CorR Regulates Multiple Components of Virulence in *Pseudomonas syringae* pv. *tomato* DC3000

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The phytotoxin coronatine (COR) is produced by various pathovars of *Pseudomonas syringae*, including *P. syringae* pv. *tomato* DC3000, which is pathogenic on crucifers and tomato, and *P. syringae* pv. *glycinea* PG4180, a soybean pathogen. The COR molecule contains two distinct components, coronafacic acid (CFA) and coronamic acid (CMA), which are intermediates in the COR biosynthetic pathway. In *P. syringae* pv. *tomato* DC3000, it is not clear whether *corR*, which encodes a response regulator, positively regulates CFA and CMA synthesis as it does in *P. syringae* pv. *glycinea* PG4180. In this study, a *corR* mutant of *P. syringae* pv. *tomato* DC3000 was constructed and was shown to be defective in the production of COR, CFA, and CMA. Furthermore, disease severity was greatly reduced in tomato plants inoculated with the *corR* mutant compared with wild-type *P. syringae* pv. *tomato* DC3000. We also showed that a mutation in *hrpL*, which encodes an alternate RNA polymerase sigma factor (σ^L) required for the expression of genes encoding components of the type III secretion system, abrogated production of COR in *P. syringae* pv. *tomato* DC3000. The presence of a potential *hrp* box, the recognition site for σ^L , upstream of *corR* suggested that *corR* might be regulated by *hrpL*. This was confirmed in reverse-transcription polymerase chain reaction experiments showing that the upstream effector gene *holPtoAA*, which was associated with the *hrp* box, was cotranscribed with *corR*. Furthermore, studies also were conducted to investigate whether mutations in *corR* had effects on the expression of *hrpL*. The *corR* mutant of *P. syringae* pv. *tomato* DC3000 showed both a reduction and delay in the expression of *hrpL* and was impaired in its ability to elicit a hypersensitive response on *Nicotiana benthamiana*. A putative CorR-binding site was identified upstream of *hrpL*, and gel shift studies confirmed the binding of CorR to this region. These results indicate that *corR* directly impacts the expression of the *hrp* regulon in *P. syringae*.

host plants, including ryegrass, soybean, tomato, and several crucifers (Bender et al. 1987; Brooks et al. 2004; Budde and Ullrich 2000; Mittal and Davis 1995; Sato et al. 1983). COR consists of two distinct structural components: i) the polyketide coronafacic acid (CFA) and ii) coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine (Ichihara et al. 1977; Mitchell 1985; Parry et al. 1994) (Fig. 1A). In general, COR is the predominant coronafacoyl compound synthesized by COR producers and also the most toxic; however, other coronafacoyl compounds may be synthesized that contain other amino acids conjugated to CFA via an amide linkage (Mitchell and Ford 1998; Mitchell and Young 1985).

COR biosynthesis has been thoroughly investigated in *P. syringae* pv. *glycinea* PG4180, where the 32.8-kb COR gene cluster is borne on a 90-kb plasmid designated p4180A (Bender et al. 1993). The structural genes for CFA and CMA biosynthesis are located on opposing ends of the COR gene cluster in *P. syringae* pv. *glycinea* PG4180 and are separated by a region containing the regulatory genes *corR* and *corS*. The translational product of *corR* shows relatedness to response regulators in two-component regulatory systems, whereas *corS* shows similarity to histidine protein kinases (HPKs) (Ullrich et al. 1995). CorR has an N-terminal receiver domain that functions as the phosphorylation site and a C-terminal effector domain with a DNA-binding, helix-turn-helix (H-T-H) motif (Rangaswamy and Bender 2000; Ullrich et al. 1995). CorR functions as a positive activator of *cor* gene expression and binds to the promoter regions of the CFA and CMA biosynthesis genes in *P. syringae* pv. *glycinea* PG4180 (Penaloza-Vazquez and Bender 1998; Wang et al. 1999). CorS has characteristics consistent with phosphorylation at a histidine residue and was shown to transphosphorylate CorR in vitro (Rangaswamy and Bender 2000).

P. syringae pv. *tomato* DC3000, a pathogen of tomato, *Brassica* spp. (cabbage and cauliflower), and *Arabidopsis thaliana* (Moore et al. 1989; Wang et al. 2002; Whalen et al. 1991), is a model strain for investigating plant-microbe interactions, largely because of its genetic tractability, pathogenicity on *Arabidopsis*, and the availability of its genomic sequence. In *P. syringae* pv. *tomato* DC3000, the COR structural and regulatory genes are highly homologous (90 to 98% nucleotide identity) to those previously described in *P. syringae* pv. *glycinea* PG4180 (Buell et al. 2003). However, *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *glycinea* PG4180 differ in the amount of COR produced in vitro, the environmental cues for COR gene induction, and the genomic location and organization of the *cor* genes (Brooks et al. 2004; Moore et al. 1989; Penaloza-Vazquez et al. 2000; Weingart et al. 2004). Unlike *P. syringae* pv. *glycinea* PG4180, where the *cor* genes are plasmid encoded and clustered, the structural gene clusters encoding the CFA and CMA biosynthetic enzymes are not contiguous

Coronatine (COR) is a chlorosis-inducing phytotoxin produced by several pathovars of *Pseudomonas syringae*, including pvs. *alisalensis*, *atropurpurea*, *glycinea*, *maculicola*, *morsprunorum*, and *tomato* (Bender and Scholz-Schroeder 2004; Cintas et al. 2002). COR contributes to the multiplication of *P. syringae* in planta and lesion formation or expansion in several

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ous in *P. syringae* pv. *tomato* DC3000 and are chromosomally encoded. Thus, in *P. syringae* pv. *tomato* DC3000, the CMA structural genes map with *corRS* and are physically separated from the CFA biosynthetic region by a 26-kb intergenic region (Fig. 1B) (Brooks et al. 2004; Buell et al. 2003). Thus, it is unclear whether *corR* modulates both CMA and CFA biosynthesis in *P. syringae* pv. *tomato* DC3000, which is the case with *P. syringae* pv. *glycinea* PG4180.

In addition to *corRS*, other regulatory loci control virulence in *P. syringae*, including the type III secretion system (TTSS). In *P. syringae*, the TTSS is required for growth of *P. syringae* in susceptible host plants and the activation of plant defense in nonhost plants (Jin et al. 2003). These host responses are elicited when the *hrp/hrc*-encoded TTSS delivers effector proteins to the plant cell, presumably via the *hrp* pilus, which is encoded by the *hrpA* gene (Collmer et al. 2002; He and Jin 2003; Roine et al. 1997). The *hrpL* gene, which encodes the alternate RNA polymerase sigma factor, σ^L , is one of the primary transcription factors modulating expression of the TTSS (Xiao et al. 1994). HrpL is a member of the extracytoplasmic family of transcription factors and recognizes a conserved promoter sequence, referred to as the “hrp box”, upstream of HrpL-dependent genes (Fouts et al. 2002; Schechter et al. 2004). In *P. syringae* pv. *tomato* DC3000, evidence exists for a regulatory connection between the TTSS and COR production; for example, mutations in the TTSS had a direct effect on the levels of COR produced by *P. syringae* pv. *tomato* DC3000 (Penaloza-Vazquez et al. 2000). Further support for regulatory cross-talk was reported previously (Fouts et al. 2002), where a promoter-trapping screen was used to identify genes that are controlled by *hrpL*. Interestingly, *hrpL*-regulated insertions were identified in two COR biosynthesis genes, *cfa1* and *cfa6*. This was an intriguing result, because neither gene contained a recognizable hrp box.

In the current study, we use genetic and biochemical approaches to evaluate whether *corR* is required for the synthesis of COR, CFA, and CMA in *P. syringae* pv. *tomato* DC3000. We also investigate whether *hrpL* regulates *cor* gene expression and whether cross-talk between *hrpL* and COR is bi-directional.

RESULTS

Expression of virulence genes in Hoitink-Sinden with sucrose and *hrp*-derepressing media.

Previous studies to investigate the expression of *hrp* genes have been conducted in *hrp*-derepressing medium (HDM) (Huynh et al. 1989). However, most studies involving *cor* gene expression have utilized Hoitink-Sinden medium supplemented with either glucose (HSC) or sucrose (HSS) (Palmer and Bender 1993; Penaloza-Vazquez and Bender 1998). In the present study, *cfl*, which is the first gene to be transcribed in the CFA gene cluster (Fig. 1C) (Liyanage et al. 1995), was used as a definitive marker for CFA gene expression. *cmaB* was used to monitor transcription in the CMA gene cluster (Budde et al. 1998) (Fig. 1C). Quantitative real-time polymerase chain reaction (RT-qPCR) (discussed below) was used to monitor the expression of selected *cor* genes (*corR*, *cfl*, and *cmaB*), *hrpL*, and *rpoD* (endogenous reference gene). All *cor* genes and the *hrpL* gene showed a relative-fold increase in expression in HSS medium (Table 1). The *cor* regulatory gene, *corR*, was induced to relatively low levels in both HSS and HDM. In HDM, induction of *hrpL* expression was approximately 70-fold higher than in HSS medium. However, *cfl* and *cmaB* showed a reduced level of induction in HDM compared with HSS medium (Table 1); consequently, all subsequent experiments where *cor* gene expression was monitored were conducted using HSS medium. It is important to note that HDM remains the medium

Fig. 1. Phytotoxin coronatine (COR) biosynthetic and regulatory regions of *Pseudomonas syringae* pv. *tomato* DC3000. **A**, Structure of COR, coronafacic acid (CFA), and coronamic acid (CMA). **B**, The entire COR biosynthetic region, including the 26-kb intergenic region (IR). Abbreviations: CFA, region required for CFA biosynthesis; REG, regulatory region; and CMA, region required for CMA biosynthesis. **C**, The *cfl* and *cmaB* genes were used to monitor transcriptional activity in the CFA and CMA biosynthetic regions, respectively. The *corR* and *corS* genes are referred to throughout the text, and encode a transcriptional activator and a histidine protein kinase, respectively.

of choice for most investigators working on the TTSS; therefore, HDM was used for monitoring the expression of *hrpL*.

Quantification of COR and CFA

in *corR* and *hrpL* mutants of *P. syringae* pv. *tomato* DC3000.

In *P. syringae* pv. *tomato* DC3000, the CMA/REG and CFA gene clusters are not contiguous as they are in PG4180, and several pieces of evidence suggest that the COR genes are regulated differently in *P. syringae* pv. *tomato* DC3000 compared with *P. syringae* pv. *glycinea* PG4180 (Wang et al. 2002). Therefore, we constructed a *corR* mutant of *P. syringae* pv. *tomato* DC3000 to determine whether *corR* modulates gene expression in the CFA and CMA biosynthetic gene clusters. The *corR* mutant, designated AS1, was generated by insertion of a kanamycin resistance (Km^r) cassette into the coding region of *corR* (discussed below). In addition to the *corR* mutant, a *hrpL* mutant of *P. syringae* pv. *tomato* DC3000 (Zwiesler-Vollick et al. 2002) was included in this study because previous studies indicated that mutations in the TTSS have regulatory effects on COR (Penaloza-Vazquez et al. 2000).

P. syringae pv. *tomato* DC3000 produced COR at 1,236 ng/ml and very little free CFA (48 ng/ml of culture), presumably because most of it was converted to COR. However, COR and CFA production by the *corR* and *hrpL* mutants was either undetectable or consistently low (≤ 40 ng, the detection limit for both CFA and COR). These results indicate that both mutants were impaired in production of COR and CFA.

In exogenous feeding studies, the *corR* and *hrpL* mutants did not produce COR when supplied with exogenous CMA or CFA, which suggests that these mutants do not produce enough CFA or CMA to result in detectable levels of COR. When supplied with CFA and CMA simultaneously, AS1 and VJ202 produced COR at 53.3 and 54.2 ng/ml, respectively. This implies that a basal level of the enzyme or enzymes that ligate CMA and CFA may be present, and confirms that the mutants are impaired in the synthesis of both CMA and CFA. As controls, mutants *cfa6* (strain DB4G3, CFA⁻ and CMA⁺) and *cmaA* (strain AK7E2, CFA⁺ and CMA⁻) were supplied with exogenous CFA or CMA, respectively, and COR was produced at levels equivalent to the wild-type *P. syringae* pv. *tomato* DC3000, which is consistent with previous results (Brooks et al. 2004).

In cocultivation experiments, if one strain secretes an intermediate (e.g., CFA or CMA) that can be assimilated by a second strain that is deficient in production of that intermediate, the latter should be able to synthesize COR. Thus, when the *cfa6* (strain DB4G3, CFA⁻ and CMA⁺) and *cmaA* (strain AK7E2, CFA⁺ and CMA⁻) mutants were cocultivated, COR production was partially restored, which is consistent with previous results (Brooks et al. 2004). However, AS1 and VJ202 did not produce COR when cocultivated with either the *cfa6* or *cmaA* mutants, suggesting that AS1 and VJ202 do not synthesize sufficient amounts of CFA or CMA, respectively, to result in COR production. Collectively, these results suggest that *corR* and *hrpL* regulate the biosynthesis of both CFA and CMA. Furthermore, when *hrpL* was overexpressed from the *lac* promoter in *P. syringae* pv. *tomato* DC3000 (pCPP2385) (Preston et al. 1998), the strain produced COR at 19.8 μ g/ml, which is 16-fold higher than the amount of COR produced by *P. syringae* pv. *tomato* DC3000.

Complementation experiments.

To confirm that impaired COR production in AS1 and VJ202 is caused by the disruptions in *corR* and *hrpL*, respectively, these genes were cloned into stable, replicating plasmids and introduced into the mutants, and the resulting transformants were analyzed for COR production. pBBR1MCS

containing *corR* (pAS2.1) and pVSP61 containing *hrpL* (pHrpL) in trans were introduced into AS1 and VJ202, respectively. Introduction of the wild-type genes restored COR production in both mutants, with AS1 (pAS2.1) and VJ202 (pHrpL) producing COR at $3,802 \pm 394$ and $1,045 \pm 110$ ng/ml, respectively. In these experiments, COR production by *P. syringae* pv. *tomato* DC3000 was $1,236 \pm 524$ ng/ml, indicating that all mutants were fully complemented for COR production.

Symptom expression and growth

of *P. syringae* pv. *tomato* DC3000 and mutants in tomato.

The effect of the *corR* and *hrpL* mutations on virulence was examined by recording the percentage of tomato leaves exhibiting typical disease symptoms (chlorosis or necrosis) as described below. Leaves from plants inoculated with *P. syringae* pv. *tomato* DC3000 exhibited 50 to 75% disease severity (Fig. 2A). In contrast, most of the leaves inoculated with the *corR* (AS1) and *cfa6* (DB4G3) mutants showed only 10 to 25% disease severity (Fig. 2B and D). Interestingly, leaves inoculated with the *corR* and *cfa6* mutants showed a small amount of chlorosis. This likely is due to factors other than COR, because this phenotype previously was demonstrated with other COR mutants (Bender et al. 1987). Leaves inoculated with the *hrpL* mutant (VJ202) (Fig. 2F) and an *hrp* structural mutant of *P. syringae* pv. *tomato* DC3000 (*P. syringae* pv. *tomato* DC3000-*hrcC*) (Fig. 2C) remained symptomless.

To determine whether the reduction in disease symptom production was correlated with reduced multiplication in plant tissue, the total populations of the *corR* and *hrpL* mutants were compared with the wild-type *P. syringae* pv. *tomato* DC3000 in spray-inoculated tomato leaves from 0 to 10 days after inoculation. The total population of *P. syringae* pv. *tomato* DC3000 exceeded 10^{10} CFU/g of tissue beginning 3 days after inoculation and was at least 100-fold higher than the mutants on days 7 and 10 (data not shown). The total population of the *corR* mutant increased for the first 3 days after inoculation and then declined to a level 100-fold lower than the wild-type at days 7 and 10 (data not shown). These data suggest that *corR* is not essential for the early stages of pathogen multiplication but, instead, is critical for persistence in the later stages of infection. The total population of the *hrpL* mutant increased slightly during the first 24 h after inoculation but remained at least 100-

Table 1. Quantitative real-time polymerase chain reaction experiments to evaluate expression of selected genes in *Pseudomonas syringae* pv. *tomato* DC3000 grown in either Hoitink-Sinden medium supplemented with sucrose (HSS) or *hrp*-derepressing medium (HDM)

Gene ^a	HSS medium (R) ^b	HDM (R) ^b
<i>hrpL</i>	49.5 $\pm$ 27.2	3,622 $\pm$ 655
<i>corR</i>	35.6 $\pm$ 1.2	25 $\pm$ 2.7
<i>cmaB</i>	202 $\pm$ 100	10.7 $\pm$ 2
<i>cfl</i>	874 $\pm$ 237	6.7 $\pm$ 2.9

^a Values for *corR* and *cmaB* indicate expression 4 h after shifting cells from King's medium B (KB) to HSS or HDM. For *hrpL* and *cfl*, the values shown indicate expression 2 and 6 h after transfer to HSS or HDM, respectively. These times were chosen because they represent the highest point of relative-fold induction of gene expression.

^b R = the relative-fold increase in target gene expression 2 to 6 h after transfer from KB to HSS relative to the gene expression immediately after transfer and was calculated as described previously (Pfaffl 2001). The threshold cycle value for *rpoD* (whose expression remained constant under the experimental conditions designed for the target gene) was 14.9 ± 2.35 and was used as the normalizer. The target gene expression is denoted as the relative-fold increase in the transcript level with respect to the gene expression at time zero (calibrator). Values represent the mean of three experiments; the number following $\pm$ indicates the standard deviation. All experiments were performed three times with similar results.

fold lower than the wild-type throughout the experiment, as expected for a mutant defective for the TTSS (Deng et al. 1998).

The complemented mutants AS1(pAS2.1) and VJ202(pHrpL) were restored for their ability to cause chlorosis (Fig. 2E and G). Although the size of the necrotic lesions was less than that observed for *P. syringae* pv. *tomato* DC3000 (Fig. 2A), the number of lesions was visibly higher in leaves inoculated with AS1(pAS2.1) and VJ202(pHrpL). Thus, the leaves selected from plants inoculated with the complemented mutants exhibited 50 to 75% disease severity, which was similar to leaves infected with *P. syringae* pv. *tomato* DC3000. The complemented mutants also were restored for growth in planta (data not shown).

Expression of *cor* genes in *P. syringae* pv. *tomato* DC3000 and the *corR* mutant.

The results described above indicated that *corR* is required for the regulation of COR biosynthesis in *P. syringae* pv. *tomato* DC3000. This was investigated further by monitoring the induction of *cfl* and *cmaB* expression in *P. syringae* pv. *tomato* DC3000 and the *corR* mutant using RT-qPCR. Preliminary studies were conducted to determine the expression profile for selected *cor* genes (*corR*, *cfl*, and *cmaB*) in *P. syringae* pv. *tomato* DC3000 (Fig. 3) and the *corR* mutant (data not shown).

Induction of gene expression was evaluated 0, 2, 4, 6, and 12 h after transfer from King's medium B (KB) to HSS medium, and the sampling time where induction of gene expression was highest in *P. syringae* pv. *tomato* DC3000 was selected for further analysis. Maximal induction of *cmaB* and *cfl* expression in *P. syringae* pv. *tomato* DC3000 occurred at 4 and 6 h after transfer from KB to HSS, respectively, and these two times were selected for further comparative studies (Table 2; Fig. 3). Maximal induction of *corR* expression in *P. syringae* pv. *tomato* DC3000 occurred at 4 h (Fig. 3) and was chosen for further analyses. The *cfl* and *cmaB* transcriptional activity was significantly impaired (approximately 100-fold lower) in the *corR* mutant compared with *P. syringae* pv. *tomato* DC3000, indicating that *corR* is required for expression of CFA and CMA genes, respectively (Table 2).

Expression of *cor* gene in the *hrpL* mutant.

As mentioned above, the *hrpL* mutant was defective in COR production; therefore, the relative-fold induction of *cfl*, *cmaB*, and *corR* expression was evaluated in the *hrpL* mutant to determine whether the *hrpL*-dependence of COR synthesis was due to impaired expression of the *cor* regulatory or structural genes. Expression of *cfl* and *cmaB* in the *hrpL* mutant was approximately 700- and 50-fold lower, respectively, than *P. syringae* pv.

Fig. 2. Symptoms on tomato leaves sprayed with **A**, *Pseudomonas syringae* pv. *tomato* DC3000; **B**, DB4G3-*cfa6* (*cfa6* mutant); **C**, DC3000-*hrcC* (*hrcC* mutant); **D**, AS1-*corR* (*corR* mutant); **E**, AS1(pAS2.1) (*corR* mutant with *corR* in trans); **F**, VJ202-*hrpL* (*hrpL* mutant); and **G**, VJ202(pHrpL) (*hrpL* mutant with *hrpL* in trans). Bacteria were sprayed (10^8 CFU/ml) onto tomato leaves until surfaces were uniformly wet. After inoculation, all plants were incubated in a growth chamber with a 12-h photoperiod at 90% relative humidity (RH) for the initial 48 h and 70% RH thereafter. Photographs were taken 7 days after inoculation.

tomato DC3000 (Table 2), indicating that *hrpL* modulates transcription of the CFA and CMA gene clusters. Expression of *corR* in the *hrpL* mutant also was reduced with a relative-fold induction approximately 5.5-fold lower than the wild type (Table 2). These results indicate that *hrpL* modulates expression of *corR*, although it was not clear whether *hrpL*-mediated regulation of *corR* was direct or indirect (e.g., via another *hrpL*-regulated gene). However, the putative *hrp* box located approximately 490 bp upstream of *corR* suggested that transcription of *corR* might be modulated by *hrpL*. This was confirmed in RT-PCR experiments showing that the upstream effector gene *holP-toAA*, which is located between the *hrp* box and *corR* (Fig. 1C), was cotranscribed with *corR*, thus constituting an operon (*holP-toAA-corR*) (Fig. 4). This result indicates the existence of a regulatory cascade in *P. syringae* pv. *tomato* DC3000 where *hrpL* modulates expression of *corR*, which in turn controls expression of the CFA and CMA biosynthetic clusters.

Expression of *hrpL* in the *corR* mutant.

To gain further insight into regulatory interactions between the *cor* and *hrp* genes, the relative-fold induction of *hrpL* expression was evaluated in *P. syringae* pv. *tomato* DC3000 and the *hrpL* and *corR* mutants. As described previously, the relative fold induction of *hrpL* expression was evaluated at 0, 2, 4, 6, and 12 h after transferring cells from KB medium to HDM (Fig. 5). In *P. syringae* pv. *tomato* DC3000, the relative-fold induction of *hrpL* expression was highest at 2 h (Fig. 5); *hrpL* transcriptional activity was negligible in the *hrpL* mutant (data not shown). In the *corR* mutant, *hrpL* expression was reduced, and there was a 2-h delay in the activation of *hrpL* transcription when compared with *P. syringae* pv. *tomato* DC3000 (Fig. 5), indicating that *corR* modulates the expression of *hrpL*.

To evaluate the biological significance of the delay in *hrpL* expression in the *corR* mutant, we assessed the ability of the *corR* mutant to elicit a hypersensitive response (HR) on *Nicotiana benthamiana*. Leaves of *N. benthamiana* were infiltrated with four different concentrations (optical density at 600 nm [OD₆₀₀] = 0.1, 0.01, 0.001, and 0.0001) of *P. syringae* pv. *tomato* DC3000 and the *corR* and *hrpL* mutants (an OD₆₀₀ of 0.1 contained 10⁸ CFU/ml). As expected, the *hrpL* mutant failed to elicit an HR, regardless of the inoculum concentration. *P. sy-*

ringae pv. *tomato* DC3000 elicited an HR at inoculum concentrations ranging from OD₆₀₀ = 0.1 to 0.001. The HR elicited by the *corR* mutant was similar to that observed with *P. syringae* pv. *tomato* DC3000 at the higher concentrations (OD₆₀₀ = 0.1 and 0.01). However, the *corR* mutant was impaired in its ability to elicit an HR on tobacco when compared with *P. syringae* pv. *tomato* DC3000 at OD₆₀₀ = 0.001. Thus, the delayed expression of *hrpL* in the *corR* mutant is biologically significant in the context of the HR. At 48 h after infiltration, electrolyte leakage was measured from leaf disks removed from *N. benthamiana* leaves inoculated with *P. syringae* pv. *tomato* DC3000, AS1 (*corR* mutant), VJ202 (*hrpL* mutant) (all strains were inoculated at OD₆₀₀ = 0.001), and water (negative control). Electrolyte leakage from leaves inoculated with *P. syringae* pv. *tomato* DC3000 was the highest, with a conductivity of 52.5 ± 0.7 μS, followed by leaves inoculated with the *corR* mutant (conductivity = 42.5 ± 0.7 μS). *N. benthamiana* leaves inoculated with the *hrpL* mutant and water had the least electrolyte leakage, with conductivities of 27 ± 1.4 and 26.5 ± 0.7 μS, respectively.

The reduction in the levels and delay in the induction of *hrpL* expression in the *corR* mutant (Fig. 5) suggested that mutations in the *cor* regulatory genes can impact expression of

Table 2. Quantitative real-time polymerase chain reaction experiments to evaluate expression of selected genes in *Pseudomonas syringae* pv. *tomato* DC3000, AS1 (*corR* mutant), and VJ202 (*hrpL*)^a

Target gene	Strains and relative-fold increase in target gene expression ^b		
	DC3000	AS1 (<i>corR</i> mutant)	VJ202 (<i>hrpL</i> mutant)
<i>cfl</i>	779 ± 134	0.6 ± 0.0	1.2 ± 1.3
<i>cmaB</i>	229 ± 39	2.1 ± 1	4.1 ± 2.1
<i>corR</i>	33 ± 5	4.3 ± 0.6	5.9 ± 0.9

^a For all genes except *cfl*, the values shown indicate expression at 4 h after shifting cells from King's medium B to Houtink-Sinden medium supplemented with sucrose (HSS) medium. For *cfl*, the values shown indicate expression 6 h after transfer to HSS medium.

^b All values indicate relative-fold increase in target gene expression as described in Table 1. Values represent the highest levels of gene expression and are the mean of three experiments; the number following ± indicates the standard deviation. All experiments were performed three times with similar results.

Fig. 3. Expression of *cor* genes in *Pseudomonas syringae* pv. *tomato* DC3000 0, 2, 4, 6, and 12 h after transfer from King's medium B to Houtink-Sinden medium supplemented with sucrose. Total RNA was isolated from *P. syringae* pv. *tomato* DC3000 and used to synthesize cDNA for quantitative real-time polymerase chain reaction (RT-qPCR).

hrp genes. We previously demonstrated that mutations in the *hrp* genes can impact *cor* gene expression (Penaloza-Vazquez et al. 2000); however, to our knowledge, this is the first evidence that *cor* regulatory mutations can impact transcriptional activity in the *hrp* gene cluster. Mechanistically, we reasoned that CorR, which binds upstream of *cor* transcripts in *P. syringae* pv. *glycinea* PG4180 (Penaloza-Vazquez and Bender 1998; Wang et al. 1999), might modulate *hrp* gene expression by binding to the regulatory region of one of the *hrp* regulatory

Fig. 4. Reverse-transcription polymerase chain reaction (RT-PCR) amplification of the *holPtoAA-corR* transcript. Total RNA was isolated from *Pseudomonas syringae* pv. *tomato* DC3000, treated with DNaseI, and used to synthesize cDNA for RT-PCR as described in Experimental procedures. RT-PCR was conducted using primers spanning from 30 bp downstream of the *holPtoAA* translational start site to 52 bp upstream of the *corR* translational stop. Lanes 1 and 5, 1-kb ladder (Invitrogen); lane 2, 920-bp fragment amplified from *P. syringae* pv. *tomato* DC3000 genomic DNA (positive control); lane 3, 920-bp fragment amplified from the cDNA template; and lane 4, *P. syringae* pv. *tomato* DC3000 total RNA without reverse transcriptase (negative control). The numbers on the left indicate the size of the fragments in the 1-kb ladder; the band shown on the right (821 bp) is the predicted size of the amplified cDNA if *holPtoAA* and *corR* are co-transcribed.

Fig. 5. Expression of *hrpL* in *Pseudomonas syringae* pv. *tomato* DC3000 and the *corR* mutant 0, 2, 4, 6, and 12 h after transfer from King's medium B to *hrp*-derepressing medium. Total RNA was isolated from *P. syringae* pv. *tomato* DC3000 and the *corR* mutant and used to synthesize cDNA for quantitative real-time polymerase chain reaction.

genes (e.g., *hrpL*). When we searched for CorR-binding sites in the upstream region of *hrpL*, a putative CorR-binding site was identified 656 bp upstream of the *hrpL* translational start site. The potential binding of CorR to this region was investigated in gel retardation assays using a fusion protein consisting of the maltose-binding protein (MBP) fused to CorR (Penaloza-Vazquez and Bender 1998). When a 0.8-kb *HindIII-XbaI* fragment containing the *hrpL* upstream region was incubated with 16 ng of MBP-CorR, migration of the labeled fragment was markedly reduced (Fig. 6, lanes 2, 3, and 4) compared with the labeled fragment alone (Fig. 6, lanes 1 and 8). The specificity of complex formation between MBP-CorR and the *hrpL* upstream region was investigated by adding increasing amounts of the unlabeled 0.8-kb *HindIII-XbaI* fragment to the reaction mixture. When unlabeled fragment was added as a competitor in amounts of 200 ng or higher, binding was either significantly reduced or completely abolished (Fig. 6, lanes 5 through 7). However, when poly(dI-dC) was added to the reaction mixture, binding was not altered (Fig. 6, lanes 2 through 4). When MBP was used in gel retardation assays, migration of the *hrpL* upstream region was not altered (data not shown). Thus, these results indicate that CorR specifically binds to the *hrpL* upstream region.

DISCUSSION

COR biosynthesis is regulated by *corR* in *P. syringae* pv. *tomato* DC3000.

Previously, *corR* was shown to control the expression of the CFA and CMA gene clusters in *P. syringae* pv. *glycinea* PG4180 (Liyanage et al. 1995; Ullrich et al. 1995); however, this assumption could not be made in *P. syringae* pv. *tomato* DC3000 because of the numerous differences in COR regulation between these two strains (Bender and Scholz-Schroeder

Fig. 6. Gel shift assays using the maltose-binding protein (MBP)-CorR fusion protein and the 800-bp *HindIII-XbaI* fragment containing the *hrpL* upstream region of *Pseudomonas syringae* pv. *tomato* DC3000. Lanes 1 and 8 show approximately 2 ng of end-labeled target DNA (800-bp *HindIII-XbaI* fragment) and 0 ng of MBP-CorR. The addition of the nonspecific competitor poly(dI-dC) is shown in lanes 2, 3, and 4, which contain 50, 100, and 200 ng of poly(dI-dC), respectively, along with 16 ng of MBP-CorR. Specific inhibition of binding was investigated by adding the following amounts of unlabeled target fragment: lane 5, 200 ng; lane 6, 400 ng; and lane 7, 600 ng.

2004). In the present study, we demonstrate that the *corR* mutant of *P. syringae* pv. *tomato* DC3000 (AS1) was defective in COR biosynthesis and expression of structural genes in the CFA (*cfl*) and CMA (*cmaB*) biosynthetic regions (Table 2). Furthermore, we show that *corR* and *hrpL* regulate each other, indicating that the expression of two key virulence factors, COR and the TTSS, is interconnected. A model describing the signal transduction cascade for activation of the *cor* genes and interaction with *hrpL* is presented below.

COR production

in *P. syringae* pv. *tomato* DC3000 requires *hrpL*.

In the current study, we show that an *hrpL* mutant of *P. syringae* pv. *tomato* DC3000 was defective in COR production, and the overexpression of *hrpL* resulted in 16-fold higher levels of COR in *P. syringae* pv. *tomato* DC3000. These results, in addition to those obtained by RT-qPCR, indicate a role for *hrpL* in COR biosynthesis and suggest that σ^L governs the expression of COR production. This also has important evolutionary implications because both COR and products of the TTSS have been shown to suppress the plant defense response (Abramovitch and Martin 2005; Brooks et al. 2005; Mudgett 2005; Oguiza and Asensio 2005), and coordinated regulation of these virulence systems could be critical in the pathogenesis of *P. syringae* pv. *tomato* DC3000.

Bioinformatic analysis revealed no candidate *hrp* boxes upstream of the CFA and CMA gene clusters. However, a putative *hrp* box and its associated effector gene, *holPtoAA*, mapped 483 and 151 bp upstream of *corR*, respectively (Buell et al. 2003), suggesting that σ^L may regulate an operon containing *holPtoAA* and *corR* (Fig. 7B). This was supported by RT-PCR experiments where *holPtoAA* was shown to be cotranscribed with *corR*, thereby forming a *holPtoAA-corR* operon (Fig. 4). Furthermore, the relative-fold expression of *corR* was significantly lower in the *hrpL* mutant compared with the wild-type *P. syringae* pv. *tomato* DC3000 (Table 2). Thus, HrpL, which is known to regulate the expression of effector genes and components of the TTSS, also regulates COR production by modulating the expression of *corR*.

In a previous study, Fouts and associates (2002) showed a potential role for *hrpL* in regulating COR production. However, these researchers were unable to demonstrate *hrpL*-dependent

modulation of *corR*. Using a hidden Markov model (HMM) to identify potential *hrp* promoters in the *P. syringae* pv. *tomato* DC3000 genome, an *hrp* box with a relatively insignificant E (expectation) value was identified upstream of *corR* (Fouts et al. 2002). Additional assays, including RNA blots and microarray analysis, were performed to assess the validity of HMM E values to predict *hrp* promoters; however, these assays failed to identify *corR* as being regulated by *hrpL*. Thus, the RNA blots performed previously may have lacked sufficient sensitivity to detect *hrpL*-mediated *corR* expression. Microarray analysis also failed to detect the expression of *corR*. As shown by our results, the timing of RNA isolation is critical to accurate detection of *cor* gene expression in *P. syringae* pv. *tomato* DC3000 (Fig. 3). Induction of *corR* expression was low throughout our experiments, with maximal expression at 4 h after transfer to a minimal medium (Fig. 3). After 6 h, *corR* levels decreased to approximately the same levels observed at 0 h. These parameters are important because the RNA used for microarrays and blots by Fouts and associates (2002) was isolated 6 h after inoculation; thus, it is possible that *corR* expression was below the level of detection in the previous study.

corR modulates expression of *hrpL*.

Our results showed that *hrpL* regulates the expression of genes in the COR biosynthetic cluster via *corR*. Hence, we also evaluated whether a mutation in *corR* had a regulatory effect on *hrpL*; our results demonstrated that the relative-fold expression of *hrpL* was both reduced and delayed in the *corR* mutant AS1 (Fig. 5). Thus, *corR* impacts the level and timing of *hrpL* expression. Our results showed that the HR induced by the *corR* mutant was diminished at lower inoculum concentrations when compared with *P. syringae* pv. *tomato* DC3000, indicating that the effect of *corR* on *hrpL* is biologically significant.

A working model for the regulation

of COR and *hrpL* in *P. syringae* pv. *tomato* DC3000.

Regulation of the TTSS occurs primarily at the level of the *hrpL* promoter and involves σ^{54} , the enhancer binding proteins HrpR and HrpS, and Lon-mediated degradation of HrpR (Bretz et al. 2002) (Fig. 7A). In this study, we demonstrate that *hrpL* also regulates COR synthesis. Activation of *hrpL* transcription in *P. syringae* pv. *tomato* DC3000 occurred within 2 h

Fig. 7. Model representing potential regulatory cascade for activation of *hrp* and *cor* genes. Times in legend represent hours following shift from rich (King's B) to minimal (Hoitink-Sinden supplemented with sucrose or *hrp*-derepressing) media. **A**, Expression of *hrpL* occurs quickly (1 to 2 h) and requires σ^{54} , HrpR/HrpS, and Lon protease (Bretz et al. 2002); **B**, σ^L , the product of *hrpL*, binds upstream of the *holPtoAA-corR-corS* region and initiates transcription (2 to 4 h); **C**, CorR activates transcription of the *cfa* and *cma* gene clusters (2 to 10 h); and **D**, CorR is required for early (within 1 to 2 h) activation of *hrpL* transcription.

after shifting cells to HDM (Fig. 5). The relative-fold induction of *corR* expression was optimal at 4 h (Fig. 3), which is consistent with a downstream signaling event where σ^L recognizes and binds to the *hrp* box upstream of the *holPtoAA-corR* operon (Fig. 7B). Optimal induction of *cmaB* and *cfl* expression occurred 4 and 6 h, respectively, after shifting to HSS medium (Fig. 3), and presumably involves transcriptional activation by CorR (Fig. 7C).

Mutations in *corR* reduced and delayed the induction of *hrpL* expression (Fig. 5; Table 2), which implies that CorR may stimulate the early induction of *hrpL* expression (Fig. 5). Furthermore, a putative CorR-binding site was identified upstream of *hrpL*, and the binding of CorR to this region was confirmed (Fig. 6). Thus, CorR may bind the *hrpL* promoter region and function to enhance early transcription of *hrpL* (Fig. 7D). In conclusion, our data imply that the levels of σ^L and CorR are dynamic and influenced by signals that control both the TTSS and COR synthesis. In a previous study, a polar mutation in *hrcC* led to the overproduction of COR, which was restored to wild-type levels by complementation with *hrpV* (Penaloza-Vazquez et al. 2000), a negative regulator of genes in the *hrp* cluster (Preston et al. 1998). Therefore, in addition to the regulation of *hrpL* by *corR*, additional cross-talk exists between additional regulatory loci in the *hrp* and COR gene clusters. Exactly how these signals are transduced and coordinated between these two virulence systems is not clear, and studies to elucidate the mechanistic basis for bidirectional cross-talk are underway.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

The bacterial strains and plasmids used in this study are described in Table 3. *P. syringae* strains were grown on mannitol-glutamate (MG) medium at 28°C (Keane et al. 1970) or KB (King et al. 1954). *Escherichia coli* strains were maintained on Luria-Bertani medium (Sambrook et al. 1989) at 37°C. Antibiotics

used for selection of *P. syringae* strains included rifampicin at 100 µg/ml, kanamycin at 25 µg/ml, spectinomycin at 10 or 25 µg/ml, and chloramphenicol at 25 µg/ml. Antibiotics used for selection of *E. coli* included ampicillin at 100 µg/ml, kanamycin at 10 or 25 µg/ml, chloramphenicol at 25 µg/ml, and spectinomycin at 25 µg/ml. Plasmids were introduced into *P. syringae* recipients by electroporation (Sambrook et al. 1989) or by triparental matings using pRK2013 (Figurski and Helinski 1979).

In COR extraction and gene expression experiments, *P. syringae* derivatives were grown in HSS (Penaloza-Vazquez et al. 2000). *P. syringae* pv. *tomato* DC3000 and AS1 were grown in HSS or HDM (Huynh et al. 1989) for RT-qPCR studies comparing the induction of selected *cor* genes (*cfl*, *cmaB*, and *corR*) and *hrpL* expression. For RT-qPCR studies, *P. syringae* pv. *tomato* DC3000 was grown for 24 h on KB media and then transferred to either HSS or HDM broth and incubated at 18°C for 0, 2, 4, 6, or 12 h. Induction of *corR*, *cfl*, *cma*, and *hrpL* expression in *P. syringae* pv. *tomato* DC3000 was analyzed using RT-qPCR as described below.

DNA manipulation and sequencing.

Routine DNA manipulations and plasmid isolations were performed as described (Sambrook et al. 1989). *P. syringae* pv. *tomato* DC3000 genomic DNA was prepared using the Wizard Genomic DNA Purification kit (Promega Corp., Madison, WI, U.S.A.). DNA fragments were isolated from agarose gels by electroelution and labeled with [α -³²P]dCTP using the Rad Prime DNA Labeling System (Gibco-BRL, Gaithersburg, MD, U.S.A.). Hybridizations and posthybridization washes were conducted under high-stringency conditions (Sambrook et al. 1989).

Mutant construction.

A *corR* mutant was constructed using the Km^R cassette in pBSL14 (Alexeyev 1995). A fragment containing *corR* was amplified from construct pH6 using the forward primer 5'

Table 3. Bacterial strains and vectors used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source
<i>Pseudomonas syringae</i> pv. <i>tomato</i>		
DC3000	Derivative of NCPPB1106; Rif ^r	Cuppels 1986
DB4G3	Rif ^r Km ^r ; <i>cfa6</i> ::Tn5 <i>uidA</i> ; CFA ⁻ CMA ⁺ COR ⁻	Brooks et al. 2004
AK7E2	Rif ^r Sm/Sp ^r ; <i>cmaA</i> ::Tn5 <i>uidA</i> ; CFA ⁺ CMA ⁻ COR ⁻	Brooks et al. 2004
AS1	Rif ^r Km ^r ; DC3000 containing Km ^R cassette in <i>corR</i>	This study
VJ202	Rif ^r Sm/Sp ^r ; <i>hrpL</i> :: Ω	Zwiesler-Vollick et al. 2002
<i>Escherichia coli</i>		
DH5 α	<i>recA lacZAM15</i>	Bethesda Research Labs
SM10	λ - <i>pir</i>	Miller and Mekalanos 1988
Plasmids		
pUC119	Ap ^r ; cloning vector	Vieira and Messing 1987
pBSL14	Ap ^r Km ^r , contains 1.2-kb Km ^r cassette	Alexeyev 1995
pBluescript SK	Ap ^r ; cloning vector	Stratagene
pBBR1MCS	Cm ^r ; 4.7-kb broad-host-range cloning vector	Kovach et al. 1994
pVSP61	Km ^r , pVS1 replicon	Loper and Lindow 1994
pRK2013	Km ^r Tra ⁺ , helper plasmid for triparental matings	Figurski and Helinski 1979
pJP5603	Km ^r ; R6K-based mobilizable suicide vector	Penfold and Pemberton 1992
pCR2.1	Ap ^r Km ^r ; 3.9-kb cloning vector	Invitrogen
pH6	Tc ^r ; 35-kb genomic fragment from <i>P. syringae</i> pv. <i>tomato</i> DC3000 with CMA and regulatory genes; in pRK7813	C. L. Bender
pAS1	Ap ^r ; contains <i>corR</i> as a 1.214-kb <i>XbaI</i> / <i>EcoRI</i> fragment in pUC119	This study
pAS1.Km	Km ^r Ap ^r ; pAS1 with <i>nptII</i> cassette from pBSL14 in <i>Bam</i> HI site of <i>corR</i>	This study
pAS2	Ap ^r ; contains <i>corR</i> as a 2-kb <i>EcoRI</i> fragment in pBluescript SK	This study
pAS2.1	Cm ^r ; contains <i>corR</i> as a 2-kb <i>HindIII</i> / <i>XbaI</i> fragment in pBBR1MCS	This study
pCPP2385	Ap ^r Sp ^r Sm ^r ; contains <i>hrpL</i> overproduced in pUCP18	Preston et al. 1998
pAP06.415	Ap ^r Tc ^r ; contains <i>malE::corR</i> in pRK415	Penaloza-Vazquez et al. 1996
pHrpL	Km ^r , contains <i>hrpL</i> in pVSP61	B. N. Kunkel

^a Rif^r, Km^r, Sm^r, Ap^r, Tc^r, Cm^r, and Sp^r indicate resistance to rifampicin, kanamycin, streptomycin, ampicillin, tetracycline, chloramphenicol, and spectinomycin, respectively; CFA = coronafacic acid, CMA = coronamic acid, and COR = coronatine.

GGAATTCGCGCCCGACAATTCCCTCTA (*Eco*RI site is underscored) and the reverse primer 5' GCTCTAGACTTAA CCCGCCAGCGTCCAG (*Xba*I site is underscored). The 1.2-kb fragment was subcloned in pUC119, resulting in pAS1. A 1.2-kb *Bam*HI-derived Km^r cassette from pBSL14 (Alexeyev 1995) then was inserted into a unique *Bam*HI site that maps within *corR*, resulting in pAS1.Km. Construct pAS1.Km was electroporated into *P. syringae* pv. *tomato* DC3000, and the resulting colonies were selected for insertion of Km^r into *corR* and screened for loss of ampicillin resistance (marker on vector) by replica plating. Insertion of the Km^r cassette into *corR* was verified by PCR analysis using the primer set that was utilized for construction of the mutant. The *corR* mutant selected for further study was designated AS1.

Complementation studies.

corR was subcloned from *P. syringae* pv. *tomato* DC3000 and introduced into AS1 (*corR* mutant) to restore COR production. A 1,562-bp fragment containing the entire *corR* gene along with *holPtoAA* was amplified from wild-type *P. syringae* pv. *tomato* DC3000 using the forward primer 5' ACCTTCCCTAACC GAACCAC and the reverse primer 5' GGGCAATTCGCGACAGTATT. The amplified *corR* gene initially was cloned in pCR2.1 and then subcloned into a unique *Eco*RI site in pBluescript SK+, resulting in pAS2. The construct was digested with *Hind*III and *Xba*I to liberate the insert and subcloned into pBBR1MCS, resulting in pAS2.1, which contains *corR*. pAS2.1 was used to electroporate the *corR* mutant, resulting in AS1(pAS2.1). The resulting colonies were selected for chloramphenicol resistance, confirmed as transformants by plasmid isolation, and analyzed for COR production as described previously (Brooks et al. 2004).

Construction of the *P. syringae* pv. *tomato* DC3000 *hrpL* mutant, VJ202, was described previously (Zwiesler-Vollick et al. 2002). VJ202 was unable to cause disease on *A. thaliana* and also failed to elicit an HR on tobacco (*N. tabacum*) (Zwiesler-Vollick et al. 2002). An 842-bp fragment containing *hrpL* was amplified from *P. syringae* pv. *tomato* DC3000 genomic DNA using the forward primer 5' CGGAATTCGTTGCCATCCGAG AGTGAGC and the reverse primer 5' CGGAATTCGTTCCCT TGCGAAGCTGACC, which both contain *Eco*RI sites (underscored). The amplified product was cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Gaithersburg, MD, U.S.A.), subcloned into the *Eco*RI site of pVSP61, and then mobilized into VJ202, which then was analyzed for its ability to induce symptoms on *A. thaliana*, an HR in tobacco, and for COR production.

A construct overproducing *hrpL*, which was designated pCPP2385 (Table 3), was electroporated into *P. syringae* pv. *tomato* DC3000, and the resulting transformant was assessed for COR production.

Cotranscription of *holPtoAA* and *corR* in *P. syringae* pv. *tomato* DC3000.

P. syringae pv. *tomato* DC3000 cells (OD₆₀₀ = 0.1) were grown in HSS medium at 18°C for 2 h and total RNA was extracted. cDNA was synthesized using random hexamers (Superscript First Strand Synthesis System; Invitrogen) as described below. Primers spanning from 30 bp downstream of the *holPtoAA* translational start site (5' ATTCCAGCTTCAATTAC ACGG) to 52 bp upstream of the *corR* stop site (5' CAGCAA CCCGAGCACTTG) were designed to amplify an 821-bp fragment, from the cDNA template. RT-PCR was performed to determine whether *holPtoAA* and *corR* were cotranscribed. A negative control reaction consisted of total RNA isolated from *P. syringae* pv. *tomato* DC3000 as template without the addition of reverse transcriptase.

Plant material and inoculation procedures.

Tomato (*Lycopersicon esculentum* cv. Glamour) plants were grown from seed and maintained in growth chambers as described previously (Penaloza-Vazquez et al. 2000). To assay for disease, derivatives of *P. syringae* pv. *tomato* DC3000 were grown at 28°C for 48 h on MG agar supplemented with the appropriate antibiotics, and cells were suspended to an OD₆₀₀ = 0.1 (approximately 10⁸ CFU/ml) in sterile distilled water. Tomato plants (4 to 5 weeks old) were spray inoculated with an airbrush (55.2 kPa) until leaf surfaces were uniformly wet and maintained in growth chambers as described previously (Penaloza-Vazquez et al. 2000).

Growth of *P. syringae* pv. *tomato* DC3000 (wild type) and the *cfa6* (DB4G3), *corR* (AS1), and *hrpL* (VJ202) mutants was monitored after spray inoculation. Tomato leaves were sampled 0, 1, 3, 7, and 10 days after inoculation to determine total populations. Leaf tissue was macerated using a mortar and pestle, and serial dilutions were plated on media containing the appropriate antibiotics.

The leaves were graded on a scale of 0 to 100 depending on the percentage of the leaf infected, 100 being the maximum disease incidence. It is important to note that both necrotic and chlorotic lesions were considered while grading the leaves for infection. The complemented mutants, AS1(pAS2.1) and VJ202 (pHrpL), also were monitored for the restoration of growth, symptom manifestation, and percent infection on leaves.

P. syringae pv. *tomato* DC3000 and the *corR* (AS1) and *hrpL* (VJ202) mutants also were tested for their ability to induce an HR on leaves of *N. benthamiana* plants (4 to 5 weeks old) using established methods (Schaad 1988). The cultures were infiltrated into tobacco leaves at OD₆₀₀ = 0.1, 0.01, 0.001, and 0.0001. Electrolyte leakage from *N. benthamiana* leaves inoculated with *P. syringae* pv. *tomato* DC3000, the *corR* and *hrpL* mutants (all strains inoculated at OD₆₀₀ = 0.001), and water (negative control) was evaluated as described previously (del Pozo et al. 2004). Twelve leaf disks per plant (0.5 cm in diameter) were removed from the infiltrated areas of each plant 48 h postinfiltration. The leaf disks were floated in 12 ml of distilled water and kept at room temperature with gentle shaking. The conductivity of the solution was measured after 4 h using a dual channel pH/ion conductivity meter (Accumet Research AR50; Fisher Scientific, Pittsburgh). The experiment was performed three times with similar results.

Detection of COR and CFA.

P. syringae derivatives were incubated in HSS at 18°C and culture supernatants were extracted and analyzed for COR and CFA by HPLC as described (Brooks et al. 2004). In all experiments, triplicate cultures were analyzed for COR production, and all experiments were repeated two or more times.

Exogenous feeding and cocultivation studies.

The *cfa6* (DB4G3; CFA⁻), *cmaA* (AK7E2; CMA⁻), *corR* (AS1), and *hrpL* (VJ202) mutants were supplemented with equimolar amounts (7 μM) of CFA or CMA and analyzed for COR as described previously (Brooks et al. 2004). The *corR* and *hrpL* mutants also were cocultivated with either the *cfa6* or *cmaA* mutant in HSS medium at 18°C for 7 days and the organic acids were extracted and analyzed.

RNA isolation and real-time qPCR.

P. syringae pv. *tomato* DC3000 and the *corR* and *hrpL* mutants were grown for 24 h on KB media, washed twice with sterile distilled water, and inoculated to either HDM or HSS medium (OD₆₀₀ = 0.1). The strains were incubated at 18°C for 0, 2, 4, 6, or 12 h, and bacterial cells were collected by centrifugation. The cells were suspended in 500 μl of Tris-EDTA

buffer, and total RNA was isolated as described by Maniatis and associates (1989).

The quality of RNA was analyzed by gel electrophoresis and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.). The extracted RNA was contaminated with small amounts of genomic DNA, which could be coamplified; therefore, the RNA sample was treated with 1 μ l of amplification grade DNase I (Invitrogen). DNase I was inactivated by adding 1 μ l of 25 mM EDTA and heating at 65°C for 10 min. RNA samples then were frozen at -70°C until needed. Total RNA (1 μ g) was used to synthesize the cDNA template using random hexamers with the Superscript First Strand Synthesis System (Invitrogen). A negative control (RNA without the RT enzyme) was included to eliminate any possible genomic DNA contamination. The cDNA then was quantified using the NanoDrop ND-1000 spectrophotometer and diluted with water to yield 150 ng of cDNA. The relative-fold induction of *cfl* expression (*cfl* maps at the 5' end of CFA gene cluster) (Fig. 1C), *cmaB* (located within the CMA gene cluster) (Fig. 1C), *corR*, and *hrpL* then was evaluated using RT-qPCR.

One of the primers used to amplify each gene was labeled with a fluorophore, and both the labeled and unlabeled primers were designed using LUX software (Invitrogen). All primers used for RT-qPCR were supplied by Invitrogen and were designed to amplify 70- to 100-bp fragments. With the exception of *rpoD*, all primers were labeled with the fluorophore JOE (6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein). The LUX primer for *rpoD* was labeled with FAM (5- or 6-carboxyfluorescein).

Amplification reactions were conducted using the HotStar Taq Master Mix Kit (250 U Kit; Qiagen Inc., Valencia, CA, U.S.A.) and contained HotStar Taq DNA Polymerase (250 U), MgCl₂ (final reaction concentration of 1.5 mM), and 200 μ M each dNTP. The total reaction volume for RT-qPCR was 25 μ l and contained 5 μ M each gene-specific primers (1 μ l), 12.5 μ l of the HotStar Taq Master Mix, and 600 ng of the cDNA template (4 μ l). The reactions were incubated at 95°C for 15 min, and then cycled (40 times) at 95°C for 15 s, 60°C for 34 s (with optics on), and 72°C for 34 s. Reactions were conducted in a 96-well spectrofluorometric thermal cycler (ABI PRISM 7700 Sequence Detector System; Applied Biosystems, Foster City, CA, U.S.A.). The amount of fluorescence as a function of PCR cycle was plotted using the ABI PRISM 7700 SDS software, and the threshold cycle (Ct) values for the genes *rpoD*, *corR*, *cfl*, *cmaB*, and *hrpL* were obtained 0, 2, 4, 6, and 12 h after transfer from KB to HSS or HDM. The Ct value for *rpoD* (an endogenous reference gene whose expression remained constant under the experimental conditions designed for the target gene) was used as the normalizer and subtracted from the Ct values of the target genes, thereby obtaining the Δ Ct values. Relative quantification of gene expression was evaluated using the comparative Ct method as described previously (Pfaffl 2001). The induction of target gene expression was denoted as the relative-fold increase in the transcript level with respect to the gene transcript level expression at time zero. Thus, the target gene expression at time zero was used as an arbitrary constant (calibrator) to subtract from all other Δ Ct mean values, resulting in the calculation of $\Delta\Delta$ Ct values. Fold changes in gene expression were calculated from the $\Delta\Delta$ Ct values using the formula $2^{-\Delta\Delta Ct}$, according to the $\Delta\Delta$ Ct mathematical model (Pfaffl 2001).

Gel retardation experiments.

To facilitate end-labeling with [α -³²P] dCTP and [α -³²P] dGTP, DNA fragments used for gel retardation were excised with enzymes that generate 5' overhanging ends. A 758-bp

fragment containing the *hrpL* upstream region (beginning 560 bp downstream of the *hrpJ* translational start and ending 51 bp downstream of *hrpL* start codon) was amplified from *P. syringae* pv. *tomato* DC3000 genomic DNA using the forward primer 5' GCTGTTTGAGCTGCTGTGTC and the reverse primer 5' GAAACATGGGCTTACCCTGA. The amplified product was cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) and the construct was sequenced to confirm the cloned insert and digested with *Hind*III and *Xba*I to generate fragments with 5' overhanging ends. This fragment then was separated on 5% polyacrylamide gel and end-labeled with [α -³²P] dCTP and [α -³²P] dGTP (Sambrook et al. 1989).

Plasmid pAP06.415 containing the *malE::corR* translational fusion (Penaloza-Vazquez et al. 1996) was isolated from *E. coli* DH5 α using standard procedures (Sambrook et al. 1989). This plasmid then was introduced into *P. syringae* pv. *tomato* DC3000 by electroporation and transformants were selected on MG medium containing tetracycline. The MBP-CorR fusion protein, DC3000(pAP06.415), was overproduced and purified from *P. syringae* pv. *tomato* DC3000 by affinity chromatography as described previously (Penaloza-Vazquez and Bender 1998).

The concentration of MBP-CorR used in the gel shift assay was evaluated as described (Penaloza-Vazquez and Bender 1998), and gel retardation assays were performed by incubating 16 ng of MBP-CorR with 3,000 cpm of end-labeled DNA probe in binding buffer (Penaloza-Vazquez and Bender 1998; Wang et al. 1999). The conditions for running the gels and analyzing the results have been reported (Penaloza-Vazquez and Bender 1998).

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