Chp8, a diguanylate cyclase from Pseudomonas syringae pv tomato DC3000 suppresses the PAMP flagellin, increases EPS and promotes plant immune evasion.

Chp8, a Diguanylate Cyclase from *Pseudomonas syringae* pv. Tomato DC3000, Suppresses the Pathogen-Associated Molecular Pattern Flagellin, Increases Extracellular Polysaccharides, and Promotes Plant Immune Evasion

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**ABSTRACT** The bacterial plant pathogen *Pseudomonas syringae* causes disease in a wide range of plants. The associated decrease in crop yields results in economic losses and threatens global food security. Competition exists between the plant immune system and the pathogen, the basic principles of which can be applied to animal infection pathways. *P. syringae* uses a type III secretion system (T3SS) to deliver virulence factors into the plant that promote survival of the bacterium. The *P. syringae* T3SS is a product of the hypersensitive response and pathogenicity (*hrp*) and hypersensitive response and conserved (*hrc*) gene cluster, which is strictly controlled by the codependent enhancer-binding proteins HrpR and HrpS. Through a combination of bacterial gene regulation and phenotypic studies, plant infection assays, and plant hormone quantifications, we now report that Chp8 (i) is embedded in the Hrp regulon and expressed in response to plant signals and HrpRS, (ii) is a functional diguanylate cyclase, (iii) decreases the expression of the major pathogen-associated molecular pattern (PAMP) flagellin and increases extracellular polysaccharides (EPS), and (iv) impacts the salicylic acid/jasmonic acid hormonal immune response and disease progression. We propose that Chp8 expression dampens PAMP-triggered immunity during early plant infection.

**IMPORTANCE** The global demand for food is projected to rise by 50% by 2030 and, as such, represents one of the major challenges of the 21st century, requiring improved crop management. Diseases caused by plant pathogens decrease crop yields, result in significant economic losses, and threaten global food security. Gaining mechanistic insights into the events at the plant-pathogen interface and employing this knowledge to make crops more resilient is one important strategy for improving crop management. Plant-pathogen interactions are characterized by the sophisticated interplay between plant immunity elicited upon pathogen recognition and immune evasion by the pathogen. Here, we identify Chp8 as a contributor to the major effort of the plant pathogen *Pseudomonas syringae* pv. tomato DC3000 to evade immune responses of the plant.

According to recent estimates by the Food And Agriculture Organization of the United Nations, the global demand for food is projected to rise by 50% by 2030 (1). Meeting this increasing need will be one of the major challenges of the 21st century. Diseases caused by plant pathogens represent a large agricultural burden. They decrease crop yields, resulting in significant economic losses, and threaten global food security (2, 3). Thus, by gaining mechanistic insights into the events at the plant-pathogen interface and employing this knowledge to make crops more pathogen resilient, strategies for improving crop management can be developed.

The bacterial plant pathogen *Pseudomonas syringae* infects more than 50 different cultivars, resulting in diseases such as bacterial speck, brown spot, halo blight, olive knot, wildfire, or bleeding canker in economically valuable crops such as tomato, beans, and rice (2, 3). *P. syringae* pv. tomato strain DC3000, which infects tomato crops, as well as the model plant *Arabidopsis thaliana*, has been fundamental in increasing our understanding of *P. syringae* pathogenicity. Found in seeds, soil, rotting plant material, and on leaf surfaces (2, 4), *P. syringae* pv. tomato DC3000 enters the plant through wounds or leaf stomata and then replicates within the apoplast, eventually causing chlorosis (yellowing), necrotic lesions, and programmed cell death in incompatible interactions (2, 5, 6).

As with many other Gram-negative plant and animal pathogens, the virulence of *P. syringae* relies upon a type III secretion system (T3SS)—a needlelike appendage that facilitates the delivery of virulence effectors into the host cells (5, 7). The T3SS of *P. syringae* is encoded by the hypersensitive response (HR) and pathogenicity (*hrp*) and HR and conserved (*hrc*) gene cluster (5).
that is controlled by the extracytoplasmic function sigma factor HrPL (8). The expression of HrPL is strictly controlled by sigma-54 and cooperatively activated through the enhancer binding proteins HrPRS (8, 9). Transcriptional control through HrPL and HrPRS is not limited to the hrp-hrc T3SS cluster but extends to other genes, including some which have unknown roles in P. syringae pathogenicity (10). One of these genes is PSPTO_2907, otherwise known as chp8 (co-regulated with hrp 8) (10), whose role in pathogenicity we have investigated in this study.

RESULTS

Chp8 is embedded in the Hrp regulon, and its expression is activated by plant signals. A functional genomics analysis of P. syringae pv. tomato strain DC3000 identified chp8 as a novel Hrp-regulated gene whose expression was upregulated under Hrp-inducing conditions, apparently in a hrpRS-dependent but hrpL-independent manner (10, 11). To confirm these findings, we measured the activity of the chp8 promoter in strain DC3000 in the presence and absence of hrpS (Fig. 1) or hrpl (see Fig. S1 in the supplemental material), respectively. Initially, we measured Chp8 induction in HIM (hrp-inducing medium), since it has been shown to induce hrp-hrc gene expression (Fig. 1, P hrpl), presumably by mimicking the nutritionally depleted environment encountered by DC3000 in the apoplast (12, 13). However, we could not detect upregulation of chp8 induction in DC3000 in HIM alone (Fig. 1, P chp8 HIM). We reasoned that chp8 induction may, in addition, require plant-derived signals. Indeed, the activity of the chp8 promoter was markedly increased when DC3000 was grown in a plant cell culture (Fig. 1, P chp8 plant cells). Recent studies have identified that plants produce flavonoids upon infection with P. syringae pv. tomato DC3000 and that this pathogen is susceptible to the plant flavonoid phloretin (14). To determine whether phloretin affects chp8 induction, we measured the activity of the chp8 promoter in HIM supplemented with phloretin (Fig. 1, P chp8 phloretin). As shown by the results in Fig. 1, the activity of the chp8 promoter was markedly increased in the presence of phloretin. In line with the requirement of HrPRS for chp8 induction, the positive effect of plant cells and phloretin is diminished in the absence of hrpS (Fig. 1, DC3000ΔhrpS). Extending earlier observations (10, 11), these results demonstrate that Chp8 is indeed embedded in the Hr regulon, suggesting that coregulation of Chp8 and T3SS occurs and that induction is responsive to plant signals, implying a role in the infection process.

Chp8 exhibits a functional c-di-GMP synthase activity in vivo and promotes a sessile lifestyle of P. syringae pv. tomato DC3000. In situ analyses of Chp8 (see Fig. S2 in the supplemental material) infer that it belongs to the diguanylate cyclase (DGC) and/or the phosphodiesterase (PDE) family of proteins. The presence of a GGDEF (characteristic of a DGC) and an EAL (characteristic of a PDE) domain indicates that Chp8 has active cyclic di-GMP (c-di-GMP)-synthesizing (DGC) and/or -degrading (PDE) activities (15–17). As a second messenger, c-di-GMP often controls the switch between planktonic and sessile lifestyles (15–17). DGCs, as c-di-GMP producers, promote biofilm formation and decrease motility, while PDEs, as c-di-GMP degraders, promote motility and decrease biofilm formation (15–17). To determine which of the two opposing activities of Chp8 predominates in vivo, we measured the (i) cellular c-di-GMP levels, (ii) biofilm formation, and (iii) motility of P. syringae pv. tomato DC3000 in the presence and absence (Fig. S3) and upon ectopic expression of wild-type Chp8 and two Chp8 variants with either the DGC or the PDE domain inactivated (Fig. 2). We used ectopic expression instead of phloretin-induced Chp8 expression since phloretin had such a strong effect on the phenotypes tested that it masked any Chp8-specific changes. Consistent with our earlier observations that chp8 induction required plant-derived signals, heterologous ectopic expression was needed to study Chp8 function ex planta (Fig. 2 and Fig. S3). Strikingly, cells expressing wild-type Chp8 (P. syringae pv. tomato DC3000chp8/pSEVachp8DGC−PDE+) showed a marked increase in cellular c-di-GMP (Fig. 2A), a slightly more extensive biofilm (Fig. 2B), and decreased motility (Fig. 2C), in line with net c-di-GMP production by Chp8 in vivo.

To test the activities of the DGC and PDE domains of Chp8 independently, we replaced the critical signature amino acids GGDEF and EAL of Chp8 with alanine to create Chp8DGC−PDE+ (disrupting the GGDEF but maintaining the integrity of the EAL motif) and, conversely, Chp8DGC+PDE− (disrupting the EAL but maintaining the integrity of the GGDEF motif). Mutating the Chp8 GGDEF motif (P. syringae pv. tomato DC3000Δchp8/pSEVachp8DGC−PDE+) impairs c-di-GMP production and biofilm formation (Fig. 2A), demonstrating that Chp8 indeed encodes a functional DGC domain. Interestingly, the Chp8 PDE domain appears to be functional (P. syringae pv. tomato DC3000Δchp8/pSEVachp8DGC+PDE−), causing a marked increase in the motility of the cells compared to that of the vector control (Fig. 2C). Remarkably, inactivation of the Chp8 PDE domain (P. syringae pv. tomato DC3000Δchp8/pSEVachp8DGC−PDE−) also interferes with c-di-GMP production (Fig. 2A) despite an unmodified DGC domain, indicating that both domains are required for maximal c-di-GMP synthase activity of Chp8. However, the DGC domain of Chp8 alone, in the absence of the intact PDE domain, retains its characteristic phe-
notypic impact, evident through an extensive biofilm (Fig. 2B) and decreased motility (Fig. 2C). In summary, we conclude that Chp8 is a composite diguanylate cyclase in which both the DGC and PDE domains are active and required for maximal c-di-GMP synthase activity in vivo and that Chp8 is involved in the switch toward a sessile lifestyle of *P. syringae* pv. tomato DC3000 by promoting biofilm formation and decreasing motility.

Chp8 downregulates flagellin and upregulates EPS production of *P. syringae* pv. tomato DC3000. The Chp8-dependent changes in motility prompted us to investigate the impact of Chp8 on flagellin production. Flagellin is the principal constituent of bacterial flagella, which confer bacterial motility (18). Flagellin is also one key pathogen-associated molecular pattern (PAMP) used by plants to detect the presence of a pathogen (19–23). Central to pathogenicity, therefore, is the link between pathogen detection and plant disease resistance via changes in the phytohormone homeostasis (24–26). Once detected by the PAMP system, flagellin results in the accumulation of the phytohormone salicylic acid.
(SA) and downstream SA-dependent defense responses in the plant (21–23). Consequently, Arabidopsis plants that are unable to detect flagellin exhibit more severe disease symptoms and are less resistant to infection (22). Interestingly, we found that the flagellin levels decreased significantly upon the expression of chp8 in P. syringae pv. tomato DC3000Δchp8 (Fig. 3A, pSEVAchp8DGC−/PDE−). The data are fully in line with our phenotypic observations of a Chp8-dependent decrease in the motility of strain DC3000 (Fig. 2) and point toward a role for Chp8 in undermining the SA-dependence of plant immune system.

The detection of PAMPs, such as flagellin, generates a cytosolic influx of Ca2+ into the plant cell (27). Here, Ca2+ acts as a second messenger modulating SA biosynthesis and SA-dependent immune responses (28). Bacteria, in turn, chelate Ca2+, suppressing PAMP-triggered plant immunity through the production of poly-anionic extracellular polysaccharides (EPS) (29). Notably, EPS production is c-di-GMP dependent and is thus interlinked with DGC action (30). Chp8’s DGC activity (Fig. 2) prompted us to assess the impact of Chp8 on EPS production. We utilized the observation that EPS increases the cell’s ability to retain Congo red and to form a “wrinkly” colony (31). As shown by the results in Fig. 3B, cells expressing Chp8 (Fig. 3B, pSEVAchp8DGC−/PDE−) retained more Congo red and were markedly more wrinkly in colony morphology than cells lacking Chp8 (Fig. 3B, pSEVA). Taken together, the data show that Chp8 downregulates flagellin and increases EPS production. Chp8 may therefore hinder the detection of P. syringae pv. tomato DC3000 by the plant and so help to circumvent PAMP-triggered immunity and promote DC3000’s pathogenicity.

**Chp8 promotes P. syringae pv. tomato DC3000’s pathogenicity.** Our data show that Chp8 is embedded in the same regulon as the T3SS and that its expression is induced by plant signals and causes a decrease in flagellin and an increase in EPS production. Together, these results strongly indicate a role for Chp8 in the pathogenesis of a P. syringae pv. tomato DC3000 infection. To test this proposal, we infected Arabidopsis thaliana plants with strain DC3000 or the DC3000Δchp8 mutant using a plate-flodding technique (32) and, in each case, monitored plant health postinfection. P. syringae pv. tomato DC3000 relies on motility to enter the apoplast of the host plant through openings on the surface of the leaves (e.g., stomata), and thus, the infectivity of immotile cells is markedly reduced (33–36). Since Chp8 decreases the motility of P. syringae pv. tomato DC3000, we chose to flood the plants with low-titer bacterial suspensions as an alternative to leaf wounding or infiltration methods that permit passive entry, in order to encourage an infection route that requires an active movement of the bacterial cells into the apoplast through the stomata.

One characteristic symptom of P. syringae pv. tomato DC3000 infection is yellowing (chlorosis) of leaves (2, 6). Both strains elicited chlorosis of Arabidopsis thaliana and ultimately caused plant death. However, the prevalence of disease symptoms was markedly delayed upon infection with the DC3000Δchp8 mutant compared to the results with DC3000 (Fig. 4A), indicating that Chp8 negatively affects the resilience of the plants.

To elucidate the molecular basis of the Chp8-dependent differences in disease progression, we quantified the levels of three key hormones, abscisic acid (ABA), salicylic acid (SA), and jasmonic acid (JA), employed by the plant to modulate its immune response against infection (24, 37). ABA regulates plant development in response to abiotic stresses (38, 39); it also increases the plant’s susceptibility to pathogens, and thus, P. syringae pv. tomato DC3000 employs T3SS effectors during infection to increase ABA (40, 41). Accordingly, we observed a marked increase in ABA levels upon infection with DC3000 (Fig. 4B). However, given that similar ABA levels were observed upon infection with DC3000Δchp8 (Fig. 4B), it seems that the effect Chp8 has on pathogenicity is not associated with the ABA system.

As described above, plants respond to an attack by (hemibiotrophic pathogens like P. syringae pv. tomato DC3000 by accumulating SA (42, 43) and to herbivores and necrotrophic pathogens by accumulating JA (44). High levels of either SA or JA, which are regulated antagonistically (42), trigger a range of plant immune responses to combat the infection (45–48). Unsurprisingly, SA accumulated upon infection with both strains (Fig. 4B). Strikingly, however, since larger quantities of SA were recovered from plants infected with DC3000Δchp8, it would appear that Chp8 restricts SA accumulation (Fig. 4B and see Fig. S5 in the supplemental material). This is fully in line with our observations that Chp8 decreases flagellin and increases EPS production, which are known to affect SA levels (Fig. 3) (21–23, 29). Cross-talk between SA and JA has been demonstrated (42, 44). During systemic acquired resistance, an initial wave of JA signaling precedes a wave of SA signaling (49), and JA levels then decrease (44, 49, 50). In agreement with this antagonism between SA and JA (42), we found that JA levels were higher in plants infected with DC3000 (Fig. 4C and Fig. S5) than in those infected with the DC3000Δchp8 mutant. Moreover, we conclude that Chp8 decreased JA indirectly through elevated SA levels. Elevated SA would result in negative regulation of JA and explain the observations reported here.

To investigate at what stage during the infection Chp8 is particularly important, we compared apoplastic colonization after single and coinfections with P. syringae pv. tomato DC3000 and DC3000Δchp8 (Fig. 4C). Notably, after stimulating infections with single strains, we recovered significantly more DC3000 cells than DC3000Δchp8 cells from plants 1 day postinfection (d.p.i.), despite similar initial inoculum densities (Fig. 4C). However, between day 1 and day 2 postinfection, the bacterial load increased similarly for both strains (Fig. 4C). Consistent with this outcome, after a 1:1 coinfection with both strains, the competitive index for DC3000Δchp8 on day 1 postinfection was only ~0.4 (standard error of the mean [SEM], 0.06), but it increased to ~0.8 (SEM, 0.15) on day 2 postinfection (Fig. 4C). Apparently, apoplasting colonization during early infection events is impaired in DC3000Δchp8 cells. Chp8, however, appears to have no effect on the survival of DC3000 within the apoplast at later stages of infection (Fig. 4C).

Taken together, our plant infection studies showed that Chp8 specifically affected the SA/JA hormone levels, ultimately affecting P. syringae pv. tomato DC3000 pathogenesis in a manner that was particularly apparent in the early stages of infection.

**DISCUSSION**

Plant-pathogen interactions are characterized by the sophisticated interplay between plant immunity elicited upon pathogen recognition, via PAMPs, and immune evasion by the pathogen (51–53). One of the key PAMPs through which plants, and indeed other hosts, recognize pathogens is the structural component of bacterial flagella, flagellin, and specifically, the flg22 epitope (19–23). Recognition of flagellin occurs during both epiphytic and endophytic growth of P. syringae, triggering Ca2+ influx into plant cells (28)
FIG 3  Effects of Chp8 on flagellin and EPS production in \textit{P. syringae} pv. tomato DC3000 strains. (A) The effect of Chp8 on flagellin production was measured via immunoblotting with antibodies against FliC (77). The band corresponding to flagellin was quantified via densitometry, taking into account gel loading. The results for the loading control can be found in Fig. S4 in the supplemental material. Statistical analysis using unpaired \((t\) test gave results as follows (significant if \(P\) value is <0.05): DC3000\textbackslash chp8 versus DC3000\textbackslash chp8\textbackslash pSEVA \(\text{chp8}^\text{DGC}^{+}\text{PDE}^{+}\) was significant, \(P = 0.0310\), AU, arbitrary units. (B) The effect of Chp8 on EPS production was measured via the change in absorbance at 490 nm through retention of the Congo red cell stain and visualized through the formation of wrinkly colony morphology. Statistical analysis using unpaired \((t\) test gave results as follows (significant if \(P\) value is <0.05): DC3000\textbackslash chp8 versus DC3000\textbackslash chp8\textbackslash pSEVA \(\text{chp8}^\text{DGC}^{+}\text{PDE}^{+}\) was significant, \(P = 0.0004\). DC3000\textbackslash chp8\textbackslash pSEVA, vector control; DC3000\textbackslash chp8\textbackslash pSEVA \(\text{chp8}^\text{DGC}^{+}\text{PDE}^{+}\), cells expressing wild-type Chp8. Error bars show standard errors of the means.
FIG 4 Effects of Chp8 on P. syringae pv. tomato DC3000 apoplast colonization and disease symptom development and hormonal immune responses of the plant. (A) Disease symptom development (yellowing of leaves) was followed after single infection of *Arabidopsis thaliana* with either DC3000 or DC3000Δchp8. Mock treatment was included as a negative control. Shown are representative images taken 1 and 2 d.p.i. (B) Levels of abscisic acid (ABA), salicylic acid (SA), and jasmonic acid (JA) were measured after single infection of *Arabidopsis thaliana* with either DC3000 or DC3000Δchp8. Mock treatment was included as a negative control. Shown are the levels measured 1 and 2 d.p.i. Statistical analysis using unpaired t test gave results as follows (significant if *P* value is <0.05): ABA at 1 d.p.i., DC3000 versus DC3000Δchp8 was not significant, *P* = 0.8307; ABA at 2 d.p.i., DC3000 versus DC3000Δchp8 was not significant, *P* = 0.5139; SA at 1 d.p.i., DC3000 versus DC3000Δchp8 was significant, *P* = 0.011; SA at 2 d.p.i., DC3000 versus DC3000Δchp8 was significant, *P* = 0.0134; JA at 1 d.p.i., DC3000 versus DC3000Δchp8 was significant, *P* = 0.0458; JA at 2 d.p.i., DC3000 versus DC3000Δchp8 was not significant, *P* = 0.144. (C) Chp8-dependent differences in apoplast colonization were assessed 1 and 2 d.p.i. by measuring CFU/g plant weight (left panel) after single infection with either DC3000 or DC3000Δchp8 and by calculating the competitive index (CI) after coinfection with both strains at a 1:1 ratio. For CI, the numerator is CFU/g plant recovered from the apoplast (Continued)
and SA-dependent defense mechanisms, such as stomatal closure (48), induction of pathogenesis-related (PR) antimicrobial proteins (46), increased reactive oxygen species (45), and enhanced callose deposition (47). However, since flagellar motility enhances effector fitness and enables bacteria to actively enter the apoplast (33–36), pathogens have evolved strategies to diminish flagellin-dependent detection by the plant immune system (19–23). For instance, some Xanthomonas campestris strains evade detection due to polymorphisms of the flg22 epitope (54). P. syringae suppresses flagellin-triggered immunity by reducing the expression of flagellar genes at both the transcriptional (11) and translational level (55) and by blocking formation of the FLS2-BAK1 flagellin receptor cluster of the plant (56). Our studies now show that Chp8 also contributes to the major effort of P. syringae pv. tomato DC3000 to diminish PAMP-triggered plant immune responses.

Extending the results of previous reports (10, 11), we show that Chp8 is embedded in the Hrp regulon in a way that suggests that signal transduction downstream from HrpRS bifurcates into HrpL-dependent (T3SS) and HrpL-independent (e.g., Chp8) pathways. Bifurcation thereby appears to occur in response to nutritional (HrpL-dependent pathway) (12) or plant-derived signals (HrpL-independent pathway).

Chp8 is a composite GGDEF-EAL protein. Despite the overriding activity of the Chp8 DGC domain, its EAL domain retained PDE activity (illustrated by the increased motility of the Chp8<sup>GGDEF<sub>DGC</sub>-PDE<sup>+</sup></sup> variant) but, more importantly, appeared to functionally interact with the DGC for maximal c-di-GMP production. Similar functional requirements for full DGC and/or PDE activities have also been reported for the composite GGDEF-EAL proteins FimX from Pseudomonas aeruginosa (57) and MSDGC-1 from Mycobacterium smegmatis (58). In addition to the GGDEF motif in the active (A) site, many DGCs also contain a secondary inhibitory (I) site to regulate c-di-GMP production through feedback inhibition (15, 16) (see Fig. S2 in the supplemental material). Recent in silico analyses point to a correlation between I site conservation and the presence of an EAL domain (59). These data further indicate that Chp8 acts prior to the passage of P. syringae pv. tomato DC3000 through the stomata (in line with reports that phloretin, which we show induces Chp8 expression, is a cuticular flavonoid (70)) during the early stages of infection. Recall that, compared to the results for DC3000, the onset of disease symptoms and apoplastic colonization by DC3000Δchp8 are reduced on day 1 but similar in later stages of infection.

In summary, among the host-pathogen interactions that depend on complex interplays between pathogen-triggered host immunity and pathogen evasion of the host immune response, Chp8, a composite GGDEF-EAL protein with a net c-di-GMP activity, serves to reduce flagellin production and increase EPS production, thus functioning as a contributor to pathogen survival in this finely tuned balancing act.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Unless otherwise indicated, P. syringae pv. tomato DC3000 and its derivatives were grown at 28°C in King’s B (KB) medium or Hrp-inducing minimal medium (HIM) supplemented with 50 μg/ml rifampin and additional antibiotics as appropriate (50 μg/ml kanamycin and 100 μg/ml ampicillin).

**Construction of gene deletions in P. syringae pv. tomato DC3000.** Markerless P. syringae pv. tomato strains DC3000Δchp8, DC3000ΔhrpS, and DC3000ΔhrpL were constructed via allelic exchange, utilizing a protocol adapted from reference 71. Briefly, ~2- to 700-bp sequences, corresponding to the 5' and 3' flanking regions of the target open reading frame (ORF), were PCR amplified and fused by single overlap extension PCR. The fusions were inserted into pGEM-T (Promega) to generate intermediate pFUSE vectors. A BamHI fragment containing an FRT-flanked kanamycin resistance gene (nptII) was obtained from the pGEM-T-nptII-BamHI plasmid and inserted into the pFUSE constructs to yield PKO plasmids. P. syringae pv. tomato DC3000 was electroporated with

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**Figure Legend Continued**

(DC3000Δchp8/DC3000) and the denominator is CFU in the initial inoculum (DC3000Δchp8/DC3000), and values indicate results as follows: CI < 1, mutant is less competitive than wild-type; CI = 1, mutant and wild-type are equally competitive; CI > 1, mutant is more competitive than wild-type. Statistical analysis using linear regression for single infection and unpaired t test for coinfection gave results as follows (significant if P value is <0.05): single infection, inoculum to 1 d.p.i., DC3000 (y = 8.9e<sup>6</sup> × −625,000, R<sup>2</sup> = 0.9763) versus DC3000Δchp8 (y = 2.69e<sup>6</sup> × −735,000, R<sup>2</sup> = 0.9681) was significant, P = 0.0039; single infection, 1 d.p.i. to 2 d.p.i., DC3000 (y = 3.57e<sup>6</sup> × −3.47e<sup>6</sup>, R<sup>2</sup> = 0.7377) versus DC3000Δchp8 (y = 1.66e<sup>6</sup> × −1.62e<sup>6</sup>, R<sup>2</sup> = 0.9974) was not significant, P = 0.2727; coinfection (CI < 1), 1 d.p.i. was significant, P = 0.0048, and 2 d.p.i. was not significant, P = 0.1983. Error bars show standard errors of the means.
pKOhp8, pKOhp5, and pKOhpL. Recombinants were selected on LB-kanamycin and screened for ampicillin sensitivity to distinguish allelic exchange (a double recombination event) from whole-plasmid integration (single recombination). To avoid polar transcriptional effects due to the nptII promoter, the nptII cassette was excised from the resulting Δchp8::nptII, ΔhorpS::nptII, and ΔhrpL::nptII strains via recombination at FRT sites flanking the nptII gene through the expression of FLP recombinase from the pPL2 plasmid (72). Transformants were screened for loss of kanamycin resistance. Cured mutant strains carrying pPL2 were subcultured four times in LB medium supplemented with 5% sucrose for sacB-mediated counterselection of the plasmid. Single colonies were scored for ampicillin sensitivity as an indication of plasmid loss. Deletion of chp8, horpS, and hrpS was confirmed by sequencing. All primers used are listed in Table S1 in the supplemental material.

Construction of plasmids. To create the green fluorescent protein (GFP) gene-tagged reporter for chp8 promoter activity, a 648-bp fragment upstream from the PsPTO_2907 start site was cloned into pBBR1-MCS-4 (73) containing gfp-mut3, including the rb30 ribosome binding site and transcriptional terminator (74). For ectopic expression, Chp8 was placed under the heterologous control of a lacI::Pcr gene module by cloning the PsPTO_2007 coding sequence into pSEVA224 (75) to create pSVEAchp8GGE::PDE+. This vector also contains an R2K origin of replication, conferring broad host range and low copy number. The GGDEF and EAL motifs of Chp8 were replaced with alanine residues via site-directed mutagenesis to specifically inactivate the DGC and PDE domains, respectively, leaving the rest of the protein intact. Plasmid pSVEAchp8GGE::PDE+ encodes Chp8GGDEF:AAAA, while pSVEAchp8GGE::PDE encodes Chp8EAL:AAA. Sequencing confirmed successful construction of the plasmids. All primers and plasmids used are listed in Table S1.

Measurement of chp8 promoter activity ex planta. Overnight cultures of P. syringae pv. tomato DC3000 strains carrying the pBBR1-Pchp8a-gfp reporter were washed twice with 10 mM MgCl2, and resuspended in HIM medium with 10 mM fructose to an optical density at 600 nm (OD600) of 0.25. To test the effect of phloretin, cell cultures were subcultured 10-fold every 7 days into cell suspension medium (3% sucrose, 0.44% MSMO (Murashige & Skoog medium with minimal organics), 1-naphthylacetic acid, 50 μg/ml Congo red cell stain), and the absorbance at 490 nm was measured to quantify the amount of Congo red retained by the cells. To visualize colony morphology, cells were removed, and the OD600 was measured. Sessile cells bound to the glass were washed twice with water, dried, and stained through a 15-min incubation with 0.1% crystal violet (Sigma) at room temperature. After washing twice with water, the stained cells were resuspended in 75% ethanol and the OD600 was measured. Biofilm formation was expressed as the ratio between sessile and planktonic cells (OD590/OD600). All assays were performed in triplicate.

Motility assay. For motility, cells from overnight cultures were diluted in fresh medium to an OD600 of 0.05. Five microliters of the bacterial suspension were spotted onto soft agar plates containing 0.4% agar. The plates were incubated at 28°C for 48 h, and swarming across the plate was measured as the diameter of spread. All assays were performed in triplicate.

Flagellin quantification. Flagellin production was measured via immunoblotting with antibodies against FliC as described previously (79). Briefly, bacteria were pelleted by centrifugation at 5,000 rpm for 10 min. To extract flagellin, pellets were washed and resuspended in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20 (vol/vol), 10% glycerol (vol/vol), 1 mM phenylmethylsulfonyl fluoride. Cells were sonicated, and proteins precipitated with 5% trichloroacetic acid for 1 h on ice, resuspended in the same buffer, and separated by 12% SDS-PAGE. Flagellin was detected via immunoblotting with anti-FliC antibodies (79) and quantified via densitometry. Gel loading was controlled and quantified via SYPRO ruby protein stain (Molecular Probes).

EPS quantification and colony morphology. Extracellular polysaccharide (EPS) was quantified and colony morphology visualized as described previously (30). Briefly, overnight cultures of P. syringae pv. tomato DC3000Δchp8/pSEVA and DC3000Δchp8/pSEVAchp8, respectively, were washed with fresh medium, 40 μg/ml Congo red cell stain (Alfa Aesar) was added, and the bacterial suspension was incubated at room temperature for 2 h with shaking. Cells were pelleted, washed, and resuspended in fresh medium. Cells were normalized by protein content, and the absorbance at 490 nm was measured to quantify the amount of Congo red retained by the cells. To visualize colony morphology, cells from overnight cultures were diluted in fresh medium to an OD600 of 0.05. Five-microliter amounts of the bacterial suspensions were spotted onto solid KB agar plates and incubated at 28°C for 5 days before visualizing colony morphology. All assays were performed in triplicate.

Plant infection assays. Arabidopsis Col-0 seedlings were grown on agar plates composed of 1/2 strength (2.1 g/liter) MS (Murashige & Skoog) medium, 0.546 g/liter MES [2-(N-morpholino)ethanesulfonic acid], 1% sucrose, and 1% phytagel. Seeds were vernalized for 2 days at

Measurement of cellular c-di-GMP. For c-di-GMP measurement, bacterial cells were grown on King’s B solid agar plates overnight and resuspended in King’s B growth medium at an OD600 of 1. The suspension was supplemented with 200 ng/ml cyclic XMP (cXMP) as an internal standard. Extraction of c-di-GMP and cXMP was done in acetonitrile-methanol-water (40:40:20, vol/vol/vol) as described previously (76). c-di-GMP analysis was by liquid chromatography–tandem mass spectrometry (LC-MS/MS) (76, 77). The LC-MS system was comprised of an Agilent 1100 LC system and an ABSciex 5400 Qtrap MS. c-di-GMP was separated on a Phenomenex Luna C18(2) column (100 mm by 2 mm by 3 μm) at a temperature of 35°C, utilizing a gradient solvent system comprised of solvents A (10-mM ammonium acetate and 0.1% [vol/vol] formic acid) and B (acetonitrile). The compounds were eluted at a flow rate of 400 ml/min with a gradient from 100% A to 90% A over 5 min. The column was washed with 70% B for 3 min and re-equilibrated with 100% A. Typically, 20-μl injections were used for the analysis. The MS was configured with a Turbo Spray IonDrive source; gas 1 and 2 were set to 40 and 60, respectively; the source temperature was 425°C; and the ion spray voltage was 5,500. V. c-di-GMP and cXMP were analyzed by multiple-reaction monitoring (MRM) in positive mode using the following transitions (mass-to-charge ratio [m/z]), with the collision energies (CE) used shown in parentheses after the transitions: 691.1→152 (60 eV), 691.1→248 (50 eV), 691.1→540 (40 eV), 347→153 (30 eV), and 347→136 (60 eV). The de-clustering potential, exit potential, and collision cell exit potential were set at 80 V, 10 V, and 10 V, respectively, for all transitions. c-di-GMP and cXMP eluted with retention times of 4.6 min and 5.0 min, respectively. Data acquisition and analysis were done with Analyst 1.6.1.

Biofilm formation. Biofilm formation was assayed as described previously (78). Briefly, P. syringae pv. tomato DC3000 strains were grown into KB in borosilicate glass tubes for 48 h at 28°C without shaking. Planktonic cells were removed, and the OD600 was measured. Sessile cells bound to the glass were washed twice with water, dried, and stained through a 15-min incubation with 0.1% crystal violet (Sigma) at room temperature. After washing twice with water, the stained cells were resuspended in 75% ethanol and the OD600 was measured. Biofilm formation was expressed as the ratio between sessile and planktonic cells (OD590/OD600). All assays were performed in triplicate.
4°C prior to sterilization. Seeds were sterilized as follows: 5 min of 70% ethanol, 5 min of 50% sodium hypochlorite, and 4 washes with sterile distilled water (SDW). Seedlings were then grown at 22 ± 1°C and 120 µmol photons m⁻² s⁻¹. After 2 weeks, seedlings were frozen in N2. Samples were freeze-dried in a Heto Drywiner DW1.0-60e for 24 h. Samples were extracted in 394 M of extraction solution composed of 25% methanol, 1% acetic acid in water. Internal standards were then added as follows: 2 µl jasmonic acid ([13C]JA, 5 µg ml⁻¹), 2 µl salicylic acid ([13C]SA, 100 µM), and 2 µl ascorbic acid ([6,7,8,9-[2H4]]ABA, 0.5 µg ml⁻¹). A 3 mM tungsten bead was also added. Samples were placed in a Qiagen TissueLyser at 25.5 Hz for 1 min 30 s and incubated on ice for 30 min. Samples were centrifuged at maximum speed, and the supernatant removed. Samples were re-extracted using 400 µl of extraction buffer, and both extractions were pooled and transferred to vials for LC-MS/MS analysis. An injection volume of 50 µl was used. Analysis was performed on an Agilent 1100LC coupled to an Applied biosystems Q-TRAP LC-MS/MS system. Separation of molecules based on hydrophobicity was achieved using a Phenomenex Luna C₁₈(2) column (100 mm by 2.0 mm by 3 µm) kept at 35°C. JA/SA/ABA ion pairs were monitored based on the following mass transitions: JA 209.2→59, [13C]JA 211.2→61, SA 137.1→93, [13C]SA 141.1→97, ABA 263.2→153, and [6,7,8,9-[2H4]]ABA 269.2→159. Analysis was performed using Analyst, and the means determined based on 4 technical repeats are shown. Error bars denote standard errors of the means.

Statistical analysis. Statistical analysis was performed using Graph-Pad Prism software, version 6.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl?doc_id=01168-14/-/DCSupplemental.

Figure S1, JPG file, 0.1 MB.
Figure S2, JPG file, 0.6 MB.
Figure S3, JPG file, 0.1 MB.
Figure S4, JPG file, 0.1 MB.
Figure S5, JPG file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.

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