBR) ($P \leq 0.01$).

Fig. 8. Overexpression of *mhb*, *pobA*, and *pchAB* impaired *Xanthomonas campestris* pv. *campestris* pathogenicity. **A**, Infected cabbage leaves. From left to right: leaves infected by XC1(pBBR), XC1(*mhb*), XC1(*pobA*), and XC1(*pchAB*). The infection phenotypes on cabbage are labeled with red dotted ellipses. **B**, Quantitative analysis of the lesion length in infected cabbage leaves. **C**, Quantitative analysis of the lesion length in infected radish leaves. Shown are averages for 15 leaves with standard deviation. *Statistically significant differences with respect to strain XC1(pBBR) ($P \leq 0.05$); **statistically significant differences with respect to strain XC1(pBBR) ($P \leq 0.01$).

essential for xanthomonadin biosynthesis in *X. campestris* pv. *campestris* (Zhou et al. 2013a, b), and the exogenous addition of either 3-HBA or 4-HBA to strain $\Delta xanB2$ differentially restored pigment production (He et al. 2011). Thus, analysis of the *xanB2* deletion mutant could not differentiate the possibly unique roles of 3-HBA and 4-HBA in the biosynthesis of xanthomonadins or the expression of other phenotypes, and an alternative strategy was required. In this study, we used genetic approaches to selectively eliminate 3-HBA, 4-HBA, or the production of both in *X. campestris* pv. *campestris*. The resulting strains were shown to be different in pigmentation, virulence factor production, and virulence. These findings suggest that both 3-HBA and 4-HBA are used in xanthomonadin production in *X. campestris* pv. *campestris*. When both 3-HBA and 4-HBA are present, *X. campestris* pv. *campestris* prefers 3-HBA for xanthomonadin biosynthesis. If 3-HBA is not present, then 4-HBA is used for a structurally uncharacterized xanthomonadin.

Previously, results showed that XanB2 acts on chorismate to produce similar levels of 3-HBA and 4-HBA in vitro (Zhou et al. 2013b). HPLC analysis also detected similar levels of exogenous 3-HBA and 4-HBA in either *X. campestris* pv. *campestris* or *X. oryzae* pv. *oryzae* cultures (Zhou et al. 2013a, b). Findings in 2018 showed that XanA2, a key enzyme needed for the activation of 3-HBA during the initiation of xanthomonadin biosynthesis, had an even higher activity toward 4-HBA than 3-HBA in vitro (Cao et al. 2018). These findings suggest that the *X. campestris* pv. *campestris* wild-type strain should produce similar amounts of 3-HBA-derived Xanthomonadin-A and 4-HBA-derived Xanthomonadin-B. However, this study clearly shows that the production of the 3-HBA-derived Xanthomonadin-A is dominant over the production of the 4-HBA-derived Xanthomonadin-B with the wild-type strain XC1 in vitro (Figs. 3A and 4E). The explanation for this contradiction may be that there are at least two 4-HBA-utilizing pathways in *X. campestris* pv. *campestris*: the UbiA-dependent CoQ biosynthetic pathway and the XanA2-dependent xanthomonadin biosynthetic pathway (Zhou et al. 2013b, 2019). Both pathways compete for the precursor 4-HBA. Intracellularly, UbiA may have a higher affinity toward 4-HBA than XanA2 so that most 4-HBA effluxes into the CoQ8 biosynthetic pathway. Assays to determine the affinities of XanA2 and UbiA toward 4-HBA will help to elucidate this possibility in the future.

SA, an analog of 3-HBA and 4-HBA, is an important plant hormone that regulates plant immune responses in response to pathogen infection (Pieterse et al. 2012; Yuan et al. 2007). Our previous study showed that *X. campestris* pv. *campestris* could not use SA for xanthomonadin biosynthesis (He et al. 2011). This study further shows that the exogenous addition of SA has no effect on xanthomonadin biosynthesis (Supplementary Fig. S3). This is consistent with the finding that XanA2 could not activate SA to form salicyl-ACP (Cao et al. 2018). However, this study also shows that the overexpression of *pchAB* in *X. campestris* pv. *campestris* results in endogenous SA biosynthesis and a loss of xanthomonadin biosynthesis (Fig. 6C). This does not mean that SA inhibits xanthomonadin biosynthesis. Overexpression of *pchAB* led to the excessive use of chorismate, which is an important biochemical intermediate for the synthesis of many alkaloids and other aromatic metabolites, including 3-HBA and 4-HBA (Zhou et al. 2013b). This indirectly led to limiting growth and xanthomonadin production in the strain XC1(*pchAB*). Taken together, this study further confirms that the plant defense signal SA is not involved in xanthomonadin biosynthesis and also, cannot interfere with *X. campestris* pv. *campestris* xanthomonadin biosynthesis.

All characterized *X. campestris* pv. *campestris* xanthomonadins were derived from cells that were cultured in vitro. Although detailed xanthomonadin chemical structures are still not fully elucidated, the aryl-polyene ring is derived from 3-HBA (Andrewes et al. 1973, 1976). This study shows that Xanthomonadin-A, which was identified in both the wild-type strain and strain XC1(*pchAB*) in

the presence of 3-HBA, corresponds to the known dibrominated xanthomonadins (Cao et al. 2018) (Figs. 3C and 5D). However, Xanthomonadin-B, which was produced by XC1(*mhb*) strain and strain XC1(*pchAB*) in the presence of 4-HBA, seems to be a new xanthomonadin variant (Figs. 3C and 5D). The detailed chemical structure and biological function of Xanthomonadin-B deserve further investigation.

Furthermore, as a phytopathogen, *X. campestris* pv. *campestris* has at least two very unique lifecycle stages in vivo. Initially, it lands on the host plant leaf surface, initiates an epiphytic stage, and increases its numbers. Subsequently, under favorable conditions, it may travel to the hydathodes, enter the plant, and initiate its pathogenic stage (Hugouvieux et al. 1998). Previous studies indicated that xanthomonadins may be needed by the pathogen in vivo for both the epiphytic and pathogenic stages (Poplawsky et al. 2000); however, it is not known which of the xanthomonadin variants are produced at either stage. Thus, the biological significance of these finding that *X. campestris* pv. *campestris* produces different xanthomonadins in response to the availability of 3-HBA, 4-HBA, or both deserves further investigation. The pathogen may selectively use either 3-HBA or 4-HBA in vivo to emphasize the production of the xanthomonadin variant most suited to its current life stage. We also observed that 3-HBA and 4-HBA had differential effects on the production of CoQ8 and EPS; 4-HBA seemed to have a role in promoting their production, whereas 3-HBA did not. This observation is intriguing and warrants further investigation.

ACKNOWLEDGMENTS

We thank Ningyi Zhou for providing strain *Klebsiella pneumoniae* M5a1.

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