

Biological Role of Pigment Production for the Bacterial Phytopathogen *Pantoea stewartii* subsp. *stewartii*

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***Pantoea stewartii* subsp. *stewartii*, the causal agent of Stewart's wilt of sweet corn, produces a yellow carotenoid pigment. A non-pigmented mutant was selected from a bank of mutants generated by random transposon mutagenesis. The transposon insertion site was mapped to the *crtB* gene, encoding a putative phytoene synthase, an enzyme involved in the early steps of carotenoid biosynthesis. We demonstrate here that the carotenoid pigment imparts protection against UV radiation and also contributes to the complete antioxidant pathway of *P. stewartii*. Moreover, production of this pigment is regulated by the EsaI/EsaR quorum-sensing system and significantly contributes to the virulence of the pathogen *in planta*.**

Pantoea stewartii subsp. *stewartii* (formerly *Erwinia stewartii*) is a yellow-pigmented, Gram-negative bacterial phytopathogen that causes a severe disease of sweet corn (*Zea mays*) called Stewart's wilt. The bacterium is introduced into the plant by its insect vector, the corn flea beetle (*Chaetocnema pulicaria*), where it colonizes both the apoplast and the xylem. Following systemic colonization of the plant, the bacteria exit the leaf tissue as a visible, yellow bacterial ooze. It is the preferential colonization of the xylem that blocks water flow in the plant and leads to the characteristic wilting associated with the disease. The type III secretion system, stewartan exopolysaccharide production, flagellum-based motility, and one host cell wall-degrading enzyme have been implicated as important pathogenicity or virulence factors for *P. stewartii*, but little is known about the biological role of the characteristic yellow pigment produced by *P. stewartii* (4, 10, 11, 22, 35).

Carotenoids are among the most diverse natural products; they are synthesized by many organisms, including animals, plants, and microorganisms, and absorb light in the 400- to 550-nm range, which gives them their yellow-orange color (5). Several *Erwinia* species, which are close relatives of *P. stewartii*, produce yellow carotenoid pigments and possess a conserved carotenoid biosynthesis operon consisting of the genes *crtE*, *crtX*, *crtY*, *crtI*, and *crtB* in map order. Phytoene synthase, encoded by *crtB*, is the enzyme for the first step in carotenoid biogenesis, and mutations in *crtB* render a nonpigmented phenotype (36, 40, 52). More specifically, the *P. stewartii* genome contains a conserved carotenoid biosynthesis operon found in *Erwinia* spp., where *crtE* encodes geranylgeranyl pyrophosphate synthase, *crtX* encodes 3-hydroxy- β -carotene glycosylase, *crtY* encodes lycopene cyclase, and *crtI* encodes phytoene dehydrogenase (43, 46). The *crtB* gene encodes phytoene synthase, which converts geranylgeranyl pyrophosphate to phytoene, an early step in the biosynthesis of β -carotene (41). Zeaxanthin diglucoside, a derivative of β -carotene, is the typical carotenoid produced by *Erwinia* spp. (2), and because of the high homology to the carotenoid biosynthetic operon in other *Erwinia* spp., we speculate that the *P. stewartii* likely produces zeaxanthin diglucoside or a closely related derivative.

Carotenoids are known antioxidants that can protect against various types of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radicals, and superoxide anions (7, 24, 29). Carotenoids can also quench singlet oxygen (1O_2) (9, 24, 45).

ROS can also be derived from the absorption of visible light by chlorophyll present in the plant host (47). Several plant-associated bacteria, including *Erwinia herbicola*, synthesize carotenoids, which aid in the defense against the deleterious effects of ROS generated by chlorophyll during photosynthesis (38a). Carotenoids can also act as photoprotectants against the damage incited by UV radiation, specifically, by providing protection against near-UV (320- to 400-nm) wavelengths (46–48). Not surprisingly, carotenoids also play a role in overall fitness (26) and virulence within the host for some species of pathogenic bacteria, such as *Cronobacter sakazakii* and *Staphylococcus aureus* (33).

The objective of this study was to investigate the biological role of carotenoid pigment production in *P. stewartii* subsp. *stewartii*, specifically regarding tolerance to oxidative stress, UV radiation, and virulence in the sweet corn host.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *P. stewartii* and *Escherichia coli* strains were maintained on nutrient agar (Difco) and in Miller's Luria-Bertani (LB) medium (Difco) at 28°C. When necessary, antibiotics were added to the media at the following concentrations: nalidixic acid, 30 μ g/ml; spectinomycin, 100 μ g/ml; and ampicillin, 100 μ g/ml. Descriptions of the bacterial strains and plasmids used in this study are presented in Table 1.

Identification of a transposon mutant deficient in pigment production. Wild-type (WT) strain DC283 was mutated by random transposon insertion mutagenesis utilizing the *MAR2xT7* transposon, a derivative of the *mariner* transposon *Himar1* (32). In brief, the *pMAR2xT7* suicide plasmid was introduced into WT DC283 by conjugal transfer. Transconjugants were plated onto LB agar medium containing nalidixic acid and gentamicin. Nonpigmented colonies were selected for further characterization. Arbitrary PCR was used to determine the location of the *MAR2xT7* transposon insertion in the nonpigmented mutants. This is a two-round PCR protocol that amplifies the sequence adjacent to the transposon insertion using a transposon-specific primer and an arbitrary primer. A second round of PCR uses a nested transposon-specific primer

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype ^a	Reference or source
Strains		
<i>P. stewartii</i> subsp. <i>stewartii</i>		
DC283	SS104 WT, Nal ^r	12
MM13	<i>crtB::Mar2xT7</i> Nal ^r Gm ^r	This study
MM16	MM13 harboring pBBR1:: <i>crtB</i> , Nal ^r Gm ^r Ap ^r	This study
ESN10	DC283 Δ <i>esaI</i> Nal ^r	4
<i>E. coli</i>		
DH10 β	F ⁻ <i>endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 ϕ80lacZΔM15 araD139 Δ(<i>ara leu</i>)7697 mcrA Δ(<i>mrr-hsdR MS-mcrBC</i>) λ⁻</i>	Invitrogen
S17-1 λ pir ⁺	<i>thi pro hsdR hsdM1 recA</i> RP4 2-Tc:: Mu-Km::Tn7 Sm ^r λ pir	43a
Plasmids		
pMAR2xT7	<i>mariner</i> transposon <i>Himar1</i> , Gm ^r	32
pCR8/GW/TOPO	TA cloning entry vector, Sp ^r	Invitrogen
pBBR1-MCS4-GW	pBBR1-MCS4 modified as a Gateway destination vector, Ap ^r	6a
pMOJ13	pCR8/GW/TOPO:: <i>crtB</i> Sp ^r	This study
pMOJ14	pBBR1:: <i>crtB</i> Ap ^r	This study

^a Antibiotic resistance abbreviations: Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Nal^r, nalidixic acid resistance; Sp^r, spectinomycin resistance.

and a primer that anneals to the nonrandom portion of the arbitrary primer (32). Specifically, genomic DNA from the nonpigmented mutants was extracted using the DNeasy blood and tissue kit from Qiagen (MD). The first round of PCR was performed with the TnMar1/ARB1 primer pair (Table 2) using optimized PCR conditions (32). The PCR product from the first round was used as a template for the second round of PCR with the TnMar2/ARB2 primer pair (Table 2) (32). The final PCR product was sequenced using the SeqTnMar primer (Table 2) (32).

Complementation of the nonpigmented *crtB* mutant. The *crtB* gene, encoding a putative phytoene synthase, was amplified using primer pair PSF/PSR (Table 2). The amplicon included 150 bp upstream of the open reading frame to ensure inclusion of the native promoter. The resulting PCR product was cloned into the pCR8/GW/TOPO TA cloning vector (Invitrogen) following the manufacturer's instructions to create plasmid pMOJ13. The *crtB* gene was subcloned from pMOJ13 into a Gateway-compatible version of the broad-host-range vector pBBR1-MCS4 (28) using the LR Clonase kit II (Invitrogen) according to the manufacturer's instructions to create pMOJ14. pMOJ14 was electroporated into the MM13 (*crtB::Mar2xT7*) mutant, and transformants were selected on nutrient agar plates containing nalidixic acid and ampicillin.

Pigment extraction and spectral analysis. Single colonies of WT DC283 or MM13 (*crtB::Mar2xT7*) were grown in 2 ml LB medium with

shaking (200 rpm) at 28°C for 48 h. Bacteria were harvested by centrifugation at 15,000 rpm. The resulting bacterial pellet was washed once in sterile distilled H₂O and mixed with 1 ml of 100% methanol, based on a previously described protocol (42). The suspension was heated at 85°C for 20 min to extract the pigment. This mixture was centrifuged, the supernatant was transferred to a fresh tube, and these methanol extracts were scanned at between 400 and 500 nm using a Biomate 3 spectrophotometer (Thermo Fisher).

Sensitivity to UV radiation. Sensitivity to UV radiation was tested based on a previously described protocol (39). In brief, bacterial strains were cultured in 2 ml LB broth to either mid-log phase or stationary phase, serially diluted in 1× phosphate-buffered saline (PBS), and placed in 13- by 100-mm glass tubes. The tubes were placed in a rack over a UV transilluminator and exposed to near-UV (320- to 400-nm) in an upright position for 30 s. Unexposed serial dilutions were used as a negative control. Aliquots (100 μ l) of the exposed or unexposed cells were spread plated on nutrient agar containing the appropriate antibiotics. Colonies were counted after 3 days of incubation at 28°C. Results were log transformed and expressed as percent surviving CFU normalized to that for the untreated control.

H₂O₂ and singlet oxygen sensitivity assays. For the H₂O₂ sensitivity assays, single colonies of WT DC283, MM13 (*crtB::Mar2xT7*), and MM16 (*crtB::Mar2xT7*/pMOJ14) were grown in 2 ml LB broth to either mid-log phase or stationary phase. Two hundred microliters of bacterial cell culture was diluted with 200 μ l LB broth in a sterile glass tube, and H₂O₂ was added to a final concentration of 40 mM as previously described (38). Controls were cell suspensions that were not treated with H₂O₂. The tubes were shaken at 200 rpm at 28°C for 30 min, stored on ice, and immediately serially diluted in 1× PBS. Survivability was assessed by spread plating 100- μ l aliquots of each dilution for the H₂O₂-treated or untreated cells onto nutrient agar plates containing antibiotics where needed. Colonies were enumerated after 3 days of incubation at 28°C. The results were expressed as log-transformed CFU/ml normalized to that for the untreated control.

Toluidine blue generates singlet oxygen (¹O₂) upon illumination with tungsten light (25). To test sensitivity to ¹O₂, bacterial strains were cultured in LB broth to mid-log phase or stationary phase and then serially diluted in 1× PBS buffer. Serial dilutions were treated with toluidine blue to a final concentration of 5 μ M in 13- by 100-mm glass culture tubes. The tubes were placed on a rack, positioned 60 cm from two 150-W tungsten light bulbs at 28°C, and left for 1 h with occasional shaking. Aliquots of the cell suspensions were spread plated on nutrient agar containing 30 μ g/ml nalidixic acid. Separate tubes containing the various serial dilutions were left untreated and served as negative controls. Colonies were counted 3 days after incubation at 28°C. The results were expressed as log-transformed CFU/ml normalized to that for the untreated control.

3-Oxo-C₆-AHL induction of pigment production. Single colonies of the *N*-3-(oxohexanoyl)-L-homoserine lactone (3-oxo-C₆-AHL) signal synthase mutant ESN10 (Δ *esaI*) (4) were grown in LB medium to an optical density at 600 nm (OD₆₀₀) of 0.1 at 28°C with shaking at 200 rpm. One set of cultures were supplemented with 3-oxo-C₆-AHL (Sigma-Aldrich, St. Louis, MO) to a final concentration of 10 μ M. The other set of

TABLE 2 Primer sequences used in this study

Primer	Sequence ^a	Reference or source
TnMar1	TACAGTTTACGAACCGAACAGGC	32
ARB1	GGCCAGGCCTGCAGATGATGNNNNNNNNNNNGTAT	32
TnMar2	TGTCAACTGGTTCGTGCCTTCATCCG	32
ARB2	GGCCAGGCCTGCAGATGATG	32
SeqTnMar	GACCGAGATAGGGTTGAGTG	32
PSF	TACAAAAAGCAGGCTAGCTCAATGCCTGCCAAGGTT	This study
PSR	TACAAGAAAGCTGGGTGCGCCACCGTTATCTAAACTTCA	This study

^a Primer sequences are oriented from 5' to 3'. Gateway sequences are shown in bold.

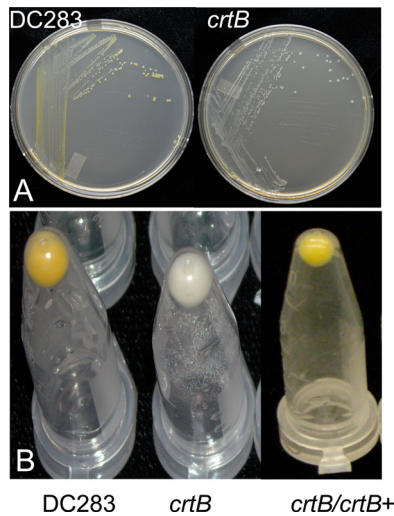


FIG 1 Pigment production by wild-type *P. stewartii* (DC283) and the *crtB::Mar2xT7* (*crtB*) mutant. (A) Single colonies of the *crtB* transposon mutant obtained were nonpigmented on nutrient agar. (B) Bacterial pellets accumulated a yellow carotenoid pigment in the wild-type strain DC283 but not the *crtB* mutant. Pigmentation was restored when a wild-type copy of *crtB* was supplied in *trans* on plasmid pMOJ14 in strain *crtB::Mar2xT7/pMOJ14* (*crtB/crtB*⁺).

cultures did not receive exogenous 3-oxo-C₆-AHL. Following this, all cultures were grown to stationary phase, and the pigment was extracted and quantified as described above.

Virulence assays. Fourteen-day-old sweet corn seedlings (var. Jubilee) (Syngenta) were inoculated by stabbing the stem below the secondary leaf using a sterile 20-gauge syringe needle and placing 5 μ l of bacterial inoculum into the wound. Inocula were prepared by growing single colonies of WT DC283, MM13 (*crtB::Mar2xT7*), and MM16 (*crtB::Mar2xT7/pMOJ14*) overnight. The cells were harvested by centrifugation, washed once with 1 \times PBS containing 0.2% Tween 20 (1 \times PBS-Tween 20), and resuspended in the same buffer to an OD₆₀₀ of 1.0. Seedlings were assessed daily for symptom development using the following arbitrary rating scale: 0, no symptoms; 1, small water-soaked lesions; 2, larger water-soaked lesions; 3, water-soaked lesions, ooze formation, and slight wilting; 4, severe wilting and ooze; and 5, dead. Seedlings inoculated with 1 \times PBS-Tween 20 buffer served as negative controls.

RESULTS

Identification of a mutation affecting pigment production. Following random transposon mutagenesis, several nonpigmented mutants were selected. We focused on one mutant in particular (Fig. 1), where the transposon mapped to the *crtB* gene (insertion at 680 bp), which encodes a putative phytoene synthase. The closest orthologs to the *P. stewartii crtB* gene are *crtB* in *Pantoea ananatis* (accession no. YP_003522457.1) ($E = 0.0$; 88% identity with 99% coverage) (15) and *crtB* in *Pantoea agglomerans* (accession no. AAA21264.1) ($E = 0.0$; 88% identity with 95% coverage) (46). A closer look at the genomic context of the *crtB* gene in the *P. stewartii* genome revealed that it was the last gene in a putative operon containing *crtEXYIB* (accession no. AY166713.1).

Carotenoid analysis. The absorption spectrum of the methanol-extracted pigment of WT *P. stewartii* (DC283) displayed a typical carotenoid spectrum in the visible range, with a peak at 451 nm, a shoulder at 475 nm, and the maximum absorbance (λ_{max}) at 450 nm, which was comparable to that of the β -carotene standard

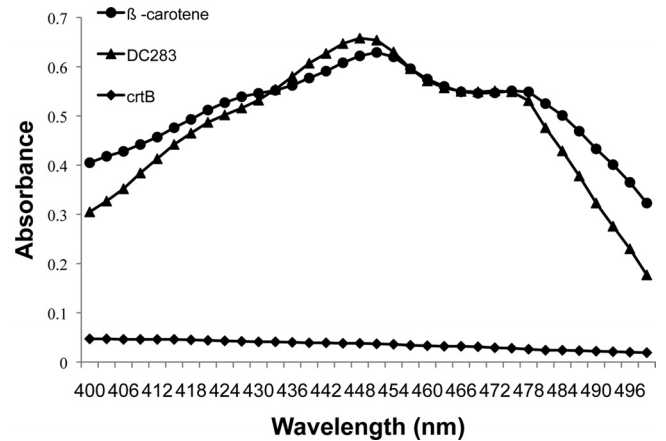


FIG 2 Spectral analysis of the *P. stewartii* carotenoid pigment. The methanol-extractable pigment from wild-type *P. stewartii* (DC283) has an absorption spectrum with a peak at 451 nm and a shoulder at 475 nm and with the maximum absorbance (λ_{max}) at 450 nm, which was similar to that of the β -carotene standard. The *crtB::Mar2xT7* (*crtB*) mutant did not have detectable pigment in the methanol extract.

(Fig. 2) (51). As expected, the *crtB* mutant had minimal detectable carotenoid in the methanol extract (Fig. 2).

The *P. stewartii* pigment functions as an antioxidant. At mid-log phase, the MM13 (*crtB::Mar2xT7*) strain was significantly more sensitive to H₂O₂ treatment than WT DC283 (Fig. 3). Survivability was quantified at 20% for the mutant and 34% for WT DC283. H₂O₂ tolerance was restored to wild-type levels in the MM13 (*crtB::Mar2xT7*) mutant when a wild-type copy of *crtB* was supplied on plasmid pMOJ14. The results represent the means \pm standard errors (SE) from at least three independent experiments, each containing three technical replicates, and a Student *t* test was used to determine the significance of differences among the different treatments. Interestingly, there was no difference in sensitivity to H₂O₂ treatment when both the MM13 (*crtB::Mar2xT7*)

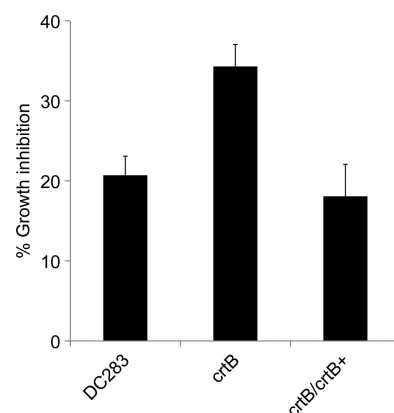


FIG 3 H₂O₂ sensitivity in the wild-type *P. stewartii* (DC283), the *crtB::Mar2xT7* (*crtB*) mutant, and the *crtB::Mar2xT7/pMOJ14* (*crtB/crtB*⁺) complemented strain. Mid-log-phase cells were treated with H₂O₂ to a final concentration of 40 mM for 30 min. Controls were left untreated with H₂O₂. CFU were enumerated, and the percent growth inhibition was normalized to that for the untreated control. The *crtB::Mar2xT7* (*crtB*) mutant was significantly more sensitive to H₂O₂ treatment than WT DC283. H₂O₂ tolerance was fully restored when a wild-type copy of *crtB* was supplied in *trans* on plasmid pMOJ14 in strain *crtB::Mar2xT7/pMOJ14* (*crtB/crtB*⁺).

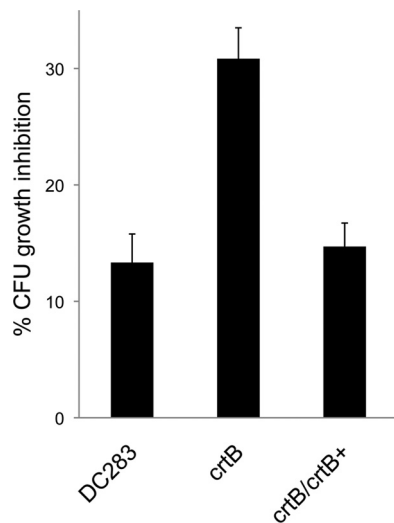


FIG 4 Tolerance to UV radiation. Wild-type *P. stewartii* (DC283), the *crtB::Mar2xT7* (*crtB*) mutant, and the *crtB::Mar2xT7/pMOJ14* (*crtB/crtB*⁺) complemented strain were grown to stationary phase in LB medium, diluted in 1× PBS, and exposed to near-UV-A irradiation (320 to 400 nm) for 30 s. CFU were enumerated, and the percent growth inhibition was normalized to that for the untreated control. The *crtB::Mar2xT7* (*crtB*) mutant was significantly more sensitive to UV radiation than the WT (DC283) strain, and UV tolerance was fully restored in the *crtB::Mar2xT7/pMOJ14* (*crtB/crtB*⁺) complemented strain.

mutant and WT DC283 were grown to stationary phase (data not shown). The loss of pigment production did not lead to a significant change in susceptibility to ¹O₂ generated by toluidine blue treatment in cells grown to mid-log or stationary phase, indicating that the carotenoid pigment does not play a significant role in quenching ¹O₂ in *P. stewartii* (data not shown).

The *P. stewartii* pigment protects against UV radiation. WT DC283 and MM13 (*crtB::Mar2xT7*) were equally susceptible to short-wave UV treatment during mid-log phase (data not shown). However, when the culture entered stationary phase, WT DC283 was significantly more tolerant to UV stress than the MM13 (*crtB::Mar2xT7*) mutant, indicating that the carotenoid pigment plays an important role in protecting the cells from the harmful effects of UV radiation during stationary phase rather than log phase (Fig. 4). The results represent the means ± SE from at least three independent experiments, each containing three technical replicates, and a Student *t* test was used to determine the significance of differences among the different treatments.

***P. stewartii* produces pigment in a quorum-sensing-dependent manner.** We observed that cultures of *P. stewartii* produced pigment in mid-log phase but accumulated the bulk of the pigment in late log/early stationary phase (data not shown), suggesting to us that pigment production occurs in a cell density-dependent manner. This prompted us to test if pigment production was dependent on the EsaI/EsaR quorum-sensing system. The quorum-sensing system depends on the production of a 3-oxo-C₆-AHL signal that is perceived by the cell density-responsive transcriptional regulator protein, EsaR. EsaI is the signal synthase, and a *ΔesaI* mutant is deficient in signal production (49). The *ΔesaI* signal synthase mutant produced significantly less pigment than WT DC283, and pigment production was fully restored when exogenous 3-oxo-C₆-AHL signal was added to the medium, demon-

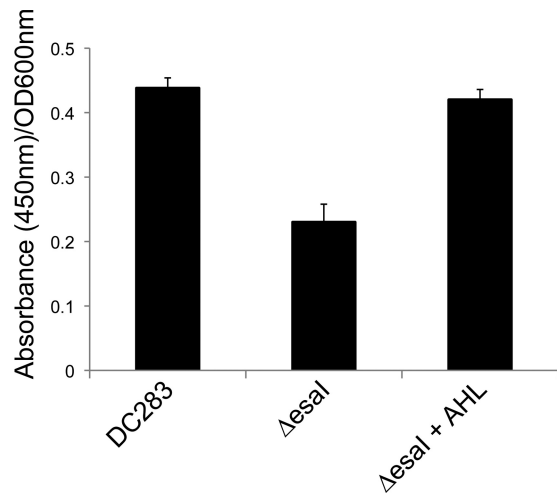


FIG 5 The EsaI/EsaR quorum-sensing system regulates carotenoid pigment production. Wild-type *P. stewartii* (DC283) and the *ΔesaI* quorum-sensing signal synthase mutant, with or without exogenous 3-oxo-C₆-AHL (AHL) signal, were grown in LB medium for 48 h. Pigment extraction and quantification were performed as described in Materials and Methods. The *ΔesaI* mutant produces less pigment, and pigment production is restored when the AHL signal is added to the medium.

strating that the EsaI/EsaR system is involved in the regulation of pigment production in *P. stewartii* either directly or indirectly (Fig. 5). The results represent the means of nine technical replicates from three independent experiments ± SE. A Student *t* test was used to determine the significance of differences among the different treatments.

Loss of pigment production leads to a reduction in virulence.

The WT DC283 parental strain was fully virulent and caused the formation of both water-soaked lesions and wilting, characteristic of the disease. At 10 days after inoculation, these plants rated a 4.2 on the arbitrary disease rating scale of 0 to 5 (Fig. 6). In contrast, plants inoculated with the MM13 (*crtB::Mar2xT7*) mutant were significantly delayed in both lesion development and wilting. The symptoms of these plants gave an average disease rating of 2.7 (Fig. 6). Virulence was fully restored when the mutant strain was complemented with a functional copy of *crtB*, carried on plasmid pMOJ14 in strain MM16 (*crtB::Mar2xT7/pBBR1::crtB*) (Fig. 6). For each experiment, the results represent the mean of 18 technical replicates from three independent experiments ± SE. A Student *t* test was used to determine the significance of differences among the different treatments. These data demonstrate that carotenoid pigment production is required for full virulence for *P. stewartii*.

DISCUSSION

ROS, such as H₂O₂ and ¹O₂ oxygen, are damaging to cellular membranes, protein, and DNA. Bacteria have evolved an effective oxidative stress response that includes the production of ROS-detoxifying enzymes such as catalases, alkyl hydroperoxidases, and superoxide dismutases (44). Another line of defense against oxidative stress includes the production of antioxidant carotenoid pigments that can effectively quench ROS (29, 30). Plants produce ROS during the host defense response as part of the oxidative burst, which is a rapid, transient response to pathogen invasion (1, 6, 21, 27, 31, 51). The oxidative burst is derived from living plant

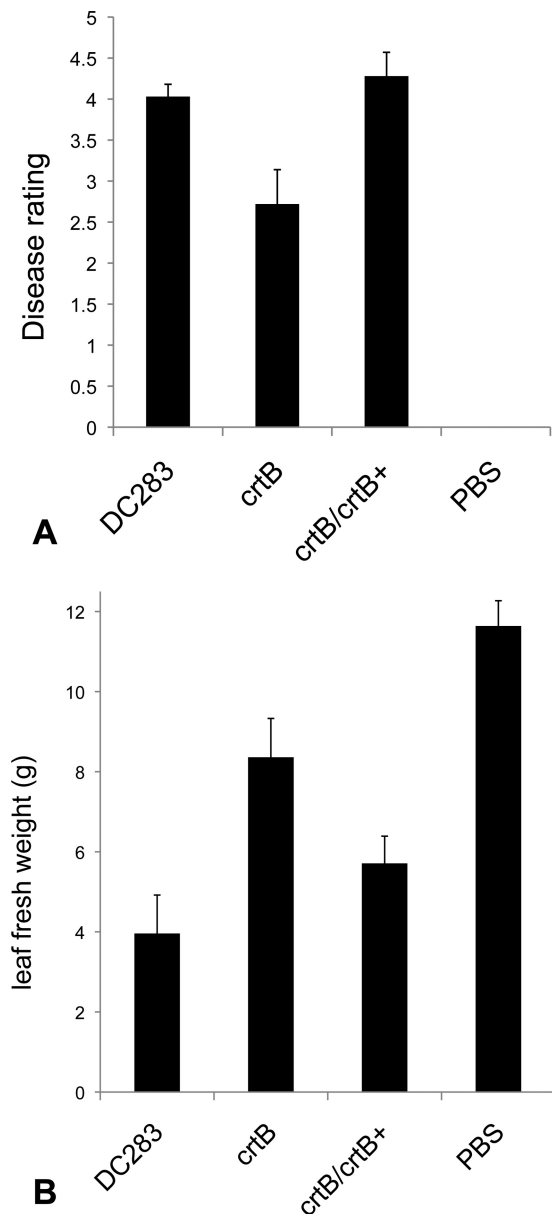


FIG 6 (A) Virulence assay in sweet corn seedlings. Fourteen-day-old sweet corn seedlings were inoculated with either the wild-type *P. stewartii* (DC283), the *crtB::Mar2xT7* (*crtB*) mutant, the *crtB::Mar2xT7/pMOJ14* (*crtB/crtB⁺*) complemented strain, or 1× PBS-Tween 20 buffer. Seedlings were stab inoculated below the secondary leaf with 5 μ l of inoculum prepared in 1× PBS-Tween 20 buffer and adjusted to an OD_{600} of 1.0. Disease ratings were assessed at 10 days postinoculation. The *crtB::Mar2xT7* (*crtB*) mutant is significantly comprised in virulence compared to the WT (DC283) strain. Virulence was fully restored in the *crtB::Mar2xT7/pMOJ14* (*crtB/crtB⁺*) complemented strain. Plants inoculated with 1× PBS-Tween 20 buffer alone did not develop any Stewart's wilt symptoms. (B) Total fresh weight (g) of sweet corn seedlings. Plants were weighed 12 days post-inoculation. The results represent the means \pm SE for 12 replicates from three independent experiments.

cells; thus, *P. stewartii* would likely encounter this stress in the early apoplastic (water-soaked lesion formation) phase of infection. In addition to the apoplastic phase, *P. stewartii* preferentially colonizes the xylem tissue as it systemically colonizes the plant, and bacterial pathogens can also incur oxidative stress in the xy-

lem (17). Xylem tissue cells are nonliving and therefore do not initiate an oxidative burst on their own. However, pathogens in the xylem do make contact with adjacent living parenchyma cells, and ultrastructural studies indicate that these cells can sense xylem-invading pathogens and mount a defense response that includes the production of ROS (23). Another source of ROS comes from differentiating thin-walled xylem cells and particular non-lignifying xylem parenchyma cells. These cells are capable of sustained H_2O_2 production, which is important for the cross-linking that occurs during the lignification process of developing xylem elements that can diffuse widely to neighboring xylem cells (3, 18, 19).

Once *P. stewartii* enters the xylem, it proliferates and reaches high cell densities ($\geq 10^8$ CFU/g tissue). Our results demonstrate that production of the carotenoid pigment by *P. stewartii* confers tolerance to H_2O_2 . Interestingly, the protective effects against H_2O_2 were observed only in mid-log-phase cells and not in cells grown to stationary phase, indicating the importance of the role of the carotenoid pigment as an antioxidant in a rapidly growing population such as occurs when the bacteria are colonizing the xylem tissue. This also suggests that other antioxidant mechanisms, perhaps controlled by the stationary-phase stress response sigma factor RpoS, are dominant to the antioxidant capabilities of the carotenoid pigment during stationary phase (8). We also demonstrated that pigmentation does not play an apparent role in quenching 1O_2 for *P. stewartii*. 1O_2 can arise as a by-product of photosynthesis. *P. stewartii* preferentially resides in the xylem tissue, which is devoid of chloroplasts; therefore, the ability to detoxify 1O_2 may not be necessary for *P. stewartii* in the specialized niche of the xylem.

Our data show that pigment production in *P. stewartii* is under the control of the EsaI/EsaR quorum-sensing system and is most abundant in a high-cell-density situation. When the bacteria are initially introduced into the plant by the corn flea beetle, they are at low cell density and would not be producing maximal amounts of pigment. Therefore, we reason that the pigment is not a large contributor to combating oxidative stress associated with the oxidative burst when small amounts of bacteria (low cell density) are initially introduced into the plant by the insect vector. Rather, we speculate that the protective antioxidant effects of the pigment are more important during the xylem colonization phase of the disease, where the bacterial population reaches the cell numbers necessary for the EsaI/EsaR quorum-sensing system to initiate gene expression of known quorum-sensing-regulated genes (50) and, specifically, pigment production. In addition to acting as potent antioxidants, carotenoids can also provide significant protection against near-UV (320 to 400 nm) and photosensitizing agents (activated by UV), which helps to prevent photodamage (16, 47). Carotenoids are hydrophobic and generally localized to hydrophobic regions of the cell, such as membranes (5). We postulate that the *P. stewartii* pigment accumulates in the membrane, where it would be optimally located to serve as a photoprotectant for the bacterial cell. Following systemic colonization of the plant to high cell density during the late stages of the *P. stewartii* infection process, substantial amounts of bright yellow bacterial ooze exits the leaf tissue and accumulates on the leaf surface. We have demonstrated that the carotenoid pigment produced by *P. stewartii* imparts tolerance to near-UV radiation in the stationary phase of growth, and we reasoned that *P. stewartii* utilizes pigment protection as a defense against the harmful effects of UV radiation as it

enters stationary phase late in the disease cycle, exits the leaf tissue, and waits to be acquired by the corn flea beetle vector (13).

Pigments, in general, can play a role in virulence in bacterial pathogens (20, 34, 37). Specifically, a carotenoid pigment called staphyloxanthin is a known virulence factor for *S. aureus* and is even referred to as its “golden coat” (14). This pigment promotes virulence via its antioxidant activity by contributing to survivability against the oxidative burst derived from human neutrophils and whole mouse blood (33). In addition, pigment-deficient mutants were less virulent in a mouse model (33, 34). Likewise, we found that absence of the carotenoid decreases virulence for *P. stewartii*, demonstrating the importance of this pigment in the pathogenic lifestyle of this bacterium.

This study indicates the importance of carotenoid pigment production in virulence for *P. stewartii*, a xylem-dwelling phytopathogen, and points at different aspects of the disease cycle where its antioxidant and UV properties may be important. We envision a model where the carotenoid pigment contributes to the antioxidant defense system of *P. stewartii* as it is colonizing the xylem and reaches the cell densities necessary for the EsaI/EsaR quorum-sensing system to trigger gene expression. Once the bacteria have heavily colonized the plant and presumably reached stationary phase, they emerge from the leaf tissue producing maximal amounts of pigment, which is visible as a bright yellow bacterial ooze and aids the cells in coping with UV light exposure encountered on the leaf surface. Future studies will test this model and may possibly identify a role of this pigment in epiphytic survival of this important pathogen of sweet corn.

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