Biosynthesis of Coenzyme Q in the Phytopathogen *Xanthomonas campestris* via a Yeast-Like Pathway

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Coenzyme Q (CoQ) is a lipid-soluble membrane component found in organisms ranging from bacteria to mammals. The biosynthesis of CoQ has been intensively studied in *Escherichia coli*, where 12 genes (*ubiA*, *-B*, *-C*, *-D*, *-E*, *-F*, *-G*, *-H*, *-I*, *-J*, *-K*, and *-X*) are involved. In this study, we first investigated the putative genes for CoQ8 biosynthesis in the phytopathogen *Xanthomonas campestris* pv. *campestris* using a combination of bioinformatic, genetic, and biochemical methods. We showed that Xc_0489 (*coq7^{Xc}*) encodes a di-iron carboxylate monooxygenase filling the *E. coli* UbiF role for hydroxylation at C-6 of the aromatic ring. Xc_0233 (*ubiJ^{Xc}*) encodes a novel protein with an *E. coli* UbiJ-like domain organization and is required for CoQ8 biosynthesis. The *X. campestris* pv. *campestris* decarboxylase gene remains unidentified. Further functional analysis showed that *ubiB* and *ubiK* homologs *ubiB^{Xc}* and *ubiK^{Xc}* are required for CoQ8 biosynthesis in *X. campestris* pv. *campestris*. Deletion of *ubiJ^{Xc}*, *ubiB^{Xc}*, and *ubiK^{Xc}* led to the accumulation of an intermediate 3-octaprenyl-4-hydroxybenzoic acid. UbiK^{Xc} interacts with UbiJ^{Xc} and UbiB^{Xc} to form a regulatory complex. Deletion analyses of these CoQ8 biosynthetic genes indicated that they are important for virulence in Chinese radish. These results suggest that the *X. campestris* pv. *campestris* CoQ8 biosynthetic reactions and regulatory mechanisms are divergent from those of *E. coli*. The variations provide an opportunity for the design of highly specific inhibitors for the prevention of infection by the phytopathogen *X. campestris* pv. *campestris*.

Coenzyme Q (CoQ) is a lipid-soluble membrane component found in organisms ranging from bacteria to mammals. It consists of a conserved aromatic ring and a polyprenyl hydrophobic tail with the number of isoprenyl units varying among

species: six in *Saccharomyces cerevisiae*, eight in *Escherichia coli*, and 10 in humans (Aussel et al. 2014b; Bentinger et al. 2010). CoQ is a key component of the electron transport chain that participates in aerobic cellular respiration within the mitochondria of eukaryotes and the plasma membrane of prokaryotes (Alcázar-Fabra et al. 2016; Kawamukai 2016; Sharma et al. 2012).

Biosynthesis of CoQ has been intensively studied in both *E. coli* and *S. cerevisiae*. CoQ8 biosynthesis in *E. coli* involves the 12 genes *ubiA*, *-B*, *-C*, *-D*, *-E*, *-F*, *-G*, *-H*, *-I*, *-J*, *-K*, and *-X*, and most of these encode enzymes that function to decorate the aromatic ring of the 4-hydroxybenzoate (4-HBA) universal precursor (Aussel et al. 2014b; Loiseau et al. 2017). The first defined step in CoQ8 biosynthesis is catalyzed by the *ubiC*-encoded chorismate-pyruvate lyase to produce 4-HBA (Supplementary Fig. S1). UbiA in *E. coli* subsequently catalyzes the prenylation of 4-HBA with the side-chain precursor octaprenyl PPi (Okada et al. 1997). Then, following condensation, the benzoate ring is modified by decarboxylation, C-hydroxylations, O-methylations, and C-methylation. Decarboxylation of the 3-octaprenyl-4-hydroxybenzoic acid (OHB) precursor is catalyzed by the decarboxylase encoded by *ubiD* and an additional gene, *ubiX*. UbiD is solely responsible for the decarboxylase activity and requires a new type of cofactor: a prenylated flavin synthesized by the associated UbiX, which is a flavin prenyltransferase (Payne et al. 2015; White et al. 2015). Subsequent methylation reactions are catalyzed by the methyltransferases encoded by *ubiG* and *ubiE*. UbiG is an S-adenosylmethionine-dependent methyltransferase, which catalyzes the two O-methylation steps (Poon et al. 1999; Wu et al. 1992), and UbiE is required for C-methylation in CoQ8 biosynthesis (Lee et al. 1997). Three hydroxylation reactions in the biosynthetic pathway introduce hydroxyl groups at positions C-1, C-5, and C-6 of the CoQ8 aromatic ring. UbiH and UbiF are proposed flavin-containing monooxygenases responsible for hydroxylation of C-1 and C-6 of the aromatic ring. Recent findings demonstrated that the C5-hydroxylation reaction was catalyzed by the UbiI protein (Hajj Chehade et al. 2013). The role of UbiB remains to be established in *E. coli*. Human mitochondrial ADCK3, the ortholog of UbiB, was shown to employ an atypical protein kinase-like fold to enable CoQ biosynthesis (Stefely et al. 2015). In addition, it was recently demonstrated that two new genes, *ubiJ* and *ubiK* (formerly *yigP* and *yqiC*, respectively, in *E. coli*), are required for efficient CoQ biosynthesis (Aussel et al. 2014a; Loiseau et al. 2017). UbiJ may serve as a carrier of the isoprenoid hydrophobic tail prior to the action of monooxygenases and methyltransferases or, alternatively, it could chaperon prenylated

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intermediates during the aerobic hydroxylation steps (Aussel et al. 2014a). Instead of being involved in a specific biosynthetic step, UbiK contributes to global CoQ8 biosynthesis by forming a complex with the C-terminal part of UbiJ (Loiseau et al. 2017).

Xanthomonas spp. are widely distributed, Gram-negative, aerobic bacteria that infect nearly 400 different plant hosts, many of which are commercially important crops such as rice, cotton, soybean, wheat, and vegetables (Leyns et al. 1984). *Xanthomonas campestris* pv. *campestris*, the causal agent of crucifer black rot, and *X. oryzae* pv. *oryzae*, the rice bacterial blight agent, are both model phytopathogens in molecular pathology (Mansfield et al. 2012). Our previous results showed that *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* produce CoQ8 as the major ubiquinone (Zhou et al. 2013a and b). Interestingly, both *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* contain no homologs of the *E. coli* chorismate lyase-encoding *ubiC* (Zhou et al. 2013a and b). Instead, they contain the gene *xanB2* (Xcc4014 in *X. campestris* pv. *campestris* and PXO_3739 in *X. oryzae* pv. *oryzae*), which encodes a novel bifunctional chorismatase needed for the biosynthesis of both 4-HBA and 3-HBA. The XanB2-dependent 4-HBA is required for CoQ8 biosynthesis (Zhou et al. 2013a and b). The other genes involved in *X. campestris* pv. *campestris* CoQ8 biosynthetic pathway remain to be elucidated.

RESULTS

Bioinformatics analysis

of *X. campestris* pv. *campestris* genome to identify the putative genes for CoQ8 biosynthesis.

CoQ8 is the major ubiquinone produced in both *X. campestris* pv. *campestris* and *E. coli*. In *E. coli*, the 12 genes required for CoQ8 biosynthesis are distributed throughout the genome, including the three gene clusters *ubiC-ubiA*, *ubiE-ubiJ-ubiB*, and *ubiH-ubiI* (Fig. 1A). Using these genes as templates, we identified seven *E. coli* homologs (Xc_0402 for *ubiA*,

Xc_0234 for *ubiB*, Xc_0673 for *ubiE*, Xc_1846 for *ubiG*, Xc_3432 for *ubiH*, Xc_3433 for *ubiI*, and Xc_3958 for *ubiK*) in *X. campestris* pv. *campestris* strain 8004 (Fig. 1B; Supplementary Fig. S2). Only two gene clusters, *ubiH* (Xc_3432)-*ubiI* (Xc_3433) and Xc_0233-*ubiB* (Xc_0234), were observed in *X. campestris* pv. *campestris* strain 8004 (Fig. 1B). Deletion of *X. campestris* pv. *campestris* *ubiH* or *ubiI* completely abolished CoQ8 biosynthesis (Supplementary Fig. S3). Because we were unable to obtain deletion mutants for *ubiA* (Xc_0402), *ubiG* (Xc_1846), or *ubiE* (Xc_0673), functional copies of these genes are probably required for *X. campestris* pv. *campestris* growth.

Xc_0489 is required for CoQ8 biosynthesis in *X. campestris* pv. *campestris*.

E. coli UbiF is a flavin-containing C6-monooxygenase with a conserved Rossmann-fold NAD(P)H/NAD(P)-binding (NADB) domain (Fig. 2A). Global domain analysis using the program SMART (Letunic et al. 2015) failed to identify an *X. campestris* pv. *campestris* gene encoding the UbiF-like NADB domain. Instead, we identified Xc_0489, which encodes a di-iron-containing four-helix-bundle domain typical of a ferritin-like superfamily (Fig. 2A). A similar ferritin-like domain is found in Coq7 of *S. cerevisiae*, another model organism for CoQ biosynthesis (Kawamukai 2016). Xc_0489 shares 35% amino-acid identity with CoQ7 in yeast (Supplementary Fig. S4) and 47.0% amino-acid identity with Coq7's homolog PA0655 in *Pseudomonas aeruginosa* PAO1 (Stenmark et al. 2001). Coq7 is a di-iron enzyme that catalyzes monooxygenation in *S. cerevisiae* CoQ6 biosynthesis (Marbois and Clarke 1996). These findings prompted us to determine whether Xc_0489 (named *coq7^{Xc}* hereafter) has CoQ7-like C6-monooxygenase activity needed for *X. campestris* pv. *campestris* CoQ8 biosynthesis.

coq7^{Xc} was deleted from *X. campestris* pv. *campestris* wild-type (WT) strain 8004, and the resulting strain $\Delta coq7^{Xc}$ displayed growth characteristics similar to those of strain 8004 in Luria-Bertani (LB) medium (Fig. 2B). The CoQ8 levels in $\Delta coq7^{Xc}$ cells at 24 h after inoculation were 26.6% of those found in WT cells. This CoQ8 deficiency could be restored by the overexpression of *coq7^{Xc}* via the vector pBBR-1-MCS (kanamycin resistant [*Kan^R*]) (Fig. 2C). An unknown electroactive compound, denoted "X", was detected in the $\Delta coq7^{Xc}$ cells but not in the WT cells. Complementation by *coq7^{Xc}* abolished the accumulation of compound X (Fig. 2C and D).

In previous work, deletion of *ubiF* from *E. coli* strain MG1655 resulted in the accumulation of demethoxy-Q8 (DMQ8) (Kwon et al. 2000); thus, we used *E. coli* strain MG1655 to generate the *ubiF* deletion strain $\Delta ubiF$ and confirmed the DMQ8 accumulation (Supplementary Fig. S5). Further comparison showed that compound X produced by *X. campestris* pv. *campestris* mutant $\Delta coq7^{Xc}$ had the same elution time and absorbance maximum as DMQ8 from *E. coli* mutant $\Delta ubiF$. Subsequent ultrahigh-performance liquid chromatography mass spectrometry (UPLC-MS) analysis verified the identity of compound X as DMQ8 (Fig. 2E).

Overexpression in strain $\Delta coq7^{Xc}$ of either *E. coli* *ubiF* or *S. cerevisiae* *coq7* via the vector pBBR-1-MCS (*Kan^R*) partially restored CoQ8 production (Fig. 3B). Conversely, overexpression of *coq7^{Xc}* in the *E. coli* strain $\Delta ubiF$ restored CoQ8 production to WT levels (Fig. 3D). Collectively, these results demonstrate that *coq7^{Xc}* is homolog of *S. cerevisiae* *coq7*, playing the same role as *E. coli* *ubiF* in *X. campestris* pv. *campestris* CoQ8 biosynthesis.

Coq7^{Xc} is a di-iron carboxylate monooxygenase with a four-helix bundle.

The *S. cerevisiae* Coq7 protein contains the conserved di-iron-binding motif EX_{n1}EXXH_{n2}EX_{n3}EXXH, which is predicted to

Fig. 1. Genetic organization and location of coenzyme Q8 (CoQ8) biosynthetic genes on the chromosome of **A**, *Escherichia coli* strain MG1655 and **B**, *Xanthomonas campestris* pv. *campestris* strain 8004. Genes indicated in a lighter shade are *X. campestris* pv. *campestris*-specific genes required for CoQ8 biosynthesis. Counterparts of *E. coli* *ubiD* and *ubiX* in *X. campestris* pv. *campestris* remain unclear.

be a di-iron center ligand (Stenmark et al. 2001). Alignment of Coq7^{Xc} and Coq7 identified a similar di-iron-binding motif. Conversion of Coq7^{Xc} key residues E96, E180, or H183 to alanine resulted in decreased CoQ8 biosynthesis (Fig. 4A), and similar analyses showed that residues G65, C68, and G75 are also essential for Coq7^{Xc} enzymatic activity (Fig. 4A). Recently, Busso et al. (2015) showed that Coq7 residues R57, V111, and S114 modulate Coq7 enzymatic activity. Among them, S114 was proposed as a new site for Coq7 phosphorylation. Sequence alignment analysis showed that the counterparts of the above three Coq7 residues in Coq7^{Xc} are R60, S112, and S115. Although point mutation of R60 and S115 significantly reduced Coq7^{Xc}'s activity for CoQ8 biosynthesis, point mutation of S112 had no effect (Fig. 4A).

Using the crystalline structure of di-iron carboxylate proteins and the proposed model of Coq7 (Busso et al. 2015; Frolow et al. 1994), we created a Coq7^{Xc} molecular model integrating information from our mutagenesis analysis with in silico analysis of residue conservation. The model consists of a four-helix bundle, with helix 2 and 3 connected by a mostly disordered loop containing a small helix segment (Fig. 4B and C).

Xc_0233 (*ubiJ^{Xc}*) is required for CoQ8 biosynthesis in *X. campestris* pv. *campestris*.

Genomic analysis of *X. campestris* pv. *campestris* strain 8004 revealed that the stop codon of Xc_0233 overlaps with the start codon of *ubiB^{Xc}*, similar to what is found with *ubiJ* and *ubiB* in *E. coli*. Although Xc_0233 and *E. coli* UbiJ have low amino-acid identity (21.8%) (Supplementary Fig. S6), both contain a similar sterol carrier protein 2 (SCP2) at their N-terminal regions (Fig. 5A). These results suggest that Xc_0233 might have *E. coli* UbiJ-like activity. Thus, we refer to Xc_0233 as *ubiJ^{Xc}* hereafter in this study.

During aerobic growth in LB medium at 28°C, the *ubiJ^{Xc}* deletion mutant Δ *ubiJ^{Xc}* exhibited a significant growth defect compared with the WT strain (Fig. 5B). Near-normal growth was restored to strain Δ *ubiJ^{Xc}* by introduction of a plasmid carrying *ubiJ^{Xc}* (Fig. 5B). The cellular CoQ8 content of strain Δ *ubiJ^{Xc}* was 37.5% of that found in the WT strain (54.8 and 145.9 pmol/mg wet weight, respectively). Overexpression of the plasmid-borne *ubiJ^{Xc}* in strain Δ *ubiJ^{Xc}* restored the CoQ8 content to WT levels (Fig. 5C and D). Because *ubiB^{Xc}* is located downstream of *ubiJ^{Xc}* in *X. campestris* pv. *campestris* (Fig. 5A), we transformed the *ubiJ^{Xc}* mutant with plasmids carrying *ubiB^{Xc}* or the *ubiJ^{Xc}-ubiB^{Xc}* gene cluster to determine the effect of the *ubiJ^{Xc}* mutation on *ubiB^{Xc}* expression. Overexpression of *ubiB^{Xc}* had little effect on CoQ8 biosynthesis in strain Δ *ubiJ^{Xc}* (Fig. 6A and B), ruling out the possibility of any polar effect of the *ubiJ^{Xc}* mutation. In contrast, overexpression of the *ubiJ^{Xc}-ubiB^{Xc}* gene cluster in strain Δ *ubiJ^{Xc}* restored CoQ8 to WT levels (Fig. 6B). Collectively, these results indicate that *ubiJ^{Xc}* is required for CoQ8 biosynthesis in *X. campestris* pv. *campestris*.

Previously, Aussel et al. (2014a) showed that the 50 C-terminal amino-acid region of UbiJ was sufficient to sustain CoQ8 biosynthesis and normal aerobic growth of the *E. coli* *ubiJ* mutant strain. In this study, partial UbiJ^{Xc} regions covering the 54 and 80 C-terminal amino acids or the 135 N-terminal amino acids were overexpressed in the *X. campestris* pv. *campestris* *ubiJ^{Xc}* mutant. Although the overexpression of the 80 C-terminal amino-acid region did restore bacterial growth and CoQ8 biosynthesis (Fig. 5B and C), the 135 N-terminal amino-acid and the 54 C-terminal amino-acid regions had no significant effect.

In an attempt to further verify that *X. campestris* pv. *campestris* UbiJ^{Xc} plays a role similar to that of *E. coli* UbiJ in CoQ8 biosynthesis, cross-complementation analyses with

Fig. 2. Xc_0489 (*coq7^{Xc}*) is required for coenzyme Q8 (CoQ8) biosynthesis in *Xanthomonas campestris* pv. *campestris*. **A**, Domain organization of UbiF, Coq7^{Xc}, and Coq7 of *Saccharomyces cerevisiae*. **B**, Growth curves of *X. campestris* pv. *campestris* (*Xcc*) strains in Luria-Bertani (LB) medium. OD₆₀₀ = optical density at 600 nm. **C**, Quantitative analysis of CoQ8 and demethoxy-Q8 (DMQ8) biosynthesis by *X. campestris* pv. *campestris* cultures at 24 h after inoculation. **D**, High-performance liquid chromatography analysis of CoQ8 and DMQ8 biosynthesis in *X. campestris* pv. *campestris* strains. **E**, Verification of the identity of DMQ8 found in the Δ coq7^{Xc} culture by ultrahigh performance liquid chromatography tandem mass spectrometry. Averages for three independent experiments with standard deviations are shown. Statistically significant differences with respect to the *X. campestris* pv. *campestris* strain are indicated by one asterisk ($P \leq 0.05$).

Fig. 3. Complementation analysis of *Xanthomonas campestris* pv. *campestris* strains $\Delta coq7^{Xc}$ and $\Delta ubiF$. **A**, Growth curves of *X. campestris* pv. *campestris* strains. **B**, Biosynthesis of coenzyme Q8 (CoQ8) and demethoxy-Q8 (DMQ8) by *X. campestris* pv. *campestris* strains at an optical density at 600 nm (OD₆₀₀) = 1.6 in LB medium. **C**, Growth curves of *Escherichia coli* strains. **D**, Biosynthesis of CoQ8 and DMQ8 by *E. coli* strains at OD₆₀₀ = 1.6. Averages for three independent experiments with standard deviation are shown. Statistically significant differences with respect to the wild-type strains of *X. campestris* pv. *campestris* or *E. coli* are indicated by one asterisk ($P \leq 0.05$).

X. campestris pv. *campestris* strain $\Delta ubiJ^{Xc}$ or *E. coli* strain $\Delta ubiJ$ were conducted. Although overexpression of $ubiJ^{Xc}$ or the $ubiJ^{Xc}$ - $ubiB^{Xc}$ gene cluster in the *X. campestris* pv. *campestris* strain $\Delta ubiJ^{Xc}$ fully restored bacterial growth and CoQ8 biosynthesis to WT levels (Figs. 5B and C and 6A and B), overexpression of *E. coli ubiJ* or the $ubiJ$ - $ubiB$ gene cluster in the same *X. campestris* pv. *campestris* strain had no effect (Fig. 6A and B). Similarly, although overexpression of $ubiJ$ or the $ubiJ$ - $ubiB$ gene cluster in the *E. coli* strain $\Delta ubiJ$ fully restored bacterial growth and CoQ8 biosynthesis (Fig. 6C and D), overexpression of $ubiJ^{Xc}$ or the $ubiJ^{Xc}$ - $ubiB^{Xc}$ gene cluster in this strain had no significant effect (Fig. 6C and D).

X. campestris pv. *campestris* $\Delta ubiJ^{Xc}$ cells accumulate the intermediate OHB.

High-performance liquid chromatography (HPLC) analysis of the $\Delta ubiJ^{Xc}$ strain CoQ extracts detected the accumulation of an unknown electroactive compound which was not present in the CoQ extracts of the WT *X. campestris* pv. *campestris* strain (Fig. 5C and D). Overexpression of $ubiJ^{Xc}$ in the mutant strain

Fig. 4. Point mutation and molecular modeling analyses indicate that $Coq7^{Xc}$ is a di-iron carboxylate monooxygenase with a four-helix bundle. **A**, Coenzyme Q (CoQ) production of *Xanthomonas campestris* pv. *campestris* strains mutated at key $Coq7^{Xc}$ residues associated with catalytic activity. **B**, $Coq7^{Xc}$ model showing a four-helix bundle and mutagenized residues. **C**, Di-iron center residues and their location inside helices. Averages for three independent experiments with standard deviation are shown. Statistically significant differences with respect to the wild-type *X. campestris* pv. *campestris* strain are indicated by one asterisk ($P \leq 0.05$).

Fig. 5. *Xc_0233* is required for coenzyme Q8 (CoQ8) biosynthesis in *Xanthomonas campestris* pv. *campestris*. **A**, Genomic localization of *ubiJ^{Xc}* in *X. campestris* pv. *campestris* strain 8004 and domain organization of UbiJ^{Xc} and UbiJ. **B**, Growth curves of *X. campestris* pv. *campestris* strains in Luria-Bertani (LB) medium. OD₆₀₀ = optical density at 600 nm. **C**, Quantitative analysis of CoQ8 and 3-octaprenyl-4-hydroxybenzoic acid (OHB) biosynthesis in *X. campestris* pv. *campestris* strains. **D**, High-performance liquid chromatography analyses of CoQ8 and OHB biosynthesis in *X. campestris* pv. *campestris* strains at OD₆₀₀ in LB medium. **E**, Verification of the identity of OHB found in strain *ΔubiJ^{Xc}* by ultrahigh-performance liquid chromatography tandem mass spectrometry. Averages for three independent experiments with standard deviation are shown. Statistically significant differences with respect to the strain *X. campestris* pv. *campestris* are indicated by one asterisk ($P \leq 0.05$).

Fig. 6. Complementation analysis of *Xanthomonas campestris* pv. *campestris* strains *ΔubiJ^{Xc}* and *ΔubiJ*. **A**, Growth curves of *X. campestris* pv. *campestris* strains in Luria-Bertani (LB) medium. OD₆₀₀ = optical density at 600 nm. **B**, Quantitative analysis of coenzyme Q8 (CoQ8) and 3-octaprenyl-4-hydroxybenzoic acid (OHB) biosynthesis in *X. campestris* pv. *campestris* strains at OD₆₀₀ = 1.6. **C**, Growth curves of *Escherichia coli* strains in LB medium. **D**, Quantitative analysis of Coq8 and 3-octaprenylphenol (OPP) biosynthesis in *E. coli* strains at OD₆₀₀ = 1.6. Averages for three independent experiments with standard deviation are shown. Statistically significant differences with respect to the strain *X. campestris* pv. *campestris* are indicated by one or two asterisks ($P \leq 0.05$ or 0.01 , respectively).

Fig. 7. Deletion of *Xanthomonas campestris* pv. *campestris* $ubiB^{Xc}$ or $ubiK^{Xc}$ significantly decreased coenzyme Q8 (CoQ8) biosynthesis, leading to accumulation of the intermediate 3-octaprenyl-4-hydroxybenzoic acid (OHB). **A**, Growth time course of *X. campestris* pv. *campestris*-derived strains. OD₆₀₀ = optical density at 600 nm. **B**, Quantitative analysis of CoQ8 and OHB biosynthesis in $\Delta ubiB^{Xc}$ -derived cultures at OD₆₀₀ = 1.6. **C**, High-performance liquid chromatography analysis of CoQ8 and OHB biosynthesis in *X. campestris* pv. *campestris*-derived strains. **D**, Quantitative analysis of CoQ8 and OHB production in $\Delta ubiK^{Xc}$ -derived cultures at OD₆₀₀ = 1.6. Averages for three independent experiments with standard deviations are shown. Statistically significant differences with respect to the strain *X. campestris* pv. *campestris* are indicated by one asterisk ($P \leq 0.05$).

$\Delta ubiJ^{Xc}$ almost completely eliminated accumulation of this compound. Further analysis of the unknown compound by UPLC-MS in positive mode gave an m/z ratio ($M+H^+$) of 681.5244, which corresponded to a chemical formula of $C_{47}H_{70}O_3$ (MW = 681.5252) (Fig. 5E). Our unpublished data showed that fragmentation of the 681.5 molecular ion yielded a characteristic tropylium-like ion. This is often found for aromatic compounds containing a benzyl unit. Taken together, the unknown compound should be one of the CoQ biosynthetic intermediates, OHB, in *E. coli* (Fig. 5E).

We then generated the *ubiJ*-in-frame deletion mutant $\Delta ubiJ$ in WT *E. coli* strain MG1655. HPLC analysis of the $\Delta ubiJ$ CoQ extract subsequently revealed a new electroactive compound not found in the WT strain. Further MS analysis indicated that this compound is the biosynthetic intermediate 3-octaprenylphenol (OPP), which has previously been shown to accumulate in *E. coli* $\Delta ubiB$ and $\Delta ubiK$ strains (Loiseau et al. 2017; Poon et al. 2000). Overexpression of *ubiJ* in strain $\Delta ubiJ$ abolished the accumulation of OPP. No OPP was found to accumulate in the *X. campestris* pv. *campestris* $\Delta ubiJ^{Xc}$ cells (Fig. 5D) and no OHB was found to accumulate in the *E. coli* $\Delta ubiJ$ cells.

$ubiB^{Xc}$ and $ubiK^{Xc}$ are involved in *X. campestris* pv. *campestris* CoQ8 biosynthesis.

X. campestris pv. *campestris* UbiB (encoded by *Xc_0234*, denoted as $ubiB^{Xc}$ hereafter) and *E. coli* UbiB have 47% amino-acid identity and a similar domain organization. Deletion of $ubiB^{Xc}$ both significantly decreased CoQ8 biosynthesis and led to an accumulation of OHB in *X. campestris* pv. *campestris* (Fig. 7B and C). Subsequently, WT levels of CoQ8 and OHB were restored to the $\Delta ubiB^{Xc}$ mutant by overexpressing $ubiB^{Xc}$ or *E. coli* *ubiB* (Fig. 7B and C).

X. campestris pv. *campestris* UbiK^{Xc} and *E. coli* UbiK have 50% amino-acid identity and have a similar domain organization. As with $ubiB^{Xc}$, deletion of $ubiK^{Xc}$ also resulted in significantly decreased CoQ8 biosynthesis and an accumulation of OHB (Fig. 7D). Likewise, overexpression of $ubiK^{Xc}$ or *E. coli* *ubiK* restored CoQ8 and OHB to WT levels (Fig. 7D).

In previous studies, the biosynthetic intermediate OPP accumulated to high levels in *E. coli* $\Delta ubiB$ and $\Delta ubiK$ strains (Loiseau et al. 2017; Poon et al. 2000). In this study, mutant strain $\Delta ubiB$ was generated in *E. coli* strain MG1655, and decreased CoQ8 biosynthesis and significant OPP accumulation were observed. Subsequently, CoQ8 and OPP were restored to WT levels by plasmids carrying either *ubiB* or $ubiB^{Xc}$ (Supplementary Fig. S7).

UbiK^{Xc} interacts with UbiJ^{Xc} and UbiB^{Xc} in *X. campestris* pv. *campestris*.

Yeast two-hybrid (Y2H) analysis was conducted in *X. campestris* pv. *campestris* to detect possible interactions between UbiK^{Xc}, UbiJ^{Xc}, and UbiB^{Xc}. There were no detectable protein level interactions between UbiB^{Xc} and the 80 C-terminal amino-acid region of either UbiJ^{Xc} or UbiK^{Xc} (Supplementary Fig. S8).

However, further investigations revealed strong binding between UbiK^{Xc} and UbiJ^{Xc}, and between UbiK^{Xc} and UbiB^{Xc}. No interactions were detected between UbiK^{Xc} and either *E. coli* UbiJ or *E. coli* UbiB.

The genes $coq7^{Xc}$, $ubiB^{Xc}$, $ubiK^{Xc}$, and $ubiJ^{Xc}$ are associated with virulence in Chinese radish.

By using the scissor clipping method of inoculation, as previously described (He et al. 2006), we showed that the lesion lengths of CoQ8 mutant strains $\Delta coq7^{Xc}$, $\Delta ubiJ^{Xc}$, $\Delta ubiB^{Xc}$, and $\Delta ubiK^{Xc}$ on Chinese radish were significantly less than that of the WT strain *X. campestris* pv. *campestris* (Fig. 8).

DISCUSSION

Biosynthesis of CoQ in *X. campestris* pv. *campestris* via a yeast-like pathway?

In *E. coli*, the products of the *ubiA*, *ubiD*, *ubiE*, *ubiF*, *ubiG*, *ubiH*, *ubiI*, and *ubiX* genes catalyze the functionalization of the phenyl ring of the 4-HBA precursor, including prenylation, decarboxylation, hydroxylation, and methylation reactions (Aussel et al. 2014a; Hajj Chehade et al. 2013; Kawamukai 2016; White et al. 2015). All enzymatic reactions necessary for Q8 biosynthesis in aerobic conditions were assigned to the corresponding Ubi proteins. The findings in this study suggest that the catalytic reactions in *X. campestris* pv. *campestris* CoQ8 biosynthetic pathway are generally similar to those in *E. coli*. First, *X. campestris* pv. *campestris* and *E. coli* have seven genes (*ubiA*, *ubiB*, *ubiE*, *ubiG*, *ubiH*, *ubiI*, and *ubiK*) in common. Individual deletion of four of them (*ubiB*, *ubiH*, *ubiI*, and *ubiK*) resulted in either the full or partial elimination of CoQ8 biosynthesis (Fig. 7). Second, although *XanB2* and *Coq7^{Xc}* in *X. campestris* pv. *campestris* are completely different from *UbiC* and *UbiF* in amino acid size and domain organization, they have similar catalytic activities. *xanB2* was able to complement *E. coli* Δ *ubiC* for CoQ8 biosynthesis (Zhou et al. 2013a and b). Δ *coq7^{Xc}* and Δ *ubiF* strains could be genetically cross-complemented by either *coq7^{Xc}* or *ubiF* (Fig. 3). In addition, *X. campestris* pv. *campestris* Δ *coq7^{Xc}* cells and *E. coli* Δ *ubiF* cells accumulated the same biosynthetic intermediate, DMQ8 (Fig. 2).

However, the present study also suggests that the order of the catalytic reactions in *X. campestris* pv. *campestris* CoQ8 biosynthesis might be different from that of *E. coli*. Decarboxylation is one of the keys steps in the bacterial CoQ biosynthetic pathway. In *E. coli*, *ubiD* and *ubiX* are responsible for the decarboxylation step (Payne et al. 2015; White et al. 2015). However, no homologs of *ubiD* and *ubiX* were identified in *X. campestris* pv. *campestris* (Fig. 1). Similar observations were also made for the CoQ6 biosynthetic pathway of *S. cerevisiae*—no gene for decarboxylation was identified (Kawamukai 2016). A comparison of the CoQ biosynthetic pathways in *E. coli* and *S. cerevisiae* shows that they diverge after the assembly of the common intermediate OHB (González-Mariscal et al. 2014; Kawamukai 2016). *E. coli* CoQ8 biosynthesis proceeds by decarboxylation of OHB followed by oxidation and O-methylation. In contrast, the *S. cerevisiae* CoQ6 biosynthetic pathway proceeds with oxidation of OHB, followed by O-methylation and then decarboxylation. Because both *X. campestris* pv. *campestris* and *S. cerevisiae* use the same type of C6-monoxygenase (*Coq7^{Xc}* and *Coq7*, respectively) in CoQ biosynthesis, and no decarboxylase was identified in either strain, it is tempting to propose that *X. campestris* pv. *campestris* might use a *S. cerevisiae*-like order of catalytic reactions for CoQ8 biosynthesis. The decarboxylation stage of the ring modification procedure could be after hydroxylation and methylation in *X. campestris* pv. *campestris*. This idea is further supported by the findings that deletion of *ubiJ^{Xc}*, *ubiB^{Xc}*, and *ubiK^{Xc}* resulted in an accumulation of the intermediate OHB in *X. campestris* pv. *campestris* (Figs. 5, 6, and 7) while deletion of the *E. coli* gene counterparts led to an accumulation of OPP.

Divergent regulatory mechanisms of CoQ biosynthesis in *X. campestris* pv. *campestris*?

Several lines of evidence indicate that UbiK, UbiJ, and UbiB may form a complex involved in the regulation of *E. coli* CoQ8 biosynthesis. First, although the products of *ubiB*, *ubiJ*, and *ubiK* are required for the efficient biosynthesis of CoQ8, none of them is predicted to carry out an enzymatic function (Hajj

Chehade et al. 2013; Lagier-Tourenne et al. 2008; Poon et al. 2000). Second, inactivation of any one of these genes drastically reduces CoQ8 levels but this leads to the accumulation of only the early intermediate OPP, which is not diagnostic of a defect in any specific biosynthetic step. Third, the UbiK protein physically interacts with UbiJ and several other Ubi proteins (Loiseau et al. 2017). *X. campestris* pv. *campestris* contains homologs of *E. coli* *ubiB* and *ubiK*, and a homolog of *E. coli* *ubiJ* (*ubiB^{Xc}*, *ubiK^{Xc}*, and *ubiJ^{Xc}*, respectively) (Fig. 1). All three of these genes are required for efficient CoQ8 biosynthesis in *X. campestris* pv. *campestris* (Figs. 5 and 7). *ubiB^{Xc}* and *ubiK^{Xc}* can complement the *E. coli* mutant strains Δ *ubiB* and Δ *ubiK*, respectively, and vice versa (Fig. 7). *UbiK^{Xc}* also interacts with *UbiJ^{Xc}* and *UbiB^{Xc}* in *X. campestris* pv. *campestris*. All of these findings support the idea that the UbiK^{Xc} complex may play roles similar to those of the *E. coli* UbiK complex to regulate CoQ8 biosynthesis in *X. campestris* pv. *campestris*.

However, the present study also suggests that the UbiK complex might act on different reaction steps of the CoQ8 biosynthetic pathway in *X. campestris* pv. *campestris* and *E. coli*. First, deletion of *ubiB^{Xc}*, *ubiJ^{Xc}*, or *ubiK^{Xc}* led to the accumulation of OHB in *X. campestris* pv. *campestris* (Figs. 5 and 7), whereas deletion of *ubiB*, *ubiJ*, or *ubiK* in *E. coli* resulted in the accumulation of OPP (Loiseau et al. 2017; Poon et al. 2000). In the proposed *E. coli* CoQ8 biosynthetic pathway, OHB is a product of UbiA while OPP is a product of OHB decarboxylation. Second, although both *UbiJ^{Xc}* and *E. coli* UbiJ contain the same SCP2 domain, *E. coli* *ubiJ* could not complement the *X. campestris* pv. *campestris* Δ *ubiJ^{Xc}* strain and vice versa (Fig. 6). There can be several reasons for this. Codon usage bias may cause a protein level difference in both *E. coli* and *X. campestris* pv. *campestris*. *UbiJ^{Xc}* may require additional unknown factors for its optimal activity or conformation in *X. campestris* pv. *campestris*. Our further results also indicate that this is probably due to the fact that *UbiK^{Xc}* interacts with *UbiJ^{Xc}* but has no binding affinity to *E. coli* UbiJ. Finally, overexpression of *E. coli* *ubiB* in the *E. coli* strain Δ *ubiB* diminished OPP accumulation to WT levels whereas overexpression of *ubiB* in the *X. campestris* pv. *campestris* strain Δ *ubiB^{Xc}* diminished OHB accumulation to WT levels (Fig. 7B). This finding suggests that *X. campestris* pv. *campestris* *UbiJ^{Xc}* and *E. coli* UbiJ may exert their roles in strain-specific manners. The roles of UbiK complex in *E. coli* and *X. campestris* pv. *campestris* CoQ biosynthesis deserve further investigation.

Fig. 8. Lesion length of *Xanthomonas campestris* pv. *campestris*-derived mutant strains Δ *coq7^{Xc}*, Δ *ubiJ^{Xc}*, Δ *ubiB^{Xc}*, and Δ *ubiK^{Xc}* in Chinese radish. Averages for three independent experiments with standard deviations are shown. Statistically significant differences with respect to the *X. campestris* pv. *campestris* wild-type strain are indicated by one or two asterisks ($P \leq 0.05$ or 0.01 , respectively).

Significance of CoQ biosynthetic gene diversity.

Previously, the gene Rv2949c was characterized as a novel chorismate lyase responsible for the direct conversion of chorismate to 4-HBA in *Mycobacterium tuberculosis* (Stadthagen et al. 2005). Pelosi et al. (2016) conducted a nice phylogenomic analysis of hydroxylases (UbiF, UbiH, and UbiI) involved in CoQ biosynthesis. Their results revealed that proteobacteria evolved a surprising diversity in the CoQ hydroxylase repertoire. In the present study, although the gene for OHB decarboxylase remains unknown, three unique genes (*xanB2*, *coq7^{Xc}*, and *ubiJ^{Xc}*) were characterized to be required for CoQ8 biosynthesis in the phytopathogen *X. campestris* pv. *campestris*. All of these findings suggest a genetic diversity in CoQ8 biosynthesis between *X. campestris* pv. *campestris* and *E. coli*.

All organisms exist in environments that vary in time and space, and genetic diversity allows populations to adapt to changing environments (Frankham 2005). The more genetic diversity a population has, the greater the likelihood that the population will be able to adapt to changing circumstances (Pullin 2002). Both *X. campestris* pv. *campestris* and *E. coli* have different life styles. *E. coli* is a facultatively anaerobic bacterium and is commonly found in the lower intestine of warm-blooded animals. It makes ATP by aerobic respiration in the presence of oxygen but is capable of switching to fermentation or anaerobic respiration if oxygen is absent (Tenaillon et al. 2010). In contrast, *X. campestris* pv. *campestris* is a strict aerobe which is known to cause black rot in crucifers (Leyns et al. 1984). It can persist in soil or on plant surfaces for over a year. As a vascular pathogen, *X. campestris* pv. *campestris* can also multiply inside plants and colonize their xylem elements (Wang et al. 2015). Thus, the question of whether other new CoQ biosynthetic genes and the putative divergent biosynthetic pathway in *X. campestris* pv. *campestris* are associated with their lifestyles deserves further investigation. Much remains to be done to fully understand the sophisticated molecular mechanisms.

The CoQ biosynthetic pathway decarboxylase gene has not yet been identified in many of the Gram-negative bacteria, *S. cerevisiae*, and all higher organisms. Because *X. campestris* pv. *campestris* and *S. cerevisiae* share similar CoQ biosynthetic pathways, *X. campestris* pv. *campestris* should be a new model organism for the study of CoQ biosynthesis, particularly the unknown gene for decarboxylation.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

X. campestris pv. *campestris* and *E. coli* strains (Supplementary Table S1) were grown in LB broth with shaking (200 rpm) at 28 and 37°C, respectively. When required, antibiotics were added to a final concentration of kanamycin at 50 mg/liter, rifampicin at 25 mg/liter, ampicillin at 100 mg/liter, tetracycline at 5 mg/liter, and gentamycin at 10 mg/liter. Solid media contained 2% agar (Sangon Biotech, Shanghai, China).

Gene deletion and functional complementation analysis.

X. campestris pv. *campestris* strain 8004 in-frame deletion mutants were generated using the methods described previously (He et al. 2006). *E. coli* gene deletion was conducted following the method previously described (Datsenko and Wanner 2000). Primers used for gene deletion are listed in Supplementary Table S2. Mutant complementation analyses were conducted by either of two methods. First, the coding region of each gene was amplified by polymerase chain reaction (PCR) and cloned into the expression vector pBBR-1-MCS (Kan^R) (Kovach et al. 1995). The resulting constructs were transferred to *X. campestris* pv. *campestris* strains through triparental mating. Alternatively, the target gene together with its promoter was

amplified by PCR, then cloned into the vector pUC18-mini-TnT7-Gm. The target gene was subsequently integrated into the *X. campestris* pv. *campestris* chromosome using the methods previously described (Jittawuttipoka et al. 2009).

In silico genome analysis and bioinformatic analysis. The whole genome of *X. campestris* pv. *campestris* strain 8004 was downloaded from the National Center for Biotechnology Information (NCBI), and the sequences of *ubiA*, -B, -C, -D, -E, -F, -G, -H, -I, -J, -K, and -X were downloaded from the genome of *E. coli* strain MG1655. The BLASTP program from NCBI was then used to identify homologs of *E. coli ubi* genes in the genome of *X. campestris* pv. *campestris* strain 8004. The homologs were selected based on two major criteria: (i) a threshold amino-acid identity of 25% and (ii) similar amino-acid size. Multiple sequence alignment analysis was performed using Clustal W, and all the nucleotide segments were translated using GENSCAN.

Site-directed mutation of the gene *coq7^{Xc}*.

coq7^{Xc} was cloned into pGEM-T-Easy vector (Promega Corp., Madison, WI, U.S.A.), and the QuikChange Site-Directed Mutagenesis Kit was used to obtain *coq7^{Xc}* point mutations. These were then verified by sequence analysis and subsequently cloned into the expression vector pBBR-1-MCS (Kan^R). In the final step of the analysis, the resulting constructs were transferred to the $\Delta coq7^{Xc}$ strain through triparental mating.

Coq7^{Xc} molecular modeling.

The Coq7^{Xc} primary sequence was obtained from the NCBI database. Molecular modeling was performed with the primary sequence as input data for protein threading in I-Tasser (Zhang 2008). All runs were performed using Auto Dock 4.0 (Morris et al. 2009), with global search exhaustiveness set to 20 and conformer maximum number set to 10. Among docking poses, lower binding energy conformations were selected for DMQ1. Figures were generated using PyMOL (DeLano 2002).

CoQ extraction, purification, and quantification by HPLC analysis.

CoQ was extracted following the previously described protocol (Zhou et al. 2013a). Briefly, 50-ml LB cultures of *X. campestris* pv. *campestris* strains were grown to an optical density (OD) of 1.6, and cells were then collected by centrifugation. Cell pellets were resuspended in 2 ml of 0.2 M acetate buffer (pH 5.6) after washing twice with 1× phosphate-buffered saline (PBS) buffer. Cryo-impacted cell homogenates were subsequently subjected to sonication for a total of 90 s, and 5 ml of hexane-acetone (1:1, vol/vol) reaction mixture was added to each cell homogenate, followed by sonication and vortexing. The hexane fractions were collected, and the solvent was removed by rotary evaporation to dryness at 40°C. The residues were 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 fractions were then collected and solvent was removed by rotary evaporation to dryness at 40°C. These residues were then dissolved in 100 µl of methanol each, and the crude extracts were further analyzed by HPLC (Agilent 1260 Infinity; Agilent Technologies, Palo Alto, CA, U.S.A.). The area under each chromatographic peak was integrated and quantified. A standard curve for CoQ8 was created by plotting the peak area versus the known concentrations. The concentration of CoQ8 in the extract was calculated based on the peak area and the standard curve. The Coq8 content in *X. campestris* pv. *campestris* was finally converted as picomoles per milligram of wet weight according to the same amount of cells taken for each measurement.

UPLC-MS analysis and quantification of CoQ8, DMQ8, OPP, and OHB.

Eluate containing the purified CoQ8 was subjected to electrospray ionization (ESI)-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. Briefly, the column temperature was maintained at 30°C and the flow rate was kept at 0.4 ml/min. In total, 5 µl of CoQ8 standard solutions or 5 µl of the extract was injected into a 1290 Infinity UPLC equipped with a Zorbax Eclipse XDB-C18 column. The signals were detected in a diode array detector at a wavelength of 220 nm with a bandwidth of ±4 nm. The UPLC elutes were further introduced into an accurate mass TOF-MS in the negative ionization mode and equipped with a Jet Stream ESI source (Agilent Technologies). The area under each chromatographic peak was integrated and quantified. A standard curve for CoQ8 was created by plotting the peak area versus the known concentrations. The concentration of CoQ8 in the extract was calculated based on the peak area and the standard curve.

Y2H assay.

Y2H assays were performed using the Matchmaker Gold Yeast Two-Hybrid System (Clontech, Mountain View, CA, U.S.A.). The test gene fragment was cloned from *X. campestris* pv. *campestris* strain 8004 by PCR amplification and ligated into the prey vector pGADT7 or bait vector pGBKT7. The resulting constructs were transformed into *S. cerevisiae* AH109 according to the manual instructions, and transformants were selected on a synthetic defined (SD) medium lacking leucine and tryptophan (SD-Leu-Trp). These strains were then suspended in 1× PBS buffer, and four 10-fold serial dilutions were placed on SD medium lacking Leu, Trp, and histidine (SD-Leu-Trp-His). Protein interactions were screened on SD medium lacking Leu, Trp, His, and adenine (SD-Leu-Trp-His-Ade). Growth of the yeast cells on SD-Leu-Trp-His-Ade plates in 2 days indicated strong interactions between the test proteins.

Virulence assay in host plant.

For the virulence assay, bacterial pellets from cultures harvested at an OD at 600 nm = 1.6 were resuspended in sterile PBS buffer to an OD of 0.05, then inoculated into adult radish plants by leaf clipping. Lesion lengths were measured 14 days after inoculation. Fifteen leaves were inoculated for each independent experiment, and each treatment was repeated three times.

Statistical analysis.

All experiments were performed at least three times. Analysis of variance for experimental data sets 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. The investigators were blinded to the group allocation by randomly selecting single colonies multiple times. No statistical methods were used to predetermine sample size.

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