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CURRENT REVIEW

Chemical Structure, Biological Roles, Biosynthesis and Regulation of the Yellow Xanthomonadin Pigments in the Phytopathogenic Genus *Xanthomonas*

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Xanthomonadins are membrane-bound yellow pigments that are typically produced by phytopathogenic bacterial *Xanthomonas* spp., *Xylella fastidiosa*, and *Pseudoxanthomonas* spp. They are also produced by a diversity of environmental bacterial species. Considerable research has revealed that they are a unique group of halogenated, aryl-polyene, water-insoluble pigments. Xanthomonadins have been shown to play important roles in epiphytic survival and host-pathogen interactions in the phytopathogen *Xanthomonas campestris* pv. *campestris*, which is the causal agent of black rot in crucifers. Here, we review recent advances in the understanding of xanthomonadin chemical structures, physiological roles, biosynthetic pathways, regulatory mechanisms, and crosstalk with other signaling pathways. The aim of the present review is to provide clues for further in-depth research on xanthomonadins from *Xanthomonas* and other related bacterial species.

Keywords: biosynthetic pathway, 3- and 4-hydroxybenzoic acids, photooxidative stress, xanthomonadin, *Xanthomonas campestris*

Xanthomonadins are membrane-bound yellow pigments that are typically produced by the phytopathogenic bacteria *Xanthomonas* spp., *Xylella fastidiosa*, *Pseudoxanthomonas* spp., and a range of environmental bacterial species. Xanthomonadins have been studied since the 1970s in the phytopathogen *Xanthomonas campestris* pv. *campestris*, which is the causal agent of black rot in crucifers. These investigations have revealed that xanthomonadins play important roles in the epiphytic survival of bacteria and in host-pathogen interactions. Further, they have revealed that xanthomonadins represent a unique group of halogenated, aryl polyene (APE), water-insoluble pigments. They are exclusively associated with the outer membranes of bacterial cell walls and it is likely that they are esterified to a phospholipid-like moiety in natural systems. The gene cluster for xanthomonadin biosynthesis has also been identified and cloned, and the five key enzymes that are essential for xanthomonadin biosynthesis have been genetically and biochemically characterized in *X. campestris* pv. *campestris*. These investigations have led to the recent proposal of a generalized xanthomonadin biosynthetic pathway. This review summarizes available information for the xanthomonadins, including chemical structures, biological roles, biosynthetic mechanisms, regulation, and crosstalk with other signaling pathways in *X. campestris* pv. *campestris*. The aim of the present review is to provide important targets for further research on xanthomonadins from *Xanthomonas* and other related bacterial species.

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THE CHEMICAL STRUCTURE OF XANTHOMONADINS

The genus *Xanthomonas* Dowson 1939 was established as a gram-negative, polarly flagellated, phytopathogenic bacteria with distinctive yellow, water-insoluble, membrane-bound pigments (Bradbury 1984) (Fig. 1). The proposed chemical structures of xanthomonadins are not stable in vitro. Several methods have been developed for pigment extraction and the latest one was described by Grammbitter et al. (2019). Briefly, *X. campestris* pv. *campestris* cultures are grown to the stationary phase (optical density at 600 nm = 2.3) in 200 ml of nutrient yeast gycerol broth medium. Cells are harvested at 10,000 rpm Q:1 for 10 min, and the pellet is then extracted with 30 ml of 1:2 (vol/vol) methanol/dichloromethane with shaking until cell debris appears nonpigmented. The debris is then removed by centrifugation at 10,000 rpm for 10 min. The supernatant of the crude extract is filtered and a saponification reaction is then performed by adding 0.5 M KOH solution (one-half of the crude extract volume) and stirring at room temperature for 1 h.

Early comparisons of nine19 yellow-pigmented *Xanthomonas* strains with 20 yellow-pigmented non-*Xanthomonas* strains from five genera indicated that the *Xanthomonas* strains all produced "*Xanthomonas* carotenoids" (i.e., xanthomonadins) exhibiting absorption maxima in petroleum ether at 418, 437, and 463 nm (Starr and Stephens 1964; Stephens and Starr 1963). Subsequent investigations of *Xanthomonas juglandis* XJ103 (since reclassified to *X. aboricola* pv. *juglandis*) revealed that xanthomonadins, which are isolated as methyl or isobutyl esters, are not carotenoids but, instead, are mixtures

of unique brominated aryl octanes (Andrewes et al. 1976). Detailed structural analysis of isobutyl xanthomonadin I further indicated that it was 17-(4-bromo-3-methoxyphenyl)-17-bromo-heptadeca-2,4,6,8,10,12,14,16-octaenoic acid (Andrewes et al. 1976) (Fig. 1). Additional studies demonstrated that xanthomonadins from different *Xanthomonas* species differ in bromination and methylation patterns that can be used to differentiate them (Starr et al. 1977).

Dianese and Schaad (1982) investigated the location of xanthomonadins among different cellular membranes and determined that xanthomonadins were probably bound to outer cellular membranes. Further analysis revealed that xanthomonadins produced by *X. oryzae* pv. *oryzae* were located in the outer membrane and that a putative cytoplasmic membrane protein was required for outer membrane localization of pigments (Goel et al. 2002). Considering the chemical structures of typical phospholipids found in cellular membranes, xanthomonadins are probably esterified to a lipid-like moiety in vivo. Accordingly, Aririatu and Kester (1985) separated the pigments of *X. juglandis* (*campestris*) ATCC 11329 on silica gel columns and identified four esters. Among these, ester types 1 and 2 comprised over 95% of the mixture. Moreover, the presence of phosphate, glycerol, and sorbitol in the pigment ester 1 and phosphate and glycerol in the pigment ester 2 indicated that they were both phospholipids. Thus, ester 1 was proposed to consist of one pigment molecule esterified to glycerophosphoryl sorbitol, while ester 2 was proposed to comprise 2 pigment molecules esterified to glycerophosphate (Fig. 1). Moser et al. (2014) also demonstrated that xanthomonadins could be extracted and separated by methods optimized for phosphatidylcholine extraction and separation, further supporting that xanthomonadins are phospholipids. In addition to the above studies, others have indicated variation among pigment molecules. For example, Cao et al. (2018) analyzed *X. campestris* pv. *campestris* pigment esters and did not observe methyl modifications of the aromatic ring. Heterologous expression of the *X. campestris* pv. *campestris* *pig* gene cluster in the plant growth–promoting strain *Pseudomonas aeruginosa* M18 yielded the production of xanthomonadin-like pigments with halogenation modifications (Cao et al. 2018). More recently, Cao et al. (2020) observed that *X. campestris* pv. *campestris* produced two xanthomonadin variants: a 3-hydroxybenzoic acid (3-HBA)-derived xanthomonadin A compound that corresponds to the published ester 17-(4-bromo-3-methoxyphenyl)-17-bromo-heptadeca-2,4,6,8,10,12,14,16-octaenoic acid and a 4-HBA-derived xanthomonadin B, whose detailed structure is unknown. Rigorous validation of the proposed structures requires the future development of a method to separate the pigment mixtures that only differ in the number of bromine atoms or the presence of hydroxyl or methyl groups on the aromatic rings.

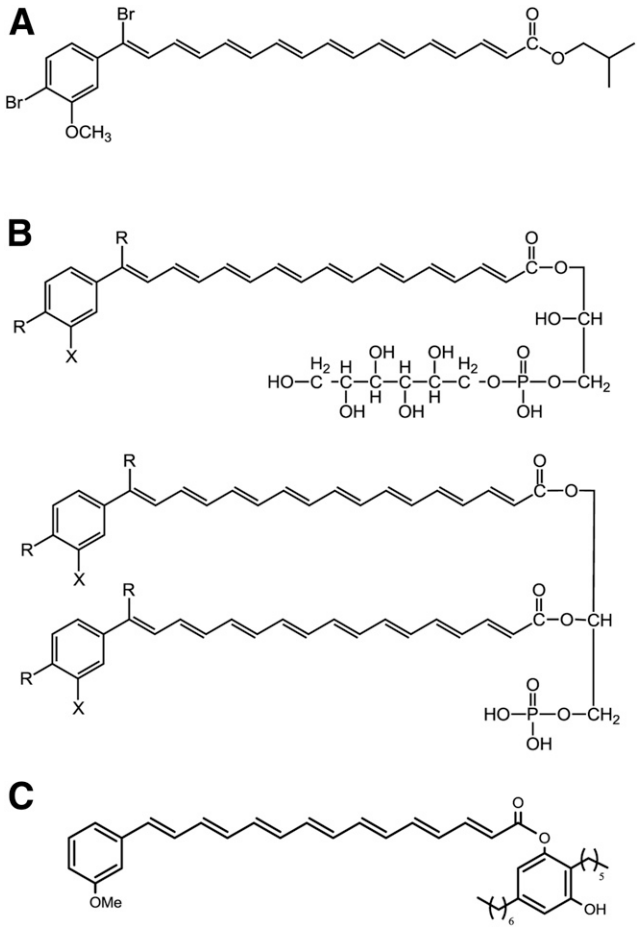

Fig. 1. The proposed chemical structures of xanthomonadin-derived esters in *Xanthomonas campestris* pv. *campestris*. **A,** Isobutyl ester of xanthomonadin I from *X. axonopodis* pv. *juglandis* (Andrewes et al. 1976). **B,** Esters I and II of the *X. campestris* ATCC11329 xanthomonadin. R = H or Br, X = –OH or –$OHCH_3$ (Aririatu and Kester 1985). **C,** Xanthomonadin-dialkylresorcinol from *Azoarcus* sp. strain BH72.

XANTHOMONADINS IN NON-*XANTHOMONAS* BACTERIA

Although a *pig*-like gene cluster is present in the genomes of all *Xanthomonas* strains that have been subjected to sequencing, the cluster has also been observed in the genomes of many non-*Xanthomonas* bacteria, including those of *Xylella fastidiosa*, *Pseudoxanthomoas spadis*, *Rhodanobacter* spp., *Frateria aurantia*, *Herminiimonas arsenicoxydans*, *Variovorax paradoxus*, an *Azoarcus* sp., *Dechloromonas aromatica* RCB, and *Lysobacter enzymogenes* (Schöner et al. 2014, 2016; Wang et al. 2013; Zhou et al. 2013b) (Table 1). The pairwise homologies of the proteins encoded by the *pig* clusters of the non-*Xanthomonas* strains ranged from 60 to 70% between some strains to 30 to 40% between others. Indeed, some of these strains produce very similar pigments. For example, the yellow-pigmented biocontrol agent *L. enzymogenes* produces non-brominated xanthomonadin analogs that are needed for protection against ultraviolet (UV) and visible light radiation (Wang et al. 2013). In addition, *Azoarcus* sp. strain BH72 and *Variovorax paradoxus* B4 were shown to produce a novel, nonbrominated, APE-dialkyl-resourcinol hybrid pigment (Schöner et al. 2014, 2016).

Bacterial strains that produce xanthomonadin-like pigments have great economic importance and industrial potential. Most importantly, all species of genera *Xanthomonas* and *Xylella* are plant pathogens. These strains infect economically important crops, such as rice, cotton, soybean, banana, grapevine, and citrus, and can lead to serious losses of yield (Hayward 1993). Second, many of these strains exhibit strong biological control of plant diseases or plant growth–promoting activities. For example, many *Lysobacter* strains exhibit strong biological control activities of plant fungal diseases (Folman et al. 2003) and *V. paradoxus* S110 is a growth-promoting endophyte that is found in many different plant hosts (Han et al. 2011). Third, some of these strains are considered "ultramicrobes" that have great potential in industrial applications. For example, *Herminiimonas arsenicoxydans* was isolated from industrial sludge and is able to oxidize the toxic chemical element arsenic to less toxic forms (Muller et al. 2006). In addition, *Pseudoxanthomonas spadix* BD-a59 and *Dechloromonas aromatica*

RCB can degrade industrial pollutant aromatics including benzene, toluene, ethylbenzene, and xylene (Coates et al. 2001; Kim et al. 2008). Further, the gram-negative bacterium *Sideroxydans lithotrophicus* ES-1 grows on $FeCO_3$ or FeS at oxic-anoxic interfaces in water under circumneutral pH and is able to oxidize Fe(II) (Emerson et al. 2007). Moreover, the highly abundant *Rhodanobacter denitrificans* strains 2APBS1 and 116-2 are active in acidic, nitrate-rich subsurface environments with high metal (e.g., uranium) concentrations. Their characteristic growth in these harsh, contaminated environments leads to concomitant bioremediation via denitrification (Prakash et al. 2012). However, little is known about the biological roles of the yellow pigments that are produced by these organisms. The determination of their pigment structures, biosynthetic pathways, and biological roles will advance our understanding of the molecular mechanisms underlying adaptations to extreme environments and further promote the industrial application of these strains.

THE ROLES OF XANTHOMONADINS IN *XANTHOMONAS*–HOST PLANT INTERACTIONS

It is well-established that many plant-pathogenic bacteria produce carotenoid pigments as photo-protectants against UV radiation damage and as antioxidants that protect against the deleterious effects of reactive oxygen species (ROS) (Goto 1992; Mohammadi et al. 2012; Tuveson et al. 1988). The similar polyene structure between carotenoids and xanthomondins has led to considerable research interest in the biological role of xanthomonadins. These investigations were initially conducted with *Xanthomonas* spp., but recent investigations of *L. enzymogenes* and *Azoarcus* sp. strain BH72 have led to an increased understanding of the biological role of xanthomonadins. Data from these studies suggest that xanthomonadin pigments play a role in establishing or maintaining commensal relationships between bacteria and their hosts.

Prevention of photodamage and enhancing epiphytic survival.

Xanthomonas species cause disease in a wide range of host plants and many can grow on plant leaf surfaces as epiphytes without affecting the host (Beattie and Lindow 1999; Hayward 1993; Hirano and Upper 1983). Once conditions are favorable, they may proliferate and increase cell densities on leaf surfaces before initiating infections. Thus, epiphytic colonization can be the first step in the *Xanthomonas* infection cycle. To survive on plant leaf surfaces, *Xanthomonas* spp. must deal with many environmental stressors, including sunlight. Visible and UV light irradiation result in the production of ROS through energy transfer by chromophores to oxygen molecules—a mechanism termed the photodynamic effect. As observed in structurally similar carotenoids, xanthomonadins absorb wavelengths ranging from UV-C to far-red light. To evaluate sensitivity to UV-C radiation, two nonpigmented *X. campestris* pv. *campestris* single-site mutation strains were compared against the pigmented parent strain (Poplawsky et al. 2000), with the two mutant strains exhibiting no other phenotypic differences from the parent strain. After UV-C exposure for 40 s, the kill curves of the three strains were indistinguishable from each other, despite that survival was reduced by almost seven log units. This indicated that xanthomonadin pigments do not protect *X. campestris* pv. *campestris* from UVr, at least not in vitro. However, these results do not preclude the possibility that xanthomonadins protect against UVr in the presence of a

Table 1. Representative bacteria producing xanthomonadin or xanthomonadin-like pigments

Family/genus and species	Descriptions	*APE* cluster[a]	Colony color	References
Xanthomonadaceae				
X. campestris pv. *campestris*	Black rot of crucifers	Yes	Yellow	Vorhölter et al. 2008
X. oryzae pv. *oryzae* strains	Rice bacterial blight	Yes	Yellow	Salzberg et al. 2008
X. perforans 91-118	Tomato leaf spot	Yes	Yellow	Potnis et al. 2011
X. sacchari NCPPB 4393	Banana bacterial wilt	Yes	Yellow	Studholme et al. 2010
X. albilineans GPE PC73	Sugarcane leaf scald	Yes	Yellow	Pieretti et al. 2012
Pseudoxanthomonas spadix BD-a59	Benzene-, toluene-, ethylbenzene-, and xylene-degrading strain	Yes	Yellow	Choi et al. 2013
Xylella fastidiosa strains 9a5c, Temecula1, and M23	Citrus canker and grape Pierce's disease	Yes	Yellow	da Silva et al. 2002
Rhodanobacter denitrificans 2APBS1	Strain capable of complete denitrification	Yes	Yellow	Prakash et al. 2012
Frateuria aurantia DSM 6220	Isolated from plant *Lilium auratum* Lindl	Yes	Yellow	Zhang et al. 2011
Lysobacter enzymogenes OH11	A biological control agent for plant diseases	Yes	Yellow	Wang et al. 2013
Fulvimonas soli LMG 19981	Soil bacterium that can degrade acetylated starch plastic	ND	Yellow	Mergaert et al. 2002
Comamonadaceae				
Variovorax paradoxus extrapolysaccharide	Aerobic soil hydrogenoxidizing bacterium	Yes	Yellow	Han et al. 2013
Variovorax paraxoxus S110	Metabolically versatile plant growth–promoting endophyte	Yes	Yellow	Han et al. 2011
Xylophilus ampelinus	Grape bacterial necrosis and canker	ND	Yellow	Willems et al. 1987
Oxalobacte aceae				
Herminiimonas arsenicoxydans	Able to oxidize the toxic chemical element arsenic	Yes	Yellow	Muller et al. 2006
Gallionellaceae				
Sideroxydans lithotrophicus ES-1	An autotrophic ironoxidizing bacterium	Yes	ND	Emerson et al. 2007
Rhodocyclaceae				
Azoarcus sp. strain BH72	Nitrogen-fixing grass endophyte	Yes	Yellow	Schöner et al. 2014
Dechloromonas aromatica RCB	Aromatics degradation strain	Yes	ND	Coates et al. 2001
Vibrionaceae				
Vibrio fischeri ES114		Yes	Yellow	Cimermancic et al. 2014
Flexibacteraceae				
Flexibacter elegans		Yes	Yellow	Fuchs et al. 2013

Q:3 [a] *APE* = aryl polyene genes; ND = not determined.

photosensitizing agent in vivo (Downum and Wen 1995). In contrast, a *L. enzymogenes* pigmented parent strain was approximately 20-fold more resistant to UV-C exposure death than were nonpigmented strains that each carried a deletion in one of three different open reading frames (ORFs)s in the *pig*-like gene cluster (Wang et al. 2013). Differences in the chemical structure or cellular location of pigment molecules could be responsible for the observed differences in the biological roles of these two pigments.

Xanthomonadins also protect against visible light damage in the presence of exogenous photosensitizers. For example, pigment-deficient mutants of several *Xanthomonas* species, including *X. juglandis*, *X. oryzae* pv. *oryzae*, and *X. campestris* pv. *campestris*, were more susceptible to photooxidative damage than their corresponding wild-type parental strains (Jenkins and Starr 1982; Poplawsky et al. 2000; Rajagopal et al. 1997) (Fig. 2A and B). Furthermore, disruption of xanthomonadin production in *X. campestris* pv. *campestris* B-24 by transposon mutagenesis resulted in an approximately 100-fold decrease in bacterial epiphytic survival following exposure to high-intensity, natural light (Poplawsky et al. 2000) (Fig. 2C). Importantly, chromosomally restored mutant strains regained epiphytic survival ability. Wang et al. (2013) further demonstrated that *Lysobacter* xanthomonadins also protect against visible light damage. Under visible light irradiation for 20 min, only 0.3 to 0.6% of the pigment-deficient strain cells survived, while the survival rate of pigmented strains was approximately 4.0% under the same conditions. Collectively, these findings support the role of xanthomonadins in maintaining the ecological fitness of bacteria by protecting bacterial cells against photooxidative stress. Nevertheless, it remains to be determined whether protection by xanthomonadins occurs by directly quenching singlet oxygen molecules or by conferring greater membrane stability. The established cellular location of xanthomonadins in membranes and the requirement for light indicates that these pigments probably stabilize cell membranes during the epiphytic phase of the pathogens. Thus, xanthomonadins probably enable the survival of xanthomonads on plant leaf surfaces by providing protection against photooxidative damage.

Antioxidant activities.

ROS including H_2O_2 and $^{1/2}O_2$ are oxidative stressors that damage cellular membranes, proteins, and DNA molecules. Oxidative stress is a common environmental stressor that bacterial pathogens encounter within various environments and,

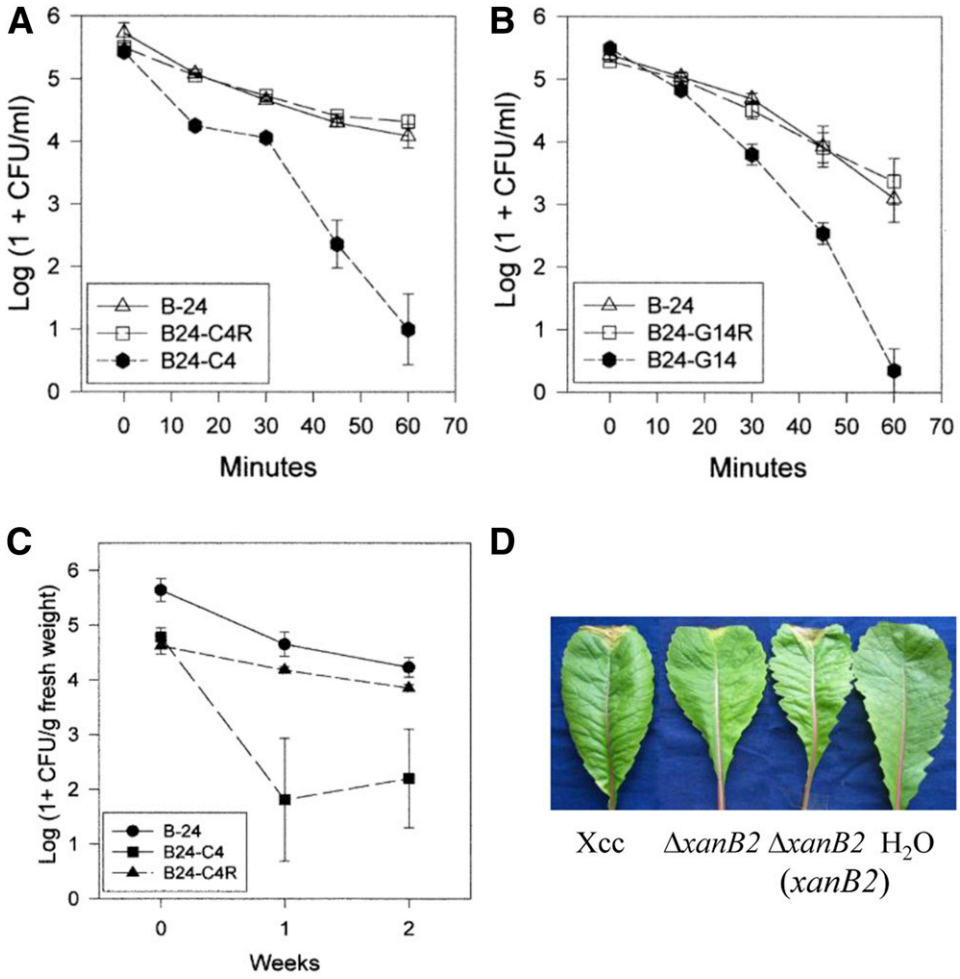

Fig. 2. Visible light sensitivity, epiphytic survival, and virulence of the *Xanthomonas campestris* pv. *campestris* wild-type strain, xanthomonadin mutants, and restored mutant strains. **A,** Survival of the *X. campestris* pv. *campestris* wild-type strain B-24, the xanthomonadin *pigC* mutant (B24-C4), and the restored mutant (B24-C4R) in the presence of visible light and toluidine blue. **B,** Survival of B-24, the xanthomonadin *pigG* mutant (B24-G14), and the restored mutant (B24-G14R) strains in the presence of visible light and toluidine blue. **C,** Epiphytic survival of B-24, the xanthomonadin mutant (B24-C4), and the restored mutant (B24-C4R) strains in greenhouse experiments. **D,** Virulence of the *X. campestris* pv. *campestris* XC1 and the derived-pigment mutants in Chinese radish plants. All data were compiled from Poplawsky et al. (2000) and He et al. (2011).

particularly, during the systemic invasion of host organisms (Bindschedler et al. 2006). Bacterial strains have consequently evolved effective oxidative stress responses, including the production of ROS-detoxifying enzymes and antioxidants such as carotenoid pigments that can effectively quench ROS (Krinsky 2001; Storz and Imlay 1999). *X. campestris* pv. *campestris* is a xylem-inhabiting phytopathogen that is likely to encounter oxidative stress in the early apoplastic phase of infections. Following the apoplastic phase of infection during systemic colonization, *Xanthomonas* preferentially colonizes xylem tissues in which bacterial cells can also experience oxidative stress. Therefore, the ability of *X. campestris* pv. *campestris* to survive oxidative stress is critical for the successful colonization of host plants. Rajagopal et al. (1997) showed that *X. oryzae* pv. *oryzae* xanthomonadins protect membrane lipids in egg-phosphatidylcholine liposomes against peroxidation in vitro. Further, XanB2 deletion mutants were xanthmomonadin-deficient and exhibited compromised production of coenzyme Q8 (CoQ8) in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* (Zhou et al. 2013a and b). The survival, H_2O_2 resistance, and systemic invasion of ΔxanB2 strain cells were all significantly compromised compared with the wild-type cells (Fig. 2D). In addition, deletion mutants of *X. campestris* pv. *campestris* 4015 (which encodes an AMP-ligase that is involved in xanthomonadin biosynthesis) were xanthomonadin-deficient and more sensitive to H_2O_2 treatment than the *X. campestris* pv. *campestris* wild-type strain but less sensitive than the ΔxanB2 strain (He et al. 2011). Xanthomonadin-dependent antioxidant activity was also observed in the strain *L. enzymogenes.* Pigment-deficient strains had a survival rate of approximately 25% after treatment with 200 μM H_2O_2, while pigmented strains had a survival rate of approximately 80% under the same conditions (Wang et al. 2013). Additionally, the APE/dialkylresorcinol hybrid yellow pigment of *Variovorax paradoxus* B4 is functionally related to antioxidative carotenoids (Schöner et al. 2016). More recently, two truncated analogs of the proposed xanthomonadin 2 by Andrewes et al. (1976) (Fig. 1) have been synthesized utilizing an iterative Heck-Mizoroki/iododeboronation cross-coupling approach (Madden et al. 2019). Further analysis showed that despite their shorter polyene chain length, incorporation of one compound into *Escherichia coli* provided photoprotective activity against singlet oxygen analogous to the natural photoprotective mechanisms employed by *Xanthomonas* bacteria. Taken together, these results suggest that xanthomonadins may confer antioxidant activities. However, direct evidence using purified xanthomonadins in biochemical assays is required to further confirm this idea.

Virulence and systemic infection.

The role of xanthomonadins in *Xanthomonas* virulence and systemic infection remain controversial. Several studies have documented that all stable, nonpleiotropic, xanthomonadin-deficient mutant strains were unaffected in pathogenicity and virulence when using wound-inoculation techniques (Poplawsky and Chun 1997; Poplawsky et al. 1993; Tsuchiya et al. 1982). Although pigment-deficient mutant strains exhibited greatly reduced hydathode infection under natural light conditions, this could be a result of the reduced epiphytic colonization observed in these experiments (Poplawsky et al. 2000) (Fig. 2). In contrast to mutant strains that were only deficient in pigment production, pleiotropic ΔxanB2 pigment-deficient *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* strains exhibited compromised virulence and systemic invasion capacities after wound inoculation (He et al. 2011; Zhou et al. 2013a). Future experiments could utilize the placement of bacteria directly into hydathode exudation droplets in order to separate epiphytic colonization from hydathode infection.

XANTHOMONADIN BIOSYNTHESIS

Identification of the xanthomonadin-encoding genomic region.

The xanthomonadin-encoding genomic region was initially identified in *X. campestris* pv. *campestris* using chemical mutagenesis, to generate pigment-deficient mutants, followed by complementation analysis with cosmid clones and subclones (Poplawsky et al. 1993). Xanthomonadin production was subsequently identified within a 25-kbp genomic clone (pIG102) that was designated the *pig* gene cluster. When transferred to *Pseudomonas fluorescens*, this clone conferred the production of a yellow pigment with an absorption maximum in methanol at 415 nm. Further transposon saturation mutagenesis analyses with the *lacZ*-encoding transposon Tn*3*HoHo1 identified seven xanthomonadin transcriptional units and their corresponding orientations within the *pig* genomic region (Poplawsky and Chun 1997). Insertional inactivation also identified a 21.0-kbp genomic region from *X. oryzae* pv. *oryzae* that was homologous to the previously described *X. campestris* pv. *campestris pig* gene cluster (Goel et al. 2002). Several *Xanthomonas* genomes have been subsequently sequenced and published since 2002. Genome sequence analysis of *X. campestris* pv. *campestris* strains ATCC33913, 8004, and B100 indicated that the *pig* cluster comprises 22 ORFs ranging from the loci *X. campestris* pv. *campestris* 3998 to *X. campestris* pv. *campestris* 4015 in the genome of the *X. campestris* pv. *campestris* strain ATCC33913 (NCBI access number) (Fig. 3A). To further confirm the role of the *pig* cluster in xanthomonadin biosynthesis, the entire *pig* gene cluster was directly cloned from *X. campestris* pv. *campestris* XC1 and was transferred to the closely related but non–xanthomonadin producing, bacterial strain *Pseudomonas aeruginosa* (Cao et al. 2018). The resultant PA (*pig*) strain produced membrane-bound dark yellow pigments when grown on Luria Bertani agar medium. High pressure liquid chromatography and subsequent mass spectrometry analysis of the methyl esters of these pigments indicated a chemical formula of $C_{24}H_{22}Cl_2O_3$. Thus, *Pseudomonas aeruginosa* heterologously expressed the *X. campestris* pv. *campestris pig* gene cluster and produced xanthomonadin-like pigments with halogenation modifications (Cao et al. 2018).

The xanthomonadin biosynthetic pathway.

Systematic deletion studies within the *pig* locus of *X. campestris* pv. *campestris* XC1 demonstrated that the genes Xcc4015 (*xanA2*), Xcc4014 (*xanB2*), Xcc4012 (*xanC*), Xcc4010 (*xanD*), Xcc4009 (*xanE*), Xcc4008 (*xanF*), Xcc4004 (*xanG*), Xcc4003 (*xanH*), Xcc4002 (*xanI*), Xcc4001 (*xanJ*), Xcc4000 (*xanK*), Xcc3999 (*xanL*), and Xcc3998 (*xanM*) are required for xanthomonadin biosynthesis (Cao et al. 2018) (Fig. 3A and B). *xanB2* was originally shown to underlie the biosynthesis of a diffusible factor (DF) in *X. campestris* pv. *campestris* (Poplawsky et al. 2005). Subsequent genetic analysis demonstrated that XanB2 operates downstream of the shikimate pathway and is essential for xanthomonadin biosynthesis. Additional in vitro biochemical assays using purified XanB2 revealed that it uses the end product of the shikimate pathway, chorismate, as a substrate to synthesize 3-hydroxybenzoic acid (3-HBA) and 4-HBA (Zhou et al. 2013a amd b). XanB2 is a 335-amino acid (aa) protein with a YjgF-like domain at the C-terminus, a conserved middle region, and an uncharacterized N-terminal domain. It is structurally different from the other known 4-HBA-producing protein, UbiC, and the 3-HBA-producing proteins Hyg5, RapK, and FkbO (Zhou et al. 2013b). Thus, XanB2 represents a novel bifunctional chorismatase for 3- and 4-HBA biosynthesis. Further analysis demonstrated that

XanB2-derived 3- and 4-HBA are the biosynthetic intermediates for xanthomonadin and CoQ8 biosynthesis, respectively (Zhou et al. 2013b). Our recent analyses indicated that 4-HBA can also be used as a precursor for xanthomonadin aryl ring biosynthesis (Cao et al. 2020).

xanC (Xcc4012) encodes an acyl carrier protein (ACP) that is essential for xanthomonadin biosynthesis, and *xanA2* (Xcc4015) encodes a 511-aa protein with a conserved AMP-binding superfamily domain. Our recent analyses demonstrated that XanA2 exhibits both 3/4-HBA:AMP ligase and 3/4-HBA-AMP:ACP ligase activities and is an ATP-dependent 3/4-HBA:ACP ligase (Cao et al. 2018). XanC and XanA2 act together to catalyze the formation of 3/4-HBA-S-ACP for initiating xanthomonadin biosynthesis via a novel type-II polyketide biosynthesis (PKS) pathway (Cao et al. 2018). Lastly, *xanH* (Xcc4003) encodes a FabG-like protein, whereas *xanK* (Xcc4000) encodes a novel glycosyltransferase. Genetic analysis demonstrated that *xanH* and *xanK* are both required for xanthomonadin biosynthesis in addition to the balanced biosynthesis of extracellular polysaccharides and diffusible signal factor (DSF)-family quorum sensing signals in *X. campestris* pv. *campestris* (Cao et al. 2018).

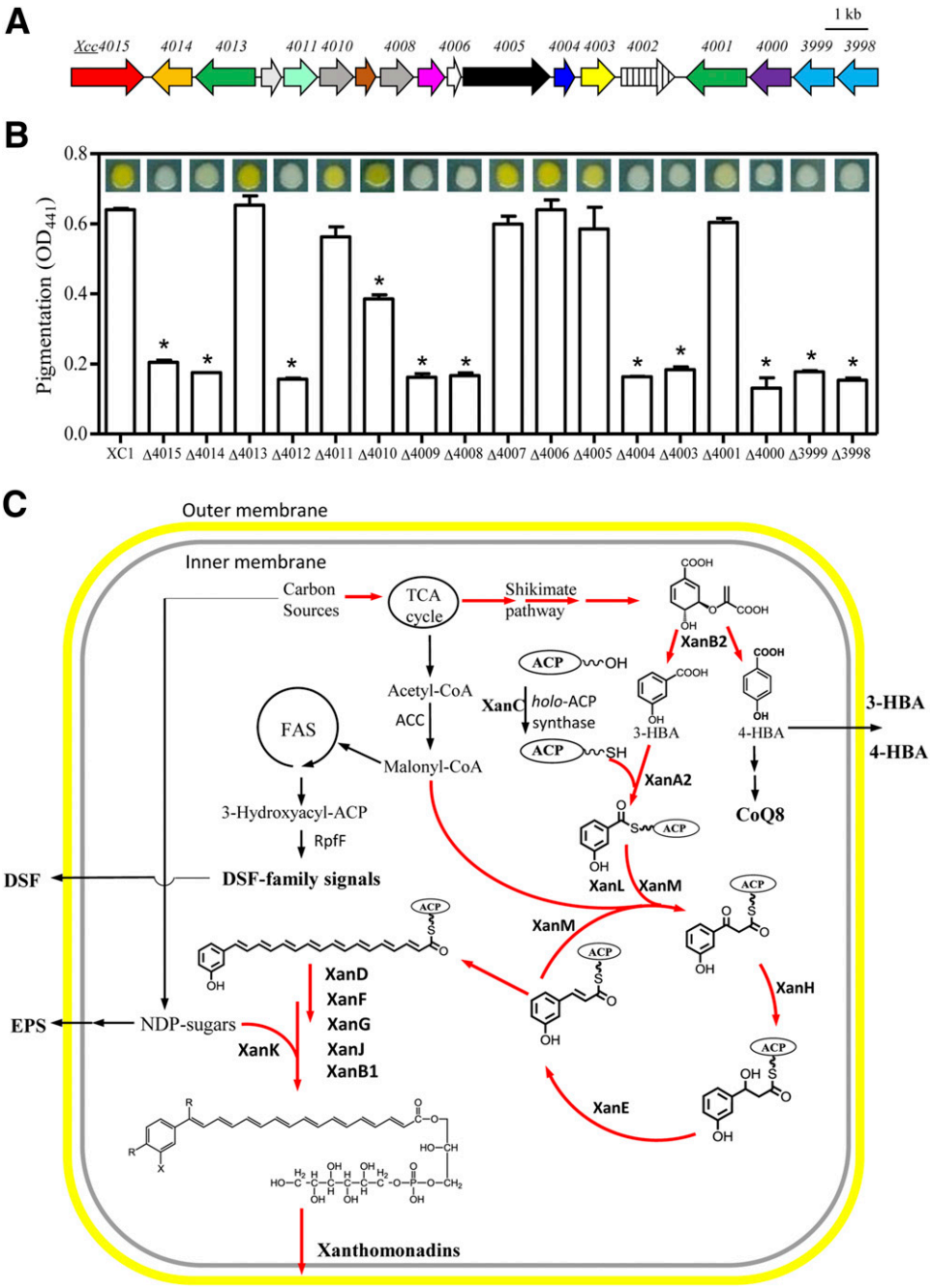

Fig. 3. Xanthomonadin biosynthetic genes and the proposed biosynthesis pathway in *Xanthomonas campestris* pv. *campestris*. **A,** Genetic organization of the xanthomonadin biosynthetic cluster in the genome of *X. campestris* pv. *campestris* ATCC33913. **B,** Pigment production by the *X. campestris* pv. *campestris* strain XC1 and XC1-derived single-gene deletion mutants. **C,** A model for xanthomonadin biosynthesis in *X. campestris* pv. *campestris*. TCA cycle = tricarboxylic acid cycle, FAS = fatty acid synthesis, ACC = acetyl-CoA carboxylase, RpfF = DSF synthetase, EPS = exopolysaccharide, NDP-sugars = nucleoside diphosphate sugars. XanA2 = 3-HBA; acyl carrier protein (ACP) ligase XanB2 = 3-hydroxybenozate/4-hydroxybenozate synthase; XanM = ketosynthase; XanL = chain length factor; XanH = ketoreductase; XanE = dehydratase; XanD and XanF = acyltransferase; XanG = FabA-like protein; XanJ = halogenase; XanK = glycosytransferase. Compiled from Cao et al. (2018) with slight modifications.

Based on the above observations, a schematic model was proposed to describe the xanthomonadin biosynthetic pathway (Fig. 3C). The proposed model involves at least four steps, including i) synthesis of the initiator compound 3-HBA-S-ACP. Synthesis of 3-HBA-S-ACP is achieved by XanB2, XanC, and XanA2 through sequential reactions that require the precursor chorismite, which is the end product of the shikimate pathway. ii) The aryl-polyene chain is elongated via a novel type II PKS pathway. XanL and XanM are responsible for catalyzing the condensation of 3-HBA-ACP with malonyl-CoA, while β-keto acyl-ACP is subsequently reduced by XanH (KR) and XanE (DH) with the concomitant formation of double bonds. iii) The aryl-polyene is further esterified to glycerol-3-phosphate or glycerophosphoryl sorbitol to form a phospholipid-like structure by the acyltransferase XanD or XanF. iv) The phospholipid-like products are further modified to form xanthomonadins. These modifications include halogenation, methylation, glycosylation, and phosphorylation reactions that are catalyzed by XanB1, XanJ, XanG, and XanK, respectively. The xanthomonadins are subsequently transported into the phospholipid bilayers of outer membranes. It should, however, be noted that the detailed mechanisms underlying modification and transport require elucidation.

Extensive crosstalk between the xanthomonadin biosynthetic pathway and other metabolite biosynthetic pathways has been observed in *X. campestris* pv. *campestris*. For instance, deletion of *xanB2* in *X. campestris* pv. *campestris* disrupted xanthomonadin biosynthesis but also caused a reduction in extrapolysaccharide (EPS) biosynthesis and an increase in the biosynthesis of the DSF signal (Poplawsky and Chun 1997; Poplawsky et al. 1998; He et al. 2011). In addition, mutation of the DSF synthase gene *rpfF* in *X. campestris* pv. *campestris* halted DSF biosynthesis but also increased xanthomonadin biosynthesis (Poplawsky et al. 1998). Furthermore, Wang et al. (2016) observed that simultaneous deletion of the *rpfB* and *rpfC* genes of *X. oryzae* pv. *oryzae* resulted in overproduction of DSF-family signals and a concomitant reduction in the biosynthesis of xanthomonadins and EPS. Recently, Cao et al. (2018) observed that deletion of *xanH* disrupted xanthomonadin biosynthesis and also increased DSF biosynthesis. Consequently, it is possible that DSF, EPS, and xanthomonadins all compete for common biosynthetic intermediates, including malonyl-CoA and sugar donors, in addition to the *holo*-ACP synthase in *X. campestris* pv. *campestris*.

Xanthomonadin-like pigment biosynthesis in non-*Xanthomonas* bacteria.

In addition to the above studies in genus *Xanthomonas*, Wang et al. (2013) identified a "yellow" locus comprising 17 ORFs that are responsible for pigment synthesis in *L. enzymogenes* OH1. The gene cluster for the xanthomonadin-like yellow pigment of OH1 is similar but not identical to that of *X. campestris* pv. *campestris*. Among the 17 ORFs, only eight are essential for pigment production. The products of these essential genes include a XanB2-type chorismatase, a ketoacyl ACP synthase, a ketoreductase, dehydratase, an acyltransferase, and other hypothetical proteins (Wang et al. 2013). Intriguingly, *L. enzymogenes* lacks the AMP ligase–encoding gene that is essential for xanthomonadin biosynthesis in *X. campestris* pv. *campestris*. Thus, the pigment biosynthetic pathway in *L. enzymogenes* may recruit proteins from other pathways for the synthesis and modification of xanthmomonadin-like pigments (Wang et al. 2013). Schöner et al. (2014) identified two gene clusters responsible for the production of the hybrid pigment arcuflavin in the genome of the β-proteobacterium *Azoarcus* sp. strain BH72 (*Rhodocyclales*). The two gene clusters encode proteins with high homology to those involved in xanthomonadin biosynthesis. ArcB, a homolog of XanB2, is also responsible for 3- and 4-HBA production and is required for arcuflavin production in *Azoarcus* sp. strain BH72. Moreover, ArcT is a homolog of XanA2 (Xcc4015) in *X. campestris* pv. *campestris* and exhibits adenylation activity that is required for arcuflavin biosynthesis. Thus, the XanB2-type chorismatase ArcB and the AMP-ligase ArcT that are encoded within the *Azoarcus* pigment biosynthetic gene clusters could perform the first two reactions of polyene assembly. Cimermancic et al. (2014) conducted a global analysis of biosynthetic gene clusters in archaea and bacteria and identified an APE family gene cluster that includes the clusters responsible for the biosynthesis of xanthomonadins, flexirubin, arcuflavin, and the APE pigments. The APE family contains more than 1,000 gene clusters that are widely distributed throughout gram-negative bacterial taxa. The distant but recognizable homology among the gene clusters in the APE family indicates that they probably share a common ancestor (Cimermancic et al. 2014).

REGULATION OF XANTHOMONADIN BIOSYNTHESIS IN *XANTHOMONAS* SPP.

***Xanthomonas* DF.**

The *Xanthomonas* DF was serendipitously identified during transposon saturation mutagenesis of the *pig* region encoded by the genome of *X. campestris* pv. *campestris* B-24 (Poplawsky and Chun 1997). Insertions into one of the seven known *pig* transcriptional units (*pigB*) resulted in reduced xanthomonadin production (14% of wild-type levels), EPS production (7% of wild type), epiphytic colonization (<1% of wild type), and host infection via hydathodes (3.1% of wild type) (Poplawsky and Chun 1998). In addition, *Xanthomonas* strains without a mutation in *pigB* could restore these traits in *pigB* mutant strains extracellularly. Consequently, it was proposed that *pigB* controls the production of a pheromone-like substance that was referred to as a DF. DF was required for epiphytic colonization and host hydathode infections but not wound-based infections (Poplawsky and Chun 1998)

The DF molecule was not functionally equivalent to known homoserine-lactone autoinducers (Fuqua et al. 2001). Rather, a tentative structure similar to the butyrolactone signals of *Streptomyces* spp. (Horinouchi 1999) was proposed (Chun et al. 1997). Subsequently, *xanB2* (Xcc4014) was shown to be part of the *pigB* transcriptional unit and responsible for DF production (Poplawsky et al. 2005). However, the proposed butyrolactone-like structure was later shown to be incorrect (Yajima et al. 2010). The DF from *X. campestris* pv. *campestris* XC1 was eventually purified and identified as 3-HBA. Moreover, the exogenous addition of 3-HBA to *xanB2* mutant strains restored pigment production (He et al. 2011). Thus, it was concluded that 3-HBA was the DF originally described by Poplawsky and Chun (1998). Subsequently, Zhou et al. (2013a and b) demonstrated that DF from *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* comprises both 3- and 4-HBA.

The roles of 3- and 4-HBA in xanthomonadin biosynthesis.

To further elucidate the roles of 3- and 4-HBA in xanthomonadin biosynthesis, Cao et al. (2020) selectively eliminated the production of either 3-HBA, 4-HBA, or both by expression of the *mhb*, *pobA*, and *pchAB* gene clusters in *X. campestris* pv. *campestris* XC1. The resultant strains were evaluated for pigmentation, virulence factor production, and virulence toward Chinese radish and cabbage. The results suggested that both 3- and 4-HBA are probably involved in xanthomonadin biosynthesis. When both 3- and 4-HBA are present, *X. campestris* pv. *campestris* preferentially uses 3-HBA for xanthomonadin A biosynthesis, as noted by the predominance of 3-HBA-derived

xanthomonadin A over 4-HBA-derived xanthomonadin in the wild-type strain XC1. If 3-HBA is not present, then 4-HBA is used in the production of a structurally uncharacterized xanthomonadin B compound. Further, exogenous addition of the plant hormone 2-HBA (salicylic acid) had little effect on xanthomonadin biosynthesis (Cao et al. 2020; He et al. 2011).

Given the above results and the presence of an aryl group in the structure of xanthomonadin A, it is likely that the freely diffusible 3- and 4-HBA are biosynthetic intermediates for xanthomonadin biosynthesis, whereas their possible roles as signals remains unresolved. It would be surprising if *X. campestris* pv. *campestris*, at great metabolic cost, produced these molecules and allowed them to diffuse into the environment with no evolutionary purpose. Can the effects of 3- and 4-HBA be explained completely by their role as intermediates in xanthomonadin biosynthesis, or do these molecules have effects that cannot be explained in this way? One way to answer this question is to compare xanB2 (*pigB* transcriptional unit) single-site mutation strains and their chromosomally restored derivatives to strains with similar mutations in a xanthomonadin biosynthetic gene (*pigC*). If a single-site mutation results in the loss or reduction of a trait and the full wild-type phenotype is restored in the chromosomally-restored mutant strain, then that mutation is responsible for the phenotypic effect.

Insertional inactivation of *pigB* resulted in a complete loss of DF (3-HBA and 4-HBA) production and reduced xanthomonadin production (14% of wild-type levels), EPS production (6.9% of wild type levels), epiphytic colonization (0.001% of wild-type levels in low, artificial light conditions), and host infection via hydathodes (3.1% of wild-type levels in low, artificial light conditions) (Table 2). Although inactivation of *pigC* resulted in the almost complete loss of xanthomonadin production, EPS and DF production were unaffected. In addition, epiphytic colonization was unaffected under low, artificial light conditions (only 100-fold reduced under high, natural light conditions), and host infection via the hydathodes was unaffected under low, artificial light conditions (only twofold reduced under high, natural light conditions) (Table 2). All phenotypes were restored in chromosomally restored derivative strains, and, in many cases with *pigB* mutant strains, they could be at least partially restored with the exogenous application of crude DF extracts (Chun et al. 1997; Poplawsky and Chun 1998). In summary, the *pigB* mutant strain was greatly reduced in EPS production, epiphytic survival, and hydathode infection, whereas the *pigC* mutant strain was unaffected in these traits. Thus, mutation of *pigB* to eliminate the production of 3- and 4-HBA clearly had numerous major effects on pathogen biology beyond those of just eliminating xanthomonadin production, and it is likely these effects were due to the freely diffusible nature of these molecules. However, studies to determine if 3- and 4-HBA affect the transcription of several of the genes controlling these traits gave negative results (He et al. 2011; A. R. Poplawsky, *unpublished*). Thus, 3- and 4-HBA probably don't exert their effects by altering gene transcription. Alternatively, it is possible that the freely diffusible 3- and 4-HBA molecules affect the biosynthesis of other bacterial products in the bacterial community through subtle effects on the availability of precursors for various other biosynthetic pathways.

X. campestris pv. *campestris* has at least two, distinctly unique, in-vivo life cycle stages as a phytopathogen: an epiphytic stage on plant surfaces and a pathogenic stage within the plant (Hugouvieux et al. 1998). Considering that xanthomonadins are needed in vivo for both the epiphytic and pathogenic life stages (He et al. 2011; Poplawsky et al. 2000) and that *X. campestris* pv. *campestris* produces different xanthomonadins in response to the availability of one or both 3-HBA and 4-HBA, the pathogen may selectively use either 3-HBA or 4-HBA in vivo to increase the production of the xanthomonadin variant most beneficial to each of its two life stages (Cao et al. 2020).

CONCLUSIONS AND PERSPECTIVES

In the present review, the current state of understanding regarding xanthomonadin and xanthomonadin-like pigments is described. The studies described above indicate that xanthomonadin-like pigments are much more widespread than originally thought. In addition to their prevalence in *Xanthomonas* spp., they also occur in a wide range of bacterial species belonging to several families. Second, these studies indicate that all xanthomonadin-like pigments seem to share a conserved aryl-polyene core structure with diverse side chain or phenyl ring modifications. Third, investigations have suggested that these pigments protect bacterial cells against one or both photo-damage and peroxidation and thereby play a role in establishing or maintaining a close relationship between bacteria and their plant hosts. Last, these studies have shown that the APE biosynthetic mechanism is conserved among these diverse bacterial taxa.

Although the study of xanthomonadins began in the early 1960s, our understanding of the characteristics of these pigments is nascent. Several aspects of the biology of xanthomonadin or xanthomonadin-like pigments remain to be addressed. First, the detailed chemical structures of these aryl-polyene pigments require further characterization. In particular, all the proposed structures shown in Figure 1 are based on incomplete experimental data. Since these pigments are located in the outer membranes of cells, it is very likely that they are a special type of phospholipid, as proposed by Aririatu and Kester (1985). Improved extraction and purification methods could help the characterization of complete structures of these pigments. Second, the detailed biosynthetic mechanisms of xanthomonadins and xanthomonadin-like pigments are still not completely understood. Consequently, further genetic and biochemical analyses of pigment biosynthetic gene clusters and their products are needed. In particular, xanthomonadin bromination needs to be further investigated. Although bioinformatics analysis identified two genes encoding putative halogenases, the exact enzymatic activities, substrates, and bromination loci remain unclear. Such investigations of the details of the biosynthetic mechanisms will provide a valuable framework for the design and development of novel plant disease control strategies, including through competitive inhibition. Finally, the roles of xanthomonadins in the adaptation of *X. campestris* pv. *campestris* to host plant environments and their associated virulence are unclear and require further

Table 2. Phenotypic characteristics of *Xanthomonas campestris* pv. *campestris pigB* and *pigC* mutant strains expressed as a percentage of the wild-type strain

Strain	Xanthomonadins	Extrapolysaccharide	Epiphytic survival[a]	Infection[b]
B-24 (WT)	100	100	100	100
pigC mutant	10	103	100	100
pigB mutant	14	6.9	0.001	3.1

[a] Under low, artificial light conditions.
[b] Via the hydathodes under low, artificial light conditions.

investigation. The xanthomondins and the related diffusible factors 3- and 4-HBA clearly play critical roles in the biology of *Xanthomonas* spp. Hopefully, future studies will clarify their involvement in the critical life processes of *Xanthomonas* plant pathogens and enable the design of new strategies for plant disease control.

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AUTHOR QUERIES

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