REGULATION OF CORONATINE BIOSYNTHESIS IN *PSEUDOMONAS SYRINGAE* PV. *TOMATO* DC3000

By

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DC3000

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CHAPTER I

Introduction

Pseudomonas syringae is an important pathogen infecting a wide range of crops and is divided into various pathovars depending on its host range. Two of the most extensively studied pathovars include *P. syringae* pv. *glycinea* strain PG4180 and *P. syringae* pv. *tomato* strain DC3000, which cause bacterial speck of tomato and bacterial blight of soybean, respectively. Various factors including the type III secretory system and the phytotoxin coronatine (COR) are required for the virulence of *P. syringae* pv. *tomato* and *P. syringae* pv. *glycinea* (Bender *et al.,* 1999; Bender and Scholz-Schroeder, 2004; Jin *et al*., 2003). COR is a non-host specific phytotoxin that consists of two distinct moieties, the polyketide coronafacic acid (CFA), and an ethylcyclopropyl amino acid derived from isoleucine, coronamic acid (CMA) (Ichihara *et al.,* 1977; Mitchell, 1991; Parry *et al.,* 1994). CFA and CMA are synthesized by separate pathways and coupled by an amide bond to form COR (Bender *et al.,* 1993; Parry *et al.,* 1994). In PG4180 and DC3000, the genes encoding for CFA and CMA biosynthesis are located on either end of the COR gene cluster and are separated by COR regulatory genes

(Bender *et al.,* 1991; Buell *et al.,* 2003; Couch *et al.,* 2004; Moore *et al.,* 1989; Ma *et al.,* 1991). The regulatory region in DC3000 and PG4180 consists of three genes, *corS*, *corR* and *corP*. In PG4180, COR regulation has been extensively studied and is modulated via a modified two component regulatory system where CorS is the sensor kinase and CorR and CorP act as response regulators (Rangaswamy and Bender, 2000; Ullrich *et al.,* 1995). COR regulation in DC3000, however, is not clearly understood and evidence suggests that there is differential regulation of COR biosynthesis between PG4180 and DC3000 (Bender *et al.,* 1991; Buell *et al.,* 2003; Moore *et al.,* 1989; Ma *et al.,* 1991; Peñaloza-Vázquez and Bender, 1998; Peñaloza-Vázquez *et al.,* 2000; Wang *et al.,* 2002). DC3000 is now considered as a model organism for studying plant-microbe interactions (Buell *et al.,* 2003), and hence, one of the objectives of this study is to determine whether the regulation of COR biosynthesis in DC3000 is modulated by the *corRS* two component regulatory system, which is the case in PG4180.

Various structural and regulatory mutants of DC3000 and PG4180 have been constructed to clearly understand the regulation and biosynthesis of COR. It is important to accurately detect the amount of CFA, CMA and COR produced by these mutants. High-performance liquid chromotography (HPLC) has been widely utilized for the detection of CFA and COR; however, detection of CMA using HPLC is more time consuming and tedious. Although CMA can be detected using HPLC, it requires a different column, extraction method and wavelength for detection than CFA and COR. Sample preparation is also time

consuming since derivatization of CMA with phenylisothiocyanate is required for detection using HPLC. In addition, large culture volumes are required in order to accurately detect and quantify CMA using HPLC. Hence, the development of a more sensitive and faster method for detection of CMA has been addressed in this study.

The type III secretion system enables *P. syringae* to grow on the surface of plants, to cause the hypersensitive response on non-host plants, and to be pathogenic on host plants (He, 1998; Hirano *et al.,* 1999). The type III secretion system is encoded by the *hrp*/*hrc* regulon, and HrpL, which encodes a sigma factor related to the extracellular factor family of alternate sigma factors, is required for the expression of several transcripts in the *hrp* gene cluster (Xiao *et al.,* 1994). Most of the genes that are regulated by HrpL contain a conserved *hrp* box, which is a conserved motif in the promoter region of *hrpL*-dependent genes (Xiao and Hutcheson, 1994; Zwiesler-Vollick *et al.,* 2002). Previous studies have shown that potential cross-talk exists between the *hrp* and the COR systems (Boch *et al.,* 2002; Fouts *et al* 2002; Peñaloza-Vázquez *et al.,* 2000*,* Preston, 1997). The presence of a potential '*hrp* box' upstream of *corR* suggests that the CFA and CMA gene clusters involved in COR biosynthesis may be indirectly regulated by the *hrp* genes (Boch *et al.,* 2002; Buell *et al*., 2003). The genetic basis for the interaction between the *hrp* and the COR gene clusters is not clearly understood and is another objective of the present study.

CHAPTER II

Literature Review

P. syringae is a gram-negative plant pathogenic bacterium that causes a wide variety of symptoms on plants, including blights, galls and leaf spots (Alfano and Collmer, 1996). *P. syringae* is divided into various pathovars (pv.) based on its host range. Two important pathovars of *P. syringae*, pvs. *glycinea* and *tomato*, cause bacterial blight of soybean and bacterial speck of tomato, respectively, and have been extensively studied.

The genetic basis for the pathogenicity and virulence of *P. syringae* includes various factors, including phytotoxins (eg: coronatine) (Bender *et al.*, 1999; Bender and Scholz-Schroeder, 2004), the type III secretion system (*hrp/hrc* cluster) (Jin *et al.*, 2003) and global regulatory proteins such as GacA and GacS (Hrabak and Willis, 1992; Rich *et al.*, 1994). Coronatine (COR) is a phytotoxin that is required for the virulence of certain pathovars of *P. syringae*, such as *maculicola*, *glycinea*, *morsprunorum* and *tomato* (Mitchell, 1982; Bender *et al.*, 1987; Brooks *et al.*, 2004; Wiebe and Campbell, 1993). The type III secretion system enables *P. syringae* to grow on the surface of plants, to cause

the hypersensitive response on non-host plants, and to be pathogenic on host plants (He, 1998; Hirano *et al.*, 1999). The GacA and GacS proteins are members of a two-component regulatory system, where GacA is a response regulator and GacS is the sensor kinase. GacS presumably senses environmental signals, is autophosphorylated at a conserved histidine residue, and phosphorylates GacA, which then activates the transcription of target genes (Heeb and Haas, 2001; Rich *et al.*, 1994).

CORONATINE

Structure and biosynthesis of COR. COR is a chlorosis-inducing, non-host-specific phytotoxin produced by some strains of *P. syringae*. COR consists of two distinct moieties, the polyketide coronafacic acid (CFA) and an ethylcyclopropyl amino acid derived from isoleucine, coronamic acid (CMA) (Ichihara *et al.*, 1977; Mitchell, 1991; Parry *et al.*, 1994).

CFA is synthesized by the polyketide pathway from one unit of pyruvate, one unit of butyrate and three acetate residues (Parry *et al.*, 1994). Studies have shown that the pyruvate is converted to α-ketoglutarate, followed by the loss of C-1, resulting in succinic semialdehyde (SSA). SSA is presumably converted into its CoA ester, which is then used as a starter for CFA synthesis (Parry *et al.*, 1996; Jiralerspong *et al*, 2001; Bender and Scholz-Schroeder, 2004). CMA is an ethylcyclopropyl amino acid derived from isoleucine (Ichihara *et al.*, 1977). L-isoleucine is first converted to L-alloisoleucine and then cyclized to form CMA

(Parry *et al.*, 1991; 1994). The formation of the cyclopropane ring of CMA is catalyzed by a 68-kDa protein, CmaA, which consists of two domains, an adenylation (A) and a thiolation (T) domain (Couch *et al.*, 2004). L-alloisoleucine is activated and linked to CmaA in the form of a thiolester and subsequently cyclized to produce covalently bound CMA. CmaT presumably hydrolyzes the covalently bound CMA to produce free CMA (Couch *et al.*, 2004). L-alloisoleucine, which is biosynthesized by *P. syringae*, is the preferred substrate for CmaA, although small amount of activation was also observed with L-leucine, L-valine and L-isoleucine (Couch *et al*, 2004).

After synthesis via two different pathways, CFA and CMA are coupled by an amide bond to form COR (Bender *et al.*, 1993; Parry *et al.*, 1994). The enzyme(s) involved in the coupling reaction lacks rigid specificity for the amino acid substrate (Fig. 1). Hence, in addition to COR, various other CFA-amino acid complexes including coronafacoylisoleucine, coronafacoylalloisoleucine, and coronafacoylvaline are also synthesized (Mitchell and Young, 1985; Mitchell, 1985, 1991; Mitchell and Ford, 1998). Among the analogues, COR is the most toxic coronafacoyl compound made by COR-producing organisms.

Mode of action of COR. COR is a non-host-specific phytotoxin that elicits diffuse chlorosis and hypertrophy, inhibits root elongation and stimulates ethylene production in a wide variety of plants (Sakai *et al.*, 1979; Volksch *et al.*, 1989; Kenyon and Turner, 1992). COR acts as a virulence factor in some *P. syringae* pathovars including *glycinea*, *tomato* and *maculicola*, in which it results in increased lesion size and bacterial multiplication (Gnanamanickam *et al.*,

1982; Bender *et al.*, 1987; Tamura *et al.*, 1998). Several studies have shown that COR has a critical role in pathogenesis (Brooks *et al.*, 2004; Mittal and Davis, 1995; Peñaloza-Vázquez *et al.*, 2000; Yao *et al.*, 2002). For example, COR^- mutants of DC3000 were used to dip-inoculate *Arabidopsis* and tomato; the mutants were compromised in their ability to induce disease symptoms *in planta*, and the multiplication of the COR^- mutants was reduced when compared to the wild-type (Mittal and Davis, 1995; Brooks *et al.*, 2004). The Mittal and Davis study showed that the accumulation of several defense related genes were higher in *Arabidopsis* leaves infiltrated with the COR^- mutant as compared to the wild-type, suggesting that COR production by the wild-type DC3000 suppresses the defense response. Uppalapati *et al.*(2005) studied the effect of COR and related compounds on tomato and demonstrated that COR affects many genes involved in the photosynthetic apparatus. In tomato seedlings, COR also inhibited root growth and induced anthocyanin accumulation. It is important to note that the role of COR in virulence may vary with the host-pathogen interaction.

COR has structural and functional similarities to methyl jasmonate, a derivative of the octadecanoid signaling pathway. Methyl jasmonate is a plant growth hormone produced in a variety of plants in response to biological stress. Various studies have suggested that COR and methyl jasmonate have a similar mode of action (Feys *et al.*, 1994). A *co*ronatine-*i*nsensitive (*coi*) mutant of *Arabidopsis* was insensitive to both COR and methyl jasmonate, which suggests that the two compounds act in a similar manner (Feys *et al.*, 1994). Both COR

and methyl jasmonate induce accumulation of proteinase inhibitors and inhibit root elongation, suggesting that COR functions as a molecular mimic of octadecanoid signaling molecules (Feys *et al.*, 1994; Weiler *et al.*, 1994; Greulich *et al.*, 1995; Koda *et al.*, 1996). However, some changes like cell wall thickening, chlorosis and intense staining of the chloroplasts were observed when tomato tissue was exposed to COR, but not to CFA or methyl jasmonate (Palmer and Bender, 1995; Uppalapati *et al.*, 2005). This suggests that the CMA moiety or the amide linkage between CFA and CMA may be involved in imparting additional biological activities to COR in tomato tissues. Recently, it was shown that both COR and endogenous MeJA modulate genes involved in JA biosynthesis (Uppalapati *et al.*, 2005).

Various studies have been conducted to understand the exact mode of action of COR (Zhao *et al.*, 2003; Block *et al.*, 2005; Uppalapati *et al.*, 2005). Zhao *et al.* (2003) have shown that COR mimics the activity of jasmonate and activates the host jasmonic acid (JA) signaling pathway, thereby increasing bacterial virulence. The JA pathway is responsible for the protection of tomato against herbivores, and Zhao *et al.* (2003) hypothesized that the activation of the JA pathway by COR is a virulence strategy of the pathogen to protect the destruction of its own growth habitat by herbivores. Thus, tomato leaves infected with *P. syringae* are less susceptible to insect feeding because of the induction of the JA pathway, which promotes enhanced resistance of tomato to herbivores.

Studies based on the *coi1* mutant of Arabidopsis show that COR and JA act through a common signaling pathway. Furthermore, an increased level of

salicylic acid (SA) was observed in the *coi1* mutant, which led to the speculation that in addition to being JA dependent, the mode of action of COR may also be SA-dependent (Block *et al*., 2005; Bender and Scholz-Schroeder, 2004). The SA pathway is responsible for resistance of plants to many pathogens including *Pseudomonas* spp. and is mutually antagonistic to the JA pathway (Kunkel and Brooks, 2002). Block *et al.* (2005), however, concluded that the action of COR as a virulence factor is not SA-dependent, although COR may lead to SA suppression by stimulating JA responsive genes. Thus, COR promotes virulence of *Pst* DC3000 by targeting the JA pathway in tomato, with resulting antagonism of the SA pathway (Zhao *et al*., 2003; Bender and Scholz-Schroeder, 2004; Block *et al*., 2005). In addition to modulating genes involved in the JA biosynthetic pathway, COR also targets other phytohormone pathways including ethylene and auxin (Uppalapati *et al.* 2005).

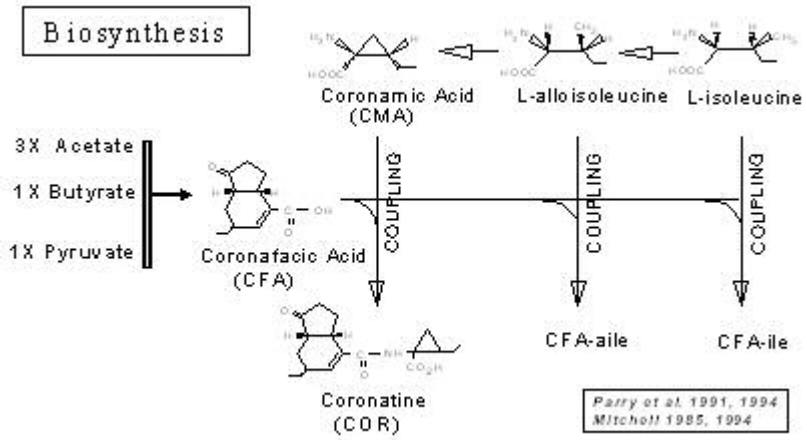

Fig. 1. Coronatine (COR) is synthesized by the coupling of two moieties, coronafacic acid (CFA) and coronamic acid (CMA) via an amide bond. CFA is a polyketide derived from three units of acetate, one unit of pyruvate and one unit of butyrate. CMA, the amino acid component of COR, is derived from isoleucine, which is first converted to alloisoleucine. CFA can also be coupled to L-alloisoleucine (aile) and L-isoleucine (ile) to form coronafacoyl analogues, CFA-aile and CFA-ile respectively.

The COR gene cluster. Research on *P. syringae* pv. *glycinea* (*Psg*) PG4180 has shown that the structural genes for COR synthesis, e.g. the CFA and CMA gene clusters, are separated by a 2.4 kb regulatory region (Fig. 2). Earlier studies have shown that the CMA region consists of four ORFs, *cmaA*, *cmaB*, *cmaT* and *cmaU*, which encode for two transcripts, the *cmaABT* operon and the monocistronic transcript containing *cmaU* (Ullrich and Bender, 1994; Budde *et al.*, 1998; Patel *et al.*, 1998). Sequence analysis has shown that *cmaA* encodes a protein with an amino acid activating domain and has characteristics typical of nonribosomal peptide synthetases (Ullrich and Bender, 1994). Recently, CmaA was shown to have two domains, an adenylation domain (A domain) and a thiolation domain (T domain). Thus, CmaA catalyzes the adenylation of L-*allo*-isoleucine and the attachment of L-*allo*-isoleucine to the CmaA T domain (Couch *et al.*, 2004). *cmaB* shows similarity to *syrB2*, which encodes a gene required for syringomycin synthesis (Budde *et al.*, 1998). Both *cmaB* and *syrB2* show relatedness to enzymes that chlorinate amino acids, and this function suggests that CmaB may chlorinate alloisoleucine, the amino acid precursor to CMA. CmaT is related to thioesterases (Ullrich and Bender, 1994), and this activity was confirmed by overproducing CmaT in *E. coli* (Patel *et al.*, 1998). Although a function for *cmaU* could not be originally deduced from sequence analysis, BLASTX has recently shown that the protein product of *cmaU* has relatedness to threonine efflux proteins. The CMA region of *Psg* PG4180 was recently resequenced, resulting in the identification of at least three previously unrecognized open reading frames, designated as *cmaC*, *cmaD*, and *cmaE*

(Couch *et al.*, 2004). CmaC showed similarity to methylmalonyl coenzyme A mutase and may be involved in the deprotonation and cyclization of CmaA-bound 6-hydroxy-alloisoleucine or 6-chloro-alloisoleucine, resulting in CmaA-bound CMA. However, the roles of CmaD (similar to acyl carrier proteins) and CmaE (similar to proteins with an α/β hydrolase fold) are not yet known.

The CFA gene transcript contains ten discrete ORFs, *cfl* and *cfa1-cfa9* (Fig. 2C) (Bender *et al.*, 1993; Liyanage *et al.*, 1995a, b; Penfold *et al.*, 1996 and Rangaswamy *et al.*, 1998 a, b). Cfl has similarity to acyl CoA ligases, and genetic experiments indicated that the *cfl* gene (1.4 kb) couples CFA and CMA via an amide linkage (Bender *et al.*, 1993); however, this function has not yet been demonstrated *in vitro* using purified Cfl. Other studies suggest that the *cfl* gene may have a role in CFA synthesis (Rangaswamy *et al.*, 1997). Sequence analysis revealed that the translational products of *cfa1*, *cfa2* and *cfa3* showed relatedness to acyl carrier proteins (ACP), fatty acid dehydratase (DH) and β-ketoacyl synthase (KS) (Penfold *et al.*, 1996). Collectively, these similarities suggest that the CFA biosynthetic region contains monofunctional polyketide synthase proteins (designated type II PKS). Although the function of *cfa4* could not be determined from database searches, *cfa5*, like *cfl*, shows similarity to acyl-CoA ligases (Penfold *et al.*, 1996). Sequence analysis of *cfa6* (8.2 kb) and *cfa7* (6.1 kb) revealed similarity to multifunctional polyketide synthases (type I PKS), such as 6-deoxyerythronolide B synthase (Rangaswamy *et al.*, 1998a). The type I PKSs are large proteins that contain domains for ACP, KS, DH, and other functions of polyketide synthesis. Thus, CFA biosynthesis requires a unique

combination of both mono- and multifunctional PKS proteins. The two remaining genes in the CFA cluster, *cfa8*and *cfa9,* show relatedness to crotonyl-CoA reductases and thioesterases, respectively (Rangaswamy *et al*., 1998a).

The regulatory region separating the CFA and CMA regions consists of three genes, *corS*, *corR* and *corP* (Fig. 2A). CorR and CorP show similarity to response regulators (RR), and the translational product of *corS* is related to histidine protein kinases (HPK). Both RR and HPKs function in two-component regulatory systems (Ullrich *et al*., 1995).

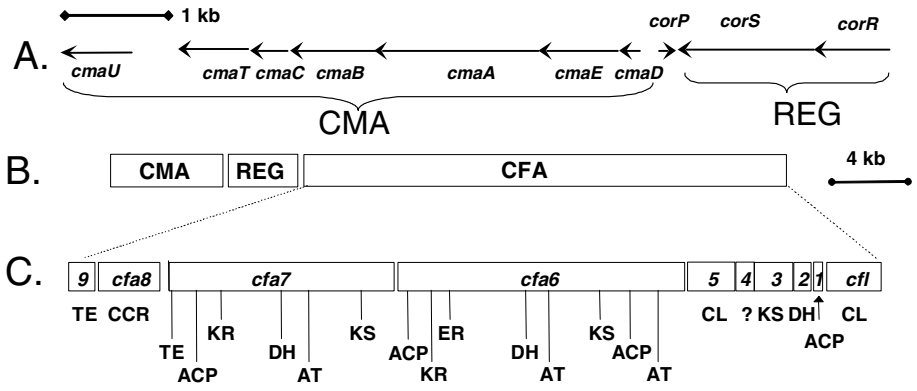

Fig. 2. The coronatine gene cluster in *P. syringae* pv. *glycinea* PG4180. **A.** Organization of the coronamic acid (CMA) gene cluster in PG4180 and the regulatory (REG) region consisting of *corR*, *corP* and *corS*. Arrows represent the direction of transcription. **B.** Functional regions of the COR gene cluster in PG4180. The CFA and CMA regions are located on either ends of the regulatory region. **C.** Expanded view of the CFA region, which consists of ten ORFs, *cfa1-cfa9* and *cfl*. Vertical lines show the catalytic domains in Cfa6 and Cfa7.

The COR regulatory system is slightly different from 'typical' two-component regulatory system in that there are two response regulators (CorR and CorP) and a single sensor (CorS). 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). However, CorP lacks the H-T-H motif. *corS* and *corR* are transcribed in the same direction, whereas *corP* is transcribed in the opposite direction. CorR functions as a positive regulator of COR gene expression by binding to the promoter regions of the *cfl- cfa* and *cmaABT* transcripts (Peñaloza-Vázquez and Bender, 1998; Wang *et al.*, 1999). This was demonstrated by using CorR in gel retardation and DNaseI footprinting assays (Peñaloza-Vázquez and Bender, 1998; Wang *et al.*, 1999). The actual role of CorP in COR synthesis is unknown, though various mechanisms of action have been suggested (Ullrich et al., 1995). The proposed mechanisms of COR regulation by CorP include a) modulating the phosphorylation and dephosphorylation of CorS b) directing the phosphorylation signal from CorS to CorR by acting as a relay C) forming a heterodimer with CorR. Rangaswamy and Bender (2000) evaluated the role of CorP as a phosphoacceptor and did not detect phosphorylation of CorP even after prolonged incubation with CorS~P. CorS, the histidine protein kinase, is autophosphorylated by [γ-^{32}P] ATP, presumably at the conserved histidine residue that functions as a phosphorylation site in other HPKs (Rangaswamy and Bender, 2000). The transphosphorylation of CorR by CorS~P was observed within 5 s, which suggests that CorR may be the cognate response regulator of CorS. A model for the regulation of COR production in *Psg* PG4180 is shown in Fig. 3.

Regulation of COR biosynthesis. Studies have shown that COR biosynthesis in *Psg* PG4180 is thermoregulated (Palmer and Bender, 1993; Rohde *et al.*, 1998, Weingart *et al.*, 2004). COR, CFA and CMA production were highest when

PG4180 was grown at 18°C, whereas negligible amounts were produced at 28°C. However, the growth of *Psg* PG4180 was not significantly different at these two temperatures. Recently, the transmembrane region of CorS was shown to be responsible for thermosensitivity in PG4180 (Smirnova and Ullrich, 2004). Similarly, COR production in *Pst* DC3000 is also regulated by temperature (Rhode *et al.*, 1998); when DC3000 was incubated at 18°C, expression of a CMA transcriptional fusion was four-fold higher as compared to expression at 28°C. According to Weingart *et al.* (2004), COR production in *Pst* DC3000 is only slightly affected by temperature, and when grown *in vitro*, the yield of COR was only 1.5-fold lower at 28°C than at 18°C.

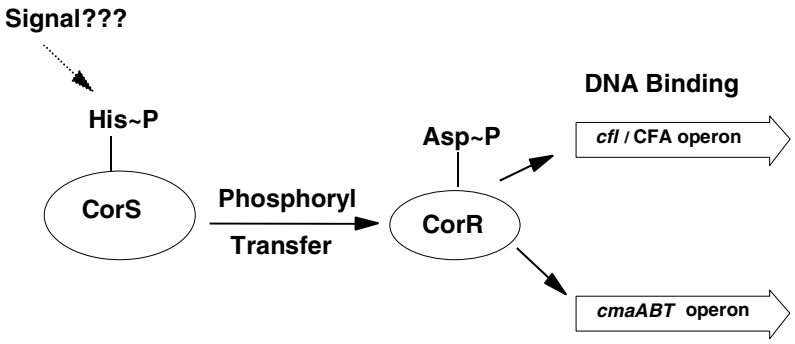

Fig. 3. Regulation of COR biosynthesis in *Psg* PG4180. The regulatory genes, *corR* and *corP* show similarity to response regulators, and *corS* shows similarity to histidine protein kinases, which function as environmental sensors in two-component regulatory systems. CorS gets autophosphorylated at the histidine residue in response to an unknown signal and CorR functions as a positive regulator of COR biosynthesis by binding to the promoter regions of the *cfl cfa* and *cmaABT* regions.

Studies have shown that COR biosynthesis in *Pst* DC3000 is plant-inducible (Li *et al.*, 1998). A study conducted by inoculating several host plants with *Pst* DC3000 revealed that among various hosts, cabbage triggered the maximum expression of COR genes (Li *et al.*, 1998). Malic, citric, shikimic and

quinic acids were identified as compounds that stimulate COR production (Li *et al.*, 1998). Furthermore, transcriptional fusion of DC3000 *cma* promoter region to a promoterless *egfp* (*cma*::*egfp*) showed higher levels of fluorescence in cells recovered from infected host plants when compared to cells grown in minimal medium (Weingart *et al.*, 2004).

Interestingly, COR production in *Psg* PG4180 is not induced by the plant host (Palmer and Bender, unpublished). The addition of extracts from soybean tissue or the supplementation of the growth medium with plant-derived secondary metabolites did not increase COR production by PG4180 *in vitro*. In addition, comparison of the transcriptional activity of CMA and CFA gene fusions failed to reveal any evidence that COR gene expression of PG4180 was higher in plant tissue when compared to expression *in vitro* (Palmer and Bender, unpublished). Moreover, a transcriptional fusion of the PG4180 *cma* promoter to promoterless *egfp* (*cma*::*egfp*) showed similar levels of fluorescence *in planta* and in minimal medium, indicating that the *cma* promoter is not plant-inducible in *Psg* PG4180 (Weingart *et al.*, 2004).

Comparison of the COR gene clusters of DC3000 and PG4180. The arrangement of the COR genes is not identical in all pathovars of *P. syringae*. In *Psg* PG4180, the genes for COR biosynthesis are located on a 90-kb plasmid designated p4180A (Bender *et al.*, 1991). The CMA and CFA biosynthetic genes are located on either ends of the cluster and are separated by a 2.4-kb regulatory region in PG4180 (Couch *et al.*, 2004). However, in *Pst* DC3000, the COR gene cluster is chromosomally-encoded (Moore *et al.*, 1989; Ma *et al.*, 1991). In

DC3000, the CMA and regulatory genes (*corRPS*) are clustered together and are physically separated from the CFA genes by a 26-kb region (Buell *et al.*, 2003) (Fig. 4). The spatial separation of the CMA/REG and CFA gene clusters may indicate that the COR genes in *Pst* DC3000 were acquired by the genome at different times and by different mechanisms. It is also important to mention that the physical separation of the regulatory region in *Pst* DC3000 from the CFA genes may indicate that CFA genes in DC3000 are not controlled by *corRPS*; this is one question that will be addressed in the proposed work.

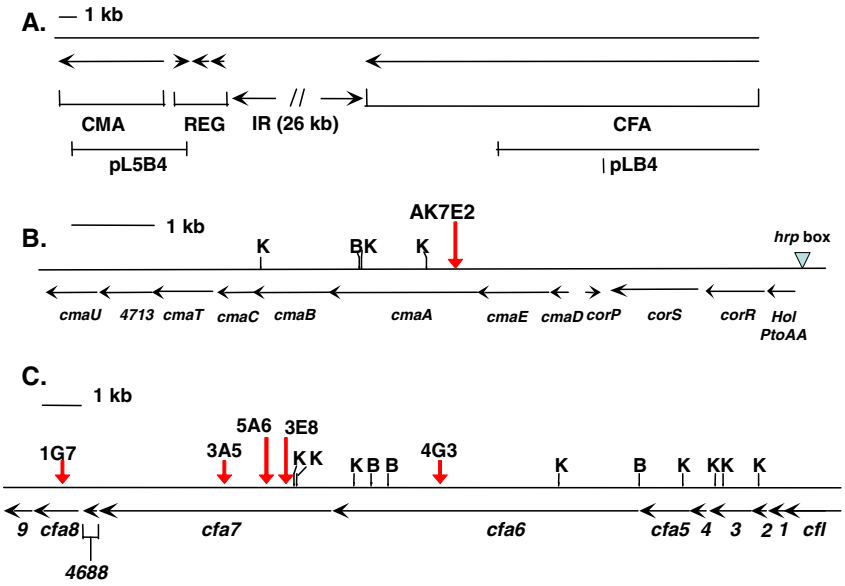

Fig. 4. The COR biosynthetic region of *Pst* DC3000. **A,** The entire COR biosynthetic region, including the 26.4 kb intergenic region (IR). Abbreviations: CMA, region required for synthesis of coronamic acid; REG, putative regulatory region consisting of *corP*, *corR*, and *corS*; CFA, region required for synthesis of coronafacic acid. Cosmids pLB4 and pL5B4 contain genomic DNA from the CFA and CMA regions, respectively, and are indicated by the solid lines (see methods). The dashed lines indicate that the extent of the inserts beyond the CMA/REG and CFA operons has not been precisely mapped in these cosmids. **B,** The CMA and regulatory (REG) region. CMA region contains *cmaD*, *cmaE*, *cmaA*, *cmaB, cmaC, cmaT* and *cmaU* genes (Couch *et al.*, 2004). The *corR* and *corS* genes in the regulatory region are transcribed in the same direction, whereas *corP* is transcribed in the opposite direction. A type III effector protein *(*encoded by *holPtoAA)* is present upstream of the *corR* gene. A 'hrp' box is also present upstream of *holPtoAA*. **C,** The CFA biosynthetic region. A predicted new gene (designated *4688*), maps within the CFA region. Red vertical arrows (panels B and C) indicate the transposon insertions characterized for COR production, and the strain designation used for each insertion mutant is indicated above the arrow. Abbreviations: K, *Kpn*I and B, *Bam*HI.

Pst DC3000 also differs from PG4180 in the amount of COR produced *in vitro*. For example, PG4180 produces 40-50 μg COR/mg protein *in vitro*, whereas DC3000 produces 1-2 μg COR/mg protein (Peñaloza-Vázquez *et al.*, 2000). This is likely due to the fact that the 'host signal' is not present in the growth medium, and consequently, *Pst* DC3000 synthesizes far less COR *in vitro* (Li *et al.*, 1998). Interestingly, serological assays using a COR-specific monoclonal antibody indicated that both PG4180 and DC3000 synthesize similar amounts of COR *in planta* (Zhao *et al.*, 2001).

Evidence suggests that there is differential regulation of COR biosynthesis in PG4180 and *Pst* DC3000. To investigate this hypothesis, the *cfl* promoter regions from PG4180 and DC3000 were fused with the *uidA* gene, which encodes glucuronidase (GUS), and the strains were incubated at 21^{o}C in COR-inducing media (Wang *et al.*, 2002). The kinetics of gene expression was clearly different in the two strains; for example, the *cfl* promoter in *Pst* DC3000 was activated faster (within 6 h) than the *cfl* promoter in PG4180 (activity barely detectable at 12 h). In DC3000, *cfl* transcriptional activity showed maximal expression prior to symptom expression in tomato and collard (Wang *et al.*, 2002). However, in soybeans inoculated with PG4180, *cfl* promoter activity continued to increase as the bacterial population multiplied in the plant and was maximal when visible chlorosis became apparent (Wang *et al.*, 2002).

It is also important to mention that the *cfl* promoter region differs in PG4180 and DC3000. In PG4180, the *cfl* promoter contains a CorR-binding site, which is absent from the *cfl* upstream region of DC3000 (Peñaloza-Vázquez and

Bender, 1998; Wang *et al*., 2002). The absence of a CorR-binding site in the *cfl* promoter of *Pst* DC3000 may indicate that the *cfl-cfa* transcript does not require CorR for transcriptional activation. This will be investigated in the proposed work.

COR biosynthesis and regulation in *Pst* DC3000 is not fully understood, although these processes have been much better described in PG4180. A better understanding of COR biosynthesis and regulation in DC3000 is now justified as this strain has become a model organism for studying plant-microbe interactions, largely because of its genetic tractability, pathogenicity on *Arabidopsis*, and the availability of its genomic sequence (Buell *et al*., 2003).

Detection of COR and COR-producing *P. syringae.* High-performance liquid chromatography (HPLC) is an analytical method that is widely used to detect and quantify COR, CFA, and CFA-amide conjugates (Palmer and Bender, 1993). In addition, monoclonal antibodies have also been used to detect COR *in vitro* (Jones *et al*., 1999; 2001). A modified form of an indirect competitive ELISA, which is much more sensitive than detection by HPLC, can be used to detect very low amounts (5-40 ng/ml) of COR in plants (Zhao *et al*., 2001). However, it is important to note that the monoclonal antisera cannot distinguish between COR and CFA-amide conjugates (Jones *et al*., 2001; Zhao *et al*., 2001). Besides using HPLC and monoclonal antibodies for the detection of COR *in vitro* and *in vivo* respectively, the COR-producing strains of *P. syringae* can be identified using PCR. Healthy and diseased leaf extracts of tomato, turnip and collard were used for PCR amplification using the *cfl* primers. An expected 0.65-kb PCR product was observed for the diseased samples, whereas no PCR product was

observed for the healthy samples, facilitating identification of COR producing strains of *P. syringae* (Zhao *et al.*, 2002).

In addition to COR and CFA, CMA can also be detected by reverse phase HPLC, using a C-18 reverse-phase column. However, it is important to note that the three compounds cannot be detected simultaneously, and require different extraction methods, different columns, and different wavelengths for detection. Also, detection of CMA by reverse-phase HPLC requires a derivatization with phenylisothiocyanate. Although HPLC can be used to detect low levels of CMA (10 picomoles), large culture volumes of *Psg* (600 ml) are needed for accurate detection. Hence, a more sensitive method is required to detect the extremely low levels of CMA produced by DC3000. This is another objective of the proposed work.

THE TYPE III SECRETION SYSTEM

Another factor that is important in the pathogenicity of *P. syringae* pv. *tomato* is the type III secretion system (TTSS), which is encoded by the *hrp*/*hrc* regulon. Some of the genes encoding for the TTSS system are highly conserved and are hence called the *hrc* genes (for *h*ypersensitive *r*esponse and *c*onserved) (Bogdanove *et al.*, 1996). Other genes encoding the TTSS are designated *hrp* (for *h*ypersensitive *r*esponse and *p*athogenicity) (He, 1998); however, the *hrp* genes are not rigidly conserved among phytopathogenic bacteria. In *P. syringae*, the *hrp* genes encode the structural and regulatory proteins associated with the TTSS. Functions for some of the genes in the *hrp*/*hrc* cluster have been

described. For example, *hrcC* plays an important role in protein translocation across the outer membrane by encoding an outer membrane protein essential for secretion via the TTSS. The *hrcC* gene is flanked by *hrpF*, *hrpG*, *hrpT* and *hrpV* (Yuan and He, 1996), and these five genes constitute an operon. *hrpF, hrpG and hrpT* encode components of the TTSS and *hrpV* functions as a negative regulator (Preston *et al.*, 1998). *hrpL* is required for the expression of several transcripts in the *hrp* gene cluster and encodes a sigma factor related to the extracellular factor family of alternate sigma factors (Xiao *et al.*, 1994). *hrpS* and *hrpR* encode response regulator members of the two-component regulatory system and are responsible for the transcriptional activation of *hrpL* (Xiao *et al.*, 1994). *hrpR* and *hrpS* are expressed as a single operon and then they interact to form a stable heterodimeric complex that positively regulates the σ^{54}-dependent *hrpL* promoter (Hutcheson *et al.*, 2001) (Fig. 5). *hrpA* encodes the structural protein of the *hrp* pilus (Roine *et al.*, 1997) and also has a regulatory role in the expression of *hrpR* and *hrpS* (Wei *et al.*, 2000).

The *hrp* pilus elongates at the distal end by the addition of HrpA pilin subunits (Li *et al.*, 2002) and acts as a conduit for protein delivery into the host cell (Jin *et al.*, 2001). Various effector proteins including the avirulence protein AvrPto (Jin *et al.*, 2001) travel through the *hrp* pilus and exit from the tip of the pilus. Proteins secreted via the TTSS are characterized by the absence of a signal peptide, the requirement for chaperones, and a requirement for host cell contact for delivery (Galan and Collmer, 1999). The TTSSs in plant pathogenic bacteria are closely related to the flagellar export systems of bacterial pathogens

of animals. Hence, it is speculated that the secretion system may have evolved as an adaptation of the flagella to secrete proteins other than flagellin. This would permit plant pathogenic bacteria to closely associate with their host cells.

Many of the effector proteins secreted by the TTSS have similar amino acid sequences in their N-terminus (Guttman *et al.*, 2002). Thirteen effectors that showed little overall homology were identified in *P. syringae* pv. *maculicola* strain ES326. The amino terminal region of these effectors had an unusually high serine content and low aspartate, leucine and lysine composition as compared to the rest of the protein. These amino terminal regions of the effectors were similar to sequences that target to the chloroplast and mitochondria; furthermore, many of the effectors delivered by the TTSS do target to the chloroplast. Guttman *et al.* (2002) utilized the unique amino acid composition of the N-terminus of effector proteins to identify 32 proteins potentially delivered by the TTSS; fifteen of these are putative novel effectors in *Pst* DC3000.

The TTSS is also important in the epiphytic colonization of *P. syringae.* Hirano *et al.* (1999) addressed the importance of the TTSS in the establishment of *P. syringae* pv. *syringae* B728a. Two mutants, *hrpC* and *hrpJ*, survived very poorly in the phyllosphere. These mutants did cause necrosis when infiltrated into leaf tissue, but disease severity was much less when compared to the wild-type.

The TTSS is environmentally regulated at both the transcriptional and post-transcriptional level, and environmental conditions such as temperature and pH play an important role in both gene expression and protein secretion (van Dijk

et al., 1999). When conducting experiments *in vitro*, the composition of the medium is important in the expression of *hrp* genes. For example, growing the bacterium in an acidic minimal salts medium that mimics *in planta* conditions induces the expression of *hrp* genes (Huynh *et al.*, 1989). A study by Bretz *et al.* (2002) reported that the TTSS in *P. syringae* is negatively regulated by Lon protease, an ATP-dependent serine protease, which is involved in the degradation of several regulatory proteins. Regulation involves Lon protease-mediated degradation of HrpR, which is reduced when *hrp* gene expression is induced. Thus, Lon protease negatively regulates the TTSS in *P. syringae* by the degradation (proteolysis) of HrpR.

The *hrp*/*hrc* gene clusters have been classified into group I and group II, based on the presence of similar genes, operon structures and regulatory systems (Alfano and Collmer, 1996). *P. syringae* and *E. amylovora* belong to group I, whereas *R. solanacearum* and *X. campestris* belong to group II. The *hrc* genes are conserved between the two groups. However, the *hrp* genes, arrangements of genes within some operons and the regulatory systems vary between the two groups (Alfano and Collmer, 1997). In group I, to which *P. syringae* belongs, the *hrp* operons are activated by HrpL.

Role of σ^{54} in COR and *hrp* gene expression. COR production and the TTSS in *P. syringae* pv. *maculicola* strain ES4326 and *P. syringae* pv. *glycinea* PG4180 are dependent on *rpoN*, which encodes the alternative σ factor, σ^{54} (Hendrickson *et al.*, 2000b; Alarcón-Chaidez *et al.*, 2003). σ^{54} controls the expression of many genes that respond to various nutritional and environmental conditions (Merrick,

1993). The *rpoN* locus in *P. syringae* pv. *glycinea* PG4180 contains *rpoN* and three additional open reading frames (ORFs), ORFA, ORFB and ORFC. These additional ORFs are also conserved in the *rpoN* locus of many genera, although their roles are not fully understood (Alarcón-Chaidez *et al.*, 2001).

The *rpoN* mutants of *P. syringae* pv. *maculicola* ES4326 and *P. syringae* pv. *glycinea* PG4180 do not induce symptoms *in planta* (Hendrickson *et al.*, 2000b; Alarcón-Chaidez *et al.*, 2003). Also, HPLC analysis showed that both strains were defective in COR production *in vitro*. Closer analysis indicated that a functional *rpoN* was required for expression of the *cmaABT* transcript in *P. syringae* pv. *maculicola* ES4326 and both the *cfl-cfa* and *cmaABT* transcripts in PG4180. Interestingly, the *cfl-cfa* and *cmaABT* transcripts in PG4180 lack the conserved recognition site that is bound by σ^{54} (Alarcón-Chaidez *et al.*, 2003). This suggests that σ^{54} mediates expression of the *cfl-cfa* and *cmaABT* promoters via another regulatory gene that is dependent on σ^{54}. The *rpoN* mutants of *P. syringae* pv. *maculicola* and *P. syringae* pv. *glycinea* PG4180 failed to multiply *in planta* and did not produce an HR (Hendrickson *et al.*, 2000a, b; Alarcón-Chaidez *et al.*, 2003). This is consistent with the model shown in Fig. 5, where σ^{54} activates transcription of *hrpL* (σ^{L}), which then activates expression of *hrp* transcripts and various avirulence genes.

Cross-talk between the COR and *hrp* systems. Recent studies have suggested potential 'cross talk' between the COR and *hrp* systems. For example, a *hrcC* mutant of *Pst* DC3000 (designated DC3000-*hrcC*) overproduced COR relative to the wild-type DC3000 (Peñaloza-Vázquez *et al.*,

2000). COR production was reduced to wild-type levels in the *hrcC* mutant when *hrpV* was expressed *in trans*, suggesting that the overproduction of COR in DC3000-*hrcC* was due to polar effects on *hrpV*, a regulatory gene in the *hrp* cluster (Peñaloza-Vázquez *et al.*, 2000). Similarly, a *hrpS* mutant also overproduced COR, suggesting that additional *hrp* genes may also be involved in the regulation of COR biosynthesis (Preston, 1997).

Most of the genes that are regulated by HrpL contain a conserved *hrp* box, which is a conserved motif in the promoter region of *hrpL*-dependent genes (Xiao and Hutcheson, 1994; Zwiesler-Vollick *et al.*, 2002). Interestingly, many of the genes containing *hrp* boxes are not up-regulated in *hrp*-inducing medium, possibly because of differences in transcript stability (Zwiesler-Vollick *et al.*, 2002). Fouts *et al.* (2002) used a promoter-trapping screen to identify genes that are controlled by *hrpL* in *Pst* DC3000. In this screen, *hrpL*-regulated insertions were identified in two COR biosynthesis genes, *cfa1* and *cfa6* (Fouts *et al.*, 2002). This was an intriguing result, since neither *cor* gene contains a recognizable '*hrp*' box. A Hidden Markov Model was utilized to look for variations in functional Hrp boxes, with the goal of finding additional *hrpL*-modulated promoters in the *Pst* DC3000 genome. This analysis indicated the potential existence of a '*hrp*' box upstream of *corR*, the response regulator known to control expression of both the CFA and CMA operons in PG4180 (Fouts *et al.*, 2002).

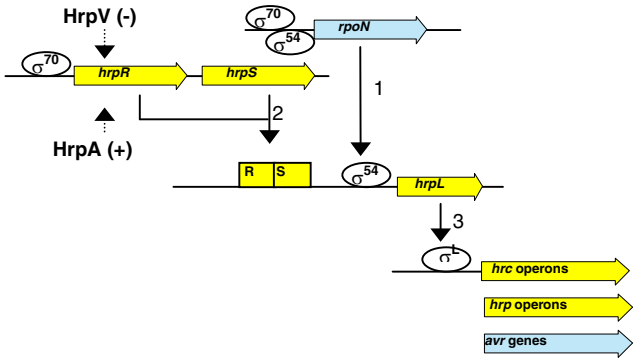

Fig. 5. Scheme showing how σ^{54} functions in activation of the *hrp* regulon. Arrow 1 shows the binding of the *rpoN* gene product (σ^{54}) to the *hrpL* promoter. Transcriptional initiation of *hrpL* may be activated by the HrpR [R] and HrpS [S] gene products (arrow 2), which show relatedness to proteins in the NtrC family. Arrow 3 depicts HrpL (σ^{L}) activation of the *hrp* and *hrc* operons and the *avr* genes, which modulate host range and fitness. HrpA and HrpV also modulate *hrp* gene expression presumably upstream of *hrpR* and *hrpS*. Aspects of the model are based on previous reports (Hutcheson *et al.*, 1996; Preston *et al.*, 1998; Wei *et al.*, 2000; Xiao *et al.*, 1994).

Another study was aimed at identifying the *Pst* DC3000 genes that are induced upon infection of Arabidopsis (Boch *et al.*, 2002). Several of the plant-induced genes in *Pst* DC3000 included several CFA structural genes, such as *cfl*, *cfa1* and *cfa*7. The *cfl*/*cfa* operon was induced within 6 h after inoculation, indicating that COR is important in the early stages of infection. The authors demonstrated that *cfa* gene expression is not directly dependent on HrpL. Instead these genes may be indirectly regulated by the Hrp system due to the presence of a potential `Hrp box` upstream of *corR* (Boch *et al.*, 2002; Buell *et al.*, 2003). No sequences resembling the Hrp box were identified upstream of the *cfl* and *cfa* genes (F. Alarcon-Chaidez and C. Bender, unpublished). In conclusion, the genetic basis for the interaction between the *hrp* and COR gene clusters is not understood and will be investigated in the proposed work.

OBJECTIVES

Objective 1: Determine whether *corR* and *corS* control the transcription of genes in the CFA and CMA gene clusters in *Pst* DC3000.

Objective 2: Determine whether COR production in *Pst* DC3000 is controlled by a signal transduction cascade in which *hrpL* controls the transcription of *corR* and *corS*.

Objective 3: Develop a sensitive, reliable method for detecting and quantifying CMA production by *P. syringae.*

Objective 4: (**a**) Characterize selected DC3000 mutants with respect to the production of COR, CFA, CMA, and coronafacoyl analogues; and (**b**) determine the expression profile of coronatine structural genes (*cfl* and *cmaB*) in a novel mutant using real-time PCR.

CHAPTER III

Evidence for Bi-Directional Regulation of the Type III Secretion System and Coronatine Biosynthesis in *Pseudomonas syringae* pv. *tomato* DC3000

Summary

The phytotoxin coronatine (COR) is produced by various pathovars of *Pseudomonas syringae*, including *P. syringae* pv. *tomato* DC3000 (*Pst* 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 both function as intermediates in the COR biosynthetic pathway. In *P. syringae* pv. *glycinea* PG4180, the genes encoding CFA and CMA are clustered and map together with a modified two-component regulatory system consisting of genes encoding two response regulators, *corR* and *corP*, and the histidine protein kinase, *corS*. *Pst* DC3000 also contains *corRPS*; however, the CFA and CMA genes in *Pst* DC3000 are physically separated in the genome, and it is not clear whether *corR, corP,* and *corS* regulate CFA and CMA synthesis as they do in PG4180. Unlike PG4180, we show in this study that *corR* and *corS* are co-

transcribed in *Pst* DC3000. However, like PG4180, *corR* and *corS* mutants of *Pst* DC3000 fail to produce COR and are also defective in the synthesis of CFA and CMA. Furthermore, studies were designed to investigate whether cross-talk exists between the type III secretion system (TTSS) and coronatine biosynthesis, which are both required for virulence in *Pst* DC3000. A mutation in *hrpL*, which encodes an alternate sigma factor (σ^L) that is required for the expression of various transcripts in the *hrp* gene cluster, abrogated production of COR in *Pst* DC3000, suggesting that mutations in the TTSS may have regulatory effects on the production of virulence factors such as COR. The presence of a potential hrp box, the recognition site for σ^L, upstream of *corR* suggested that *corRS* might be modulated by *hrpL*. This was confirmed in RT-PCR experiments showing that the upstream effector gene *holPtoAA*, which was associated with the hrp box, was co-transcribed with the *corRS*, thus constituting an operon (*holPtoAA-corR-corS*). Furthermore, we also show that mutations in *corR* and *corS* have regulatory effects on the expression of *hrpL* and *hrpA*, the latter of which encodes for the major structural unit of the *hrp* pilus. This finding was validated in gel shift studies showing that CorR binds to nucleotide sequences upstream of *hrpL*. To our knowledge, these results provide the first example showing that the *cor* regulatory system can directly impact the expression of the *hrp* regulon in *P. syringae*.

Introduction

Coronatine (COR) is a chlorosis-inducing phytotoxin produced by several pathovars of *Pseudomonas syringae* including pv. *alisalensis*, *atropurpurea*,

glycinea, maculicola, morsprunorum, and *tomato* (Cintas *et al.*, 2002, Bender and Scholz-Schroeder, 2004). COR contributes to the multiplication of *P. syringae in planta* and lesion formation or expansion in several host plants, including ryegrass, soybeans, tomatoes 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: (1) the polyketide coronafacic acid (CFA) and (2) coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine (Ichihara *et al.*, 1977; Mitchell, 1985a; Parry *et al.*, 1994) (Fig. 6A). 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, 1985b; Mitchell and Young, 1985; Mitchell and Ford, 1998).

COR biosynthesis has been thoroughly investigated in *P. syringae* pv. *glycinea* PG4180, in which 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 PG4180 and are separated by a region containing the regulatory genes *corR*, *corP* and *corS*. The translational products of *corP* and *corR* show 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

(Ullrich *et al.*, 1995; Rangaswamy and Bender, 2000). However, CorP lacks the H-T-H motif. CorR functions as a positive activator of *cor* gene expression and binds to the promoter regions of the *cor* structural/biosynthetic genes in PG4180 (Penaloza-Vazquez and Bender, 1998; Wang *et al.*, 1999). This relationship was demonstrated by using CorR in gel retardation and DNaseI footprinting assays (Penaloza-Vazquez and Bender, 1998; Wang *et al.*, 1999). The actual role of CorP in COR synthesis is unknown, though various mechanisms of action have been suggested (Rangaswamy and Bender, 2000). CorS has characteristics consistent with phosphorylation at a histidine residue and was shown to transphosphorylate CorR (Rangaswamy and Bender, 2000). COR production in PG4180 is temperature-sensitive (Palmer and Bender, 1993), and recently the transmembrane region of CorS was shown to be responsible for thermosensitivity in PG4180 (Smirnova and Ullrich, 2004).

P. syringae pv. tomato DC3000 (*Pst* DC3000), a pathogen of tomato, *Brassica* spp. (cabbage, cauliflower), and *Arabidopsis thaliana* (Moore *et al.*, 1989; Whalen *et al.*, 1991; Wang *et al.*, 2002), has become 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 *Pst* DC3000, the COR structural and regulatory genes are highly homologous (90-98% nucleotide identity) to those previously described in PG4180 (Buell *et al.*, 2003). However, *Pst* DC3000 and 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 (Moore *et al.*, 1989;

Penaloza-Vazquez *et al.*, 2000; Brooks *et al.*, 2004; Weingart *et al.*, 2004). Unlike PG4180, the structural gene clusters encoding the CFA and CMA biosynthetic enzymes are not contiguous in *Pst* DC3000, and analysis of the genomic sequence of *Pst* DC3000 has indicated that the CMA structural genes map with *corRPS* and are separated from the CFA biosynthetic region by a 26 kb intergenic region (Fig. 6B) (Buell *et al.*, 2003; Brooks *et al.*, 2004). Thus, it is unclear whether *corRPS* modulate both CMA and CFA biosynthesis in *Pst* DC3000, as it does in PG4180.

In *P. syringae*, the type III secretion system (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 (Roine *et al.*, 1997; Collmer *et al.*, 2002; He and Jin, 2003). HrpL, which encodes the alternate 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).

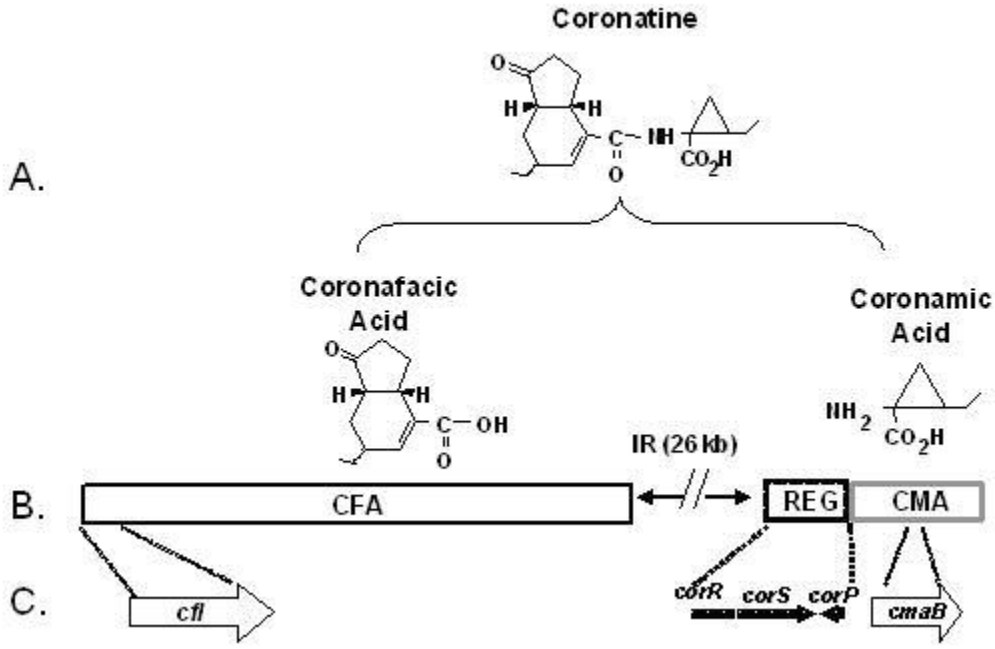

Fig. 6. The phytotoxin coronatine (COR) biosynthetic and regulatory region of *Pst* DC3000. **A,** Structure of COR, coronafacic acid (CFA), and coronamic acid (CMA). **B,** The entire COR biosynthetic region, including the 26-kb intergenic region (IR). Abbreviations: CFA, region required for CFA biosynthesis; REG, regulatory region; and CMA, region required for CMA biosynthesis. **C,** The *cfl* and *cmaB* genes were used to indicate 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.

In *Pst* DC3000, evidence exists for a regulatory connection between the TTSS and COR production (Penaloza-Vazquez et al., 2000). Further support for regulatory cross-talk was reported by Fouts *et al.* (2002), who used a promoter-trapping screen 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, since neither *cor* gene contains a recognizable Hrp box. However, a Hidden Markov Model indicated the potential existence of a Hrp box upstream of *corR* (Fouts *et al.*, 2002), suggesting that *hrpL* may modulate the expression of *cor* genes via *corR* in *Pst* DC3000.

The physical separation of the regulatory region in *Pst* DC3000 from the CFA genes may indicate that CFA genes in *Pst* DC3000 are not controlled by *corRS*, a question that we addressed in the current study. We also evaluated whether *hrpL*, *corR* and *corS* modulate CMA, CFA and COR production in *Pst* DC3000 by assaying whether these compounds are produced in mutants disrupted in each of these genes and by measuring expression of *hrp* and *cor* genes via real-time quantitative PCR. Furthermore, we provide data showing that mutations in *corR* and *corS* have regulatory effects on the expression of *hrpA* and *hrpL*, thus providing suggesting that the TTSS and COR systems show evidence of reciprocal modulation of gene expression.

Experimental Procedures

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are described in Table 1. *P. syringae* strains were grown on mannitol-glutamate (MG) medium (Keane *et al.*, 1970) or King's medium B (KB) (King *et al.*, 1954), at 28°C. *E. coli* strains were maintained on Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) at 37°C. Antibiotics used for selection of *P. syringae* strains included (in μg ml^{-1}): rifampicin, 100; kanamycin, 25; spectinomycin, 10 or 25; and chloramphenicol 25. Antibiotics used for selection of *E. coli* included (in μg ml^{-1}): ampicillin, 100; kanamycin, 10 or 25; chloramphenicol 25 and spectinomycin, 25. Plasmids were

introduced into *P. syringae* recipients by electroporation (Sambrook *et al*. 1989) or by triparental matings using pRK2013 (Figurski and Helinski, 1979).

DNA manipulation and sequencing

Routine DNA manipulations and plasmid isolations were performed as described (Sambrook *et al*., 1989). *Pst* DC3000 genomic DNA was prepared using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). DNA fragments were isolated from agarose gels by electroelution and labeled with [α-^{32}P]dCTP using the Rad Prime DNA Labeling System (Gibco-BRL). Hybridizations and post-hybridization washes were conducted under high-stringency conditions (Sambrook *et al*., 1989).

Mutant construction

A *corR* mutant was constructed using the kanamycin resistance cassette in pBSL14 (Alexeyev, 1995). A fragment containing *corR* was amplified from construct pH6 using the forward primer 5'-GGAATTCGCGCCCGACAATTCCCCTCTA (*Eco*RI site is underscored) and the reverse primer 5'-GCTCTAGACTTAACCCGGCCAGCGTCCAG (*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), was then inserted into a unique *Bam*H1 site that maps within *corR*, resulting in pAS1.Km. Construct pAS1.Km was electroporated into *Pst* DC3000, and the resulting colonies were selected for insertion of Km^R into *corR* and screened for loss of Ap^R (marker on vector) by replica plating.

Table 1. Bacterial strains and vectors used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
P. syringae pv. *tomato*		
DC3000	Derivative of NCPPB1106; Rif^R	Cuppels, 1986
DB4G3	Rif^R Km^R; *cfa6*::Tn*5 uidA*; CFA^- CMA^+ COR^-	Brooks *et al.*, 2004
AK7E2	Rif^R Sm/Sp^R; *cmaA*::Tn*5 uidA*; CFA^- CMA^+ COR^-	Brooks *et al.*, 2004
AS1	Rif^R Km^R; DC3000 containing Km^R cassette in *corR*	This study
VJ202	Rif^R Sm/Sp^R; *hrpL*::Ω	Zwiesler-Vollick *et al.*, 2002
KP430	Rif^R Sp^R/Sm^R Km^R; VJ202 containing *hrpL* in pVSP61	This study
DC3000.M1	Rif^R Km^R; contains 1.4-kb deletion in *corS* of DC3000	This study
PRE40	Rif^R Km^R; contains *cfl*: *uidA* transcriptional fusion in *Pst* DC3000	This study
PRE42	Rif^R Sp^R/Sm^R Km^R; contains *cfl*: *uidA* transcriptional fusion in VJ202 (*hrpL*::Ω)	This study
E. coli		
DH5α	*recA lacZ*ΔM15	Bethesda Research Labs
SM10	λ-*pir*	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
PCR2.1	Ap^R Km^R; cloning vector for PCR products	Invitrogen
pMKm	Ap^R Km^R; contains 1.7-kb Km^R cassette	Murillo *et al.*, 1994
pBluescript SK	Ap^R; cloning vectors;	Stratagene
pBBR1MCS	Cm^r; 4.7-kb broad-host-range cloning vector	Kovach *et al.*, 1994
pBBR.Gus	Cm^r; 6.6-kb promoter probe vector containing a promoterless *uidA* gene	Penaloza-Vazquez and Bender, 1998
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
pIPET	Km^r; contains the 1.8-kb *uidA* gene as an *Xba*I-*Nco*I fragment	Boch *et al.*, 2002
pH6	Tc^R; 35 kb genomic fragment from *Pst* DC3000 with CMA and regulatory genes; in pRK7813	C. L. Bender
pAS1	Ap^R; contains *corR* as a 1.214kb *Xba*1/*Eco*R1 fragment in pUC119	This study
pAS1.Km	Km^R Ap^R; pAS1 with *nptII* cassette from pBSL14 in *Bam*HI site of *corR*	This study
pAS2	Ap^R; contains *corR* as a 2 kb *Eco*RI fragment in pBluescript SK	This study
pAS2.1	Cm^r; contains *corR* as a 2 kb *Hin*dIII/*Xba*I fragment in pBBR1MCS	This study
pBL1	Ap^R; contains *corS* as a 1.9 kb *Eco*RI fragment in pBluescript SK	This study
pBL1.1	Cm^r; contains *corS* as a 1.9 kb *Hin*dIII/*Xba*I fragment in pBBR1MCS	This study
pCPP2385	Ap^R Sp^R Sm^R; contains *hrpL* overproduced in pUCP18	Preston *et al.*, 1998

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.

For construction of the *corS* deletion mutant, a 1.39-kb fragment beginning 736 bp upstream of the *corR* translational start site and terminating 58 bp downstream of the *corS* translational start was amplified from genomic DNA of *Pst* DC3000 using the forward primer 5'-CTAGTCTAGATGTAATGCGGAAAAACGCCT (*Xba*I site is underscored) and the reverse primer 5'-CCAGAATTCTGAGTTCGACCAGAATGAGT (*Eco*RI site is underscored). A 1.59-kb *Eco*RI-*Kpn*I fragment beginning 6 bp upstream of the

corS translational stop codon and ending in the *cmaA* gene was amplified from *Pst* DC3000 genomic DNA using the forward primer 5'-CCGGAATTCCCGTTTTAGCGCACCTCAAC (*Eco*RI site is underscored) and the reverse primer 5'- CAAGGTACCGGTACAAACGTTCGTGGCCA (*Kpn*I site is underscored). After these two PCR products were ligated into pBluescript, the 1.7-kb Km-cassette from pMKm was inserted into *Eco*RI site of the construct, resulting in a suicide vector that was used for electroporation into DC3000. After electroporation, Km^R colonies were selected on KB medium.

Complementation studies

corR and *corS* were subcloned from *Pst* DC3000 and introduced into AS1 (*corR* mutant) and DC3000.M1 (*corS*) mutants, respectively, to restore COR production. A 1,562 bp fragment containing the entire *corR* gene along with *holptoAA* was amplified from wild-type *Pst* DC3000 using the forward primer 5'-ACCTTCCCTAACCGAACCAC and the reverse primer 5'-GGGCAATTTCGCACAGTATT. Similarly, a 1,927 bp fragment containing the *Pst* DC3000 *corS* gene was amplified using the forward primer 5'-AACTGCAGGTACGGTGGAGTTTCACTCTAA (*Pst*I site underscored) and the reverse primer 5'- GGAATTCTGCGGTGGAGTTTCACTCTAAG (*Eco*RI site is underscored). The amplified *corR* and *corS* genes were initially cloned in PCR2.1 and then subcloned into a unique *Eco*R1 site in pBluescript SK+, resulting in pAS2 and pBL1, respectively. These two constructs were digested with *Hin*dIII and *Xba*I to liberate the inserts and subcloned into pBBR1MCS resulting in pAS2.1 and pBL1.1, which contain *corR* and *corS*, respectively.

pAS2.1 and pBL1.1 were used to electroporate the *corR* and *corS* mutants resulting in AS1(pAS2.1) and DC3000.M1(pBL1.1). The resulting colonies were selected for Cm^R, confirmed as transformants by plasmid isolation, and analyzed for COR production as described previously (Brooks *et al*., 2004). The orientation of insertion of the *corS* gene in pBBR1MCS was analyzed by diagnostic restriction digests.

Construction of the *Pst* 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 a hypersensitive response (HR) on tobacco (*Nicotiana tabacum*) (Zwiesler-Vollick *et al*., 2002). An 842 bp fragment containing *hrpL* was amplified from *Pst* DC3000 genomic DNA using the forward primer 5'- CGGAATTCGTTGCCATCCGAGAGTGAGC and the reverse primer 5' CGGAATTCGTTCCCTTGCGAAGCTGACC, which both contain *Eco*RI sites (underscored). The amplified product was cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen), subcloned into the *Eco*RI site of pVSP61, and then mobilized into VJ202 to generate strain KP430 (containing *hrpL in trans*). This strain was analyzed for its ability to induce symptoms on *A. thaliana*, an HR in tobacco, and for COR production.

Co-transcription of *corR* and *corS* genes in *Pst* DC3000

Pst DC3000 cells 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 220 bp upstream of the *corR* stop site (5'

TCCCGAACCGAGGACTATC) to 475 bp downstream of the *corS* start site (5' GATAAAGACTGGGCACGGC) were designed to amplify a 700 bp fragment from the cDNA template. Reverse transcriptase (RT)-PCR was performed to determine if *corR* and *corS* are co-transcribed. Another set of primers spanning 30 bp downstream of the *holptoAA* translational start site (5' ATTCCAGCTTCAATTACACGG) to 152 bp upstream of the *corR* stop site (5' CAGCAACCCGAGCACTTG) was designed to amplify a 821 bp fragment. Reverse transcriptase (RT)-PCR was performed to determine if *holptoAA* and *corR* are co-transcribed. A negative control reaction was included using total RNA as the template without reverse transcriptase.

Plant material and inoculation procedures

Tomato (*Lycopersicum esculentum* cv. 'Glamour') plants were grown from seed and maintained in growth chambers at 24-25^{o}C at 30-40% relative humidity (RH), with a photoperiod of 12 h. Plants were maintained at ≥90% RH for 48 h before inoculation. All tomato plants used for virulence studies were 4-5 weeks old. To assay for disease, derivatives of *Pst* DC3000 were grown at 28^{o}C for 48 h on MG agar supplemented with the appropriate antibiotics, and cells were suspended to an OD_{600}=0.1 (~10^{6} cfu ml^{-1}) in sterile distilled water. The plants were spray-inoculated with an airbrush (55.2 kPa) until leaf surfaces were uniformly wet. After inoculation, tomato plants were incubated in growth chambers where the RH was 90% for the initial 48 h and 70% thereafter; the photoperiod was 12 h for the duration of the experiment.

Growth of *Pst* DC3000 (wild-type), DB4G3 (*cfa6* mutant; CFA^-), AS1 (*corR* mutant), DC3000.M1 (*corS* mutant), VJ202 (*hrpL* mutant) and the complemented mutants were 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 0-100 depending on the percentage of the leaf area 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), DC3000.M1(pBL1.1) and KP430 were also monitored for the restoration of growth, symptom manifestation, and percentage infection on leaves.

Detection of COR and CFA

P. syringae derivatives were incubated in HSS at 18°C, and organic acids were extracted from culture supernatants (10 ml volumes, 3-4 replicate cultures) as described previously (Penaloza-Vazquez *et al.*, 2000). An Ultrasphere C-8 reverse phase column (Beckman Coulter, Fullerton, CA) was used for detection of COR and CFA production by *Pst* DC3000 derivatives (Brooks *et al.*, 2004). In all experiments, triplicate cultures were analyzed for COR production, and all experiments were repeated two or more times. In some experiments, COR production was normalized for differences in bacterial growth by expressing the quantity as a function of protein concentration (Penaloza-Vazquez and Bender,

1998). The protein content in bacterial cell lysates was determined with the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA).

Exogenous feeding and co-cultivation studies

Both CFA and CMA function as defined intermediates in the COR pathway and can be used in substrate feeding studies to relieve the biosynthetic block in COR^- mutants (Bender *et al.*, 1993). In the current study, DB4G3, AK7E2, AS1 and DC3000.M1 were supplemented with equimolar amounts (7 μM) of CFA or CMA as described previously (Young *et al.*, 1992). After feeding, cells were incubated an additional five days at 18°C, and organic acids were extracted and analyzed for COR as described above. The mutants AS1 and DC3000.M1 were also co-cultivated with either DB4G3 (CFA^-) or AK7E2 (CMA^-) in HSS medium at 18^0C for 7 days and the organic acids were extracted and analyzed.

Glucuronidase assays

The *cfl* gene is the first gene to be transcribed in the CFA gene cluster (Liyanage *et al.*, 1995) and was used as definitive marker for CFA gene expression (Fig. 6C). The *cfl* promoter region was amplified from *Pst* DC3000 genomic DNA using the forward primer: 5' CCTGAGCTCGCGTATCGATTGGTTGCTCCG (*Sac*I site is underscored) and reverse primer: 5' ACAGACCATGGCCATGCTAGATCACCTTTTTTTGCACC (*Nco*I site is underscored). The amplified promoter region, which contained 1002 bp upstream of the *cfl* translational start site, was digested with *Sac*I and *Nco*I. The *uidA* gene was isolated by digesting the pIPET vector with *Nco*I and *Xba*I. The *cfl* promoter and *uidA* gene were then ligated into pJP5603, which was

digested with *Sac*I and *Xba*I. Ligated DNA was transformed into *E. coli* SM10, and putative transformants were screened with diagnostic restriction digests. The fidelity of the *cfl*::*uidA* fusion in selected transformants was confirmed by DNA sequencing.

PRE40 and PRE42, which contain the *cfl::uidA* promoter fusion in *Pst* DC3000 and VJ202 (*hrpL* mutant), respectively, were grown in MG agar containing the appropriate antibiotics for two days, inoculated into HSS medium (OD_{600}=0.1), and then incubated at 18^0C. Aliquots of the cells (three replicates per sampling) were removed at 12, 24, 48, 72 and 144 h after inoculation and analyzed for GUS activity as described previously (Palmer *et al.*, 1997). GUS activity was expressed in U ml^{-1}, with 1 U equivalent to 1 nmol methylumbelliferone formed per min. *Pst* DC3000 containing a promoterless *uidA* gene in pBBR.Gus vector was used as a negative control.

RNA isolation and real-time PCR

Pst DC3000, AS1 (*corR* mutant), DC3000.M1 (*corS* mutant) and VJ202 (*hrpL* mutant) were grown in HSS medium at 18^0C, and total RNA was isolated using a method modified from (Maniatis *et al.*, 1989). Bacterial cells were collected by centrifugation and suspended in 500 μl TE. An equal volume of saturated phenol (pH 8.0) was then added to the suspension, vortexed for 1 min and centrifuged at 16100 g for 2 min. The supernatant was collected, RNA was precipitated by adding isopropanol (500 μl), and centrifuged at 16100 g for 20 min. The pellet was washed with ethanol, resuspended in 30 μl of RNase free water, and stored at –70°C.

The quality of RNA was analyzed by gel electrophoresis and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), Since the extracted RNA was contaminated with small amounts of genomic DNA, which could be co-amplified, the RNA sample was treated with 1 µl of amplification grade DNase I (Invitrogen). DNase I was inactivated by adding 1 µl of 25 mM EDTA and heating at 65^{o}C for 10 min. RNA samples were then frozen at –70^{0}C until needed. 1 µg of total RNA was used to synthesize the cDNA template using random hexamers with the Superscript^{TM} First Strand Synthesis System (Invitrogen). A negative control (RNA without the RT enzyme) was included to eliminate any possible genomic DNA contamination. The cDNA was then quantified using the NanoDrop ND-1000 spectrophotometer and diluted with water to yield 150 ng of cDNA. The expression of *cfl* (located at the 5' end of CFA gene cluster) (Fig. 6C), *cmaB* (located within the CMA gene cluster) (Fig. 6C), *corR, corS, hrpA and hrpL* genes was then evaluated using real-time PCR.

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 designer software (Invitrogen, www.invitrogen.com/lux). All primers used for real-time PCR were supplied by Invitrogen (Table 2). 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 the *rpoD* gene was labeled with FAM (5 or 6-carboxyfluorescein). The LUX primers were designed to amplify fragments ranging in size between 70-100 bp (Table 2).

Table 2. Fluorogenic LUX primer pairs used for quantitative real-time-PCR.

Target gene	Labeled LUX primer	Unlabeled primer[a]	Size (bp)
corR	For TACTTGACGACAGTCAGCGAAGCAAG[JOE]AG	Rev TGCAAATCGAGGAGGATG	92
corS	For ACGGTTGCTTTGGTATGTCGCCG[JOE]C	Rev CGTCCAGGCGGAGTAGCAAT	87
cfl	Rev CTACTACACTCCTGGTCGCCGATGTAG[JOE]AG	For GAAATCGGCGCAGTGACAGT	113
cmaB	Rev CTACGAATCCAGGTGCCGGTCG[JOE]AG	For TTCAACCGCACCCATGCT	78
rpoD	For GACCGGGCGCTGAAGAAGTTCGG[FAM]C	Rev ACCACGGACACGCTCTACCA	127
hrpA	Rev CAACAACTGTGGCACTGATCTTCTTG[JOE]TG	For TGCAGGCCCAGGAAACAAT	99
hrpL	Rev GACATTCAGAAACACGCACTGGAGAATG[JOE]C	For TGCTCAGGGCGTTTATCCAA	80

[a] Abbreviations: For, forward primer; Rev, reverse primer. All primers given in 5' orientation.

Amplification reactions were conducted using the HotStar Taq Master Mix Kit (250 U Kit; Qiagen Inc., Valencia, CA) and contained HotStar Taq DNA Polymerase (250 U), $MgCl_2$ (final reaction concentration 1.5 mM), and 200 μM each of dNTP. The total reaction volume for real-time PCR was 25 μl and each reaction contained 5 μM each of the gene specific primers (1 μl), 12.5 μl of the HotStar Taq Master Mix, and 600 ng of the cDNA template (4 μl). The reactions were incubated at 95°C for 15 min, and then cycled (40×) 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). 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 were obtained. The Ct values for all target genes were first normalized using *rpoD*, which is an endogenous reference gene (normalizer). The comparative C_T method was used for the relative quantitation of gene expression. This method compared the relative amount of target gene expression to the gene expression at time zero (calibrator). Thus, the target gene expression was denoted as the relative fold increase in the transcript level with respect to the gene expression at time zero. The relative differences in target genes were calculated according to the $\Delta\Delta C_t$ mathematical model (Pfaffl, 2001).

Gel retardation experiments

To facilitate end-labeling with [α-^{32}P] dCTP and [α-^{32}P] dGTP, DNA fragments used for gel retardation were excised with enzymes that generate 5' overhanging ends. DNA fragments were then separated on 5% polyacrylamide gels and end-labeled with [α-^{32}P] dCTP and [α-^{32}P] dGTP (Sambrook *et al.*, 1989). A maltose-binding protein MBP-CorR translational fusion was used in gel shift assays as described previously (Penaloza-Vazquez and Bender, 1998). The concentration of MBP-CorR used in gel shift assays was evaluated by loading different volumes of the soluble protein fraction from *E. coli* DH5α to 10% polyacrylamide gels containing known amounts of bovine serum albumin. Gels were stained with Coomassie blue, and the concentration of MBP-CorR was determined using a Bio-Rad GS-700 densitometer and Molecular Analyst Software (Version 1). Gel retardation assays were performed by incubating 8 ng of MBP-CorR with 3000 cpm of end-labeled DNA probe in binding buffer

(Penaloza-Vazquez and Bender, 1998). After 20 min on ice, 3 μl of loading buffer (binding buffer supplemented with 0.4% bromophenol blue and 1% glycerol) was added and the samples were loaded onto a 5% polyacrylamide gel. After electrophoresis, the gels were dried and autoradiographed.

Results

Expression of virulence genes in HSS and HDM 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 *et al*., 2000). In order to compare expression of both *hrp* and *cor* genes in a single experiment, we monitored the expression of *hrpA* and selected *cor* genes (*corR*, *corS*, *cfl* and *cmaB*) in HSS and HDM media. The *cfl* gene is the first gene to be transcribed in the CFA gene cluster (Liyanage *et al*., 1995) and was used as definitive marker for CFA gene expression; whereas *cmaB* is a strong indicator of transcription in the CMA gene cluster (Budde *et al*., 1998) (Fig. 6C). In HSS medium, both the *cor* and *hrpA* genes were expressed (Table 3). In both HSS and HDM, the *cor* regulatory genes, *corR* and *corS* were expressed at low levels. In HDM medium, *hrpA* expression was approximately two-fold higher than in HSS medium. However, *cfl* and *cmaB* were very weakly

expressed in HDM; consequently, all remaining experiments were conducted using HSS medium to facilitate optimal expression of both *cor* and *hrp* genes.

Table 3. Real-time PCR experiments to evaluate expression of selected genes in *Pst* DC3000 grown in either Hoitink-Sinden medium supplemented with sucrose (HSS) or *hrp*-derepressing medium (HDM). Values shown indicate expression 4 h after shifting cells from KB medium to the indicated minimal media (HSS or HDM), for all genes except *cfl* For *cfl* the values shown indicate expression 6 h after transfer to HSS or HDM medium

Gene	HSS Medium (R)[a]	HDM (R)[a]
hrpA	1842 ± 177[b]	4254 ± 1268
corR	33 ± 5	25 ± 2.7
corS	19 ± 4.6	12 ± 1.6
cmaB	202 ± 100	10.7 ± 2
cfl	874.1 ± 237.1	6.7 ± 2.9

a. R is the relative fold increase in target gene expression 4 h after transfer from KB to HSS relative to the gene expression immediately after transfer and was calculated as described previously (Pfaffl, 2001). The *rpoD* gene was used as the normalizer and the target gene expression at time 0 was used as the calibrator.
b. Values represent the highest levels of gene expression and are the mean of three replicates; the number following ± indicates the standard deviation. All experiments were performed twice.

Inactivation of *corR* and *corS* in *Pst* DC3000

In *P. syringae* pv. *glycinea* PG4180, CFA and CMA production requires the two-component system encoded by *corR* and *corS* (Liyanage *et al*., 1995; Ullrich *et al*., 1995). However, several pieces of evidence suggest that the COR genes are differentially regulated in *Pst* DC3000 (Wang et al., 2002), and the spatial separation of the CMA/REG and CFA gene clusters implies that they may have been acquired by the *Pst* DC3000 genome at different times and by different mechanisms. Therefore, we constructed *corR* and *corS* mutants of *Pst*

DC3000 to determine whether these two genes modulate gene expression of the CFA and CMA biosynthetic gene clusters.

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 (see Experimental Procedures). The *corR* mutant selected for further study was designated AS1. Putative *corS* mutants were screened by Southern blot analysis using labeled pBluescript and *corS* as probes. The Km^R mutant selected for further study, DC3000.M1, contained a 1.4-kb deletion in the coding region of *corS.*

Quantification of COR and CFA

The organic acids of *Pst* DC3000, AS1 (*corR* mutant), DC3000.M1 (*corS* mutant) and VJ202 (*hrpL* mutant) were analyzed for production of CFA and COR using an HPLC fractionation method developed specifically for *Pst* DC3000 (Brooks *et al*. 2004). Using this method, we found that *Pst* DC3000 produced very little free CFA (48 ng ml^{-1} of culture), presumably because most of it was converted to COR. *Pst* DC3000 produced 1236 ng ml^{-1} of COR, whereas COR production by the *corR*, *corS* and *hrpL* mutants was either undetectable or consistently low (at or around the detection limit of 40 ng). These results indicate that all three mutants were impaired in production of COR and CFA.

Exogenous feeding studies were conducted to further assess CFA and CMA synthesis in AS1, DC3000.M1 and VJ202. These mutants did not produce COR when supplied with exogenous CMA or CFA, which suggests that the *corR*, *corS*, and *hrpL* mutants, respectively, do not produce enough CFA or CMA to result in detectable levels of COR. AS1, DC3000.M1 and VJ202 produced 53.3, 70, and

54.2 ng COR ml^{-1}, respectively, when supplied with CFA and CMA simultaneously. These results imply that a basal level of the enzyme(s) that ligates CMA and CFA may be present, and confirms that the three mutants are impaired in the synthesis of both CMA and CFA. As positive controls, two *Pst* DC3000 mutants, DB4G3 (CFA^-) and AK7E2 (CMA^-), were included in these experiments. When DB4G3 and AK7E2 were supplied with exogenous CFA or CMA, respectively, COR was produced at levels equivalent to the wild-type *Pst* DC3000 (data not shown).

In co-cultivation experiments, if one strain secretes an intermediate (e.g. CFA or CMA) that can be assimilated by a second strain, the latter should be able to synthesize COR. AS1, DC3000.M1 and VJ202 did not produce COR when co-cultivated with either AK7E2 (CMA^-) or DB4G3 (CFA^-), suggesting that AS1, DC3000.M1 and VJ202 do not synthesize sufficient amounts of CFA or CMA to result in COR production. Collectively, these results suggest that *corR*, *corS* and *hrpL* regulate the biosynthesis of both CFA and CMA. However, when DB4G3 (CFA^-) and AK7E2 (CMA^-) were co-cultivated, COR production was partially restored, which is consistent with previous results (Brooks *et al.*, 2004).

Complementation experiments

To confirm that impaired COR production in AS1, DC3000.M1 and VJ202 is caused by the disruptions in *corR*, *corS* and *hrpL*, respectively, these genes were cloned into stable, replicating plasmids, introduced into the mutants, and the resulting transformants were analyzed for COR production. pBBR1MCS containing *corR in trans* (pAS2.1), pBBR1MCS containing *corS in trans* (pBL1.1),

and pVSP61 containing *hrpL in trans* were introduced into AS1 (*corR*), DC3000.M1 (*corS*) and VJ202 (*hrpL*), respectively. The transgenic strains were analyzed for COR production *in vitro*. Introduction of the wild-type genes restored COR production in all three strains. AS1 (pAS2.1), DC3000.M1 (pBL1.1) and KP430 produced 3802 ± 394, 3512 ± 131, and 1045 ± 110 ng ml^{-1} of COR, respectively. In these experiments, COR production by *Pst* DC3000 was 1236 ± 524 ng ml^{-1}, indicating that all mutants were fully complemented for COR production. The slight overproduction of COR by AS1 (pAS2.1) and DC3000.M1 (pBL1.1) may be attributed to the presence of the complementing gene on pBBR1MCS, which is a high copy-number vector (Kovach *et al.*, 1994).

Although *corR* and *corS* are independently transcribed in *P. syringae* pv. *glycinea* PG4180 (Ullrich *et al.*, 1995), we could not assume this would be true in *Pst* DC3000. Therefore, the potential polarity of the *corR* mutation on *corS* gene expression was an important consideration in the interpretation of complementation analyses. Therefore, RT-PCR experiments were conducted to evaluate whether *corR* and *corS* comprise an operon or are independently transcribed. RT-PCR amplification of cDNA from *Pst* DC3000 using primers extending 220 bp from the 3' end of *corR* to 475 bp downstream of the translational start site of *corS* yielded a 700 bp fragment, which indicated that the *corR* and *corS* genes are co-transcribed (Fig. 7, lane 3). This fragment was absent in a control using *Pst* DC3000 RNA that had not been treated with reverse transcriptase as template (Fig. 7, lane 4), confirming that there was no genomic DNA contamination in the RNA used for cDNA synthesis.

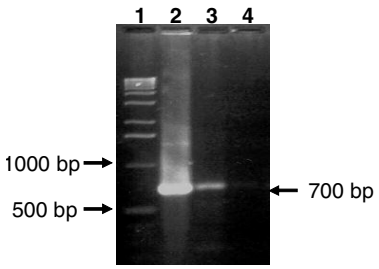

Fig. 7. RT-PCR amplification of the *corRS* transcript. Total RNA was isolated from *Pst* DC3000, treated with DNaseI, and used to synthesize cDNA for RT-PCR as described in Experimental procedures. RT-PCR was conducted using primers spanning from 220 bp upstream of the *corR* translational stop to 475 bp downstream of the *corS* translational start. Lanes: **1**, 1 kb ladder (Invitrogen); **2**, 700 bp fragment amplified from *Pst* DC3000 genomic DNA (positive control); **3**, 700 bp fragment amplified from the cDNA template; and **4**, *Pst* DC3000 total RNA without reverse transcriptase (negative control). The numbers on the left side indicate the size of the fragments in the 1 kb ladder; the band shown on the right (700 bp) is the predicted size of the amplified cDNA if *corR* and *corS* are co-transcribed.

With respect to the *corS* mutant, complementation by pBL1.1, which contains *corS* but not *corR*, was initially confusing since RT-PCR results (described above) indicated that the *corR* and *corS* genes are co-transcribed. However, when the orientation of pBL1.1 was analyzed, we discovered that the 5' end of *corS* was located adjacent to the *lacZ* promoter in pBBR1MCS. Thus it is probable that complementation of the *corS* mutant by pBL1.1 occurred due to transcription of *corS* via the *lacZ* promoter.

Symptom expression and growth of *cor* mutants in tomato

The effect of the *corR*, *corS*, and *hrpL* mutations on virulence was examined by recording the percentage area of leaves exhibiting typical disease symptoms (chlorosis and necrosis) as described in Experimental Procedures. 50-75% area of leaves selected from plants inoculated with *Pst* DC3000 exhibited disease symptoms (Fig. 8A). In contrast, in plants inoculated with AS1 (*corR*) and DB4G3, a mutant containing an insertion in a structural gene for CFA synthesis (*cfa6*), only 10-25% of leaves exhibited disease symptoms (Fig. 8B, D). Interestingly, in plants inoculated with DC3000.M1 (*corS*), only 0-10% of leaf

area exhibited disease symptoms (Fig. 8C). Leaves inoculated with VJ202 (*hrpL*), remained symptomless (data not shown).

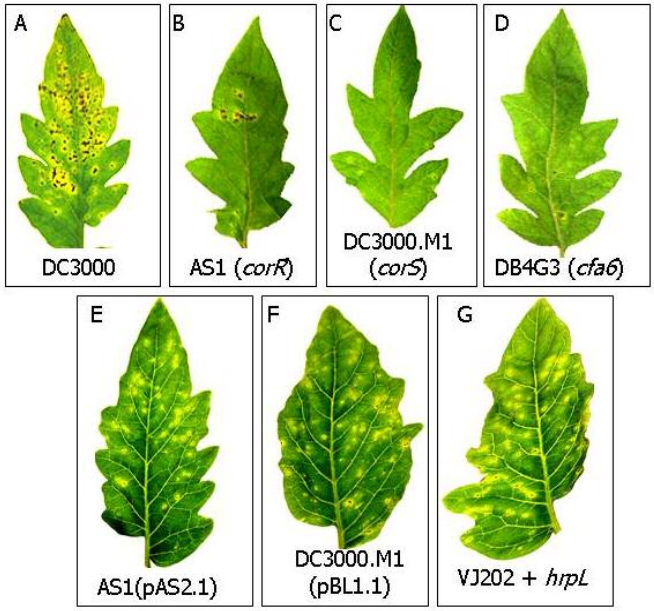

Fig. 8. Symptoms on tomato leaves sprayed with **A**, *Pst* DC3000; **B**, AS1 (*corR* mutant of *Pst* DC3000); **C**, DC3000.M1 (*corS* mutant); **D**, DB4G3 (*cfa6* mutant); **E**, AS1(pAS2.1) (AS1 containing *corR in trans*); **F**, DC3000.M1(pBL1.1) (DC3000.M1 containing *corS in trans*); and **G**, KP430 (VJ202 containing *hrpL in trans*). Bacterial inoculum was sprayed (10^6 c.f.u. ml^{-1}) 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 for the initial 48 h and 70% thereafter. Photographs were taken 7 days after inoculation.

To determine whether the reduction in disease symptom production was correlated with reduced bacterial multiplication in plant tissue, the total populations of AS1 (*corR*), DC3000.M1 (*corS*) and VJ202 (*hrpL* mutant) were compared to that of the wild-type *Pst* DC3000 in spray-inoculated tomato leaves. The total populations of *Pst* DC3000, AS1, DC3000.M1 and VJ202 were monitored from zero to ten days after inoculation (Fig. 9). The total population of *Pst* DC3000 exceeded 10^{10} cfu g^{-1} tissue beginning three days after inoculation, and remained significantly higher than the mutants for the remainder of the experiment (Fig. 9). The total population of AS1 and DC3000.M1 increased for

the first three days after inoculation and then declined; the mutant populations were significantly lower than the wild-type at the end of the sampling period (Fig. 9). The total population of VJ202 increased slightly during the first 24 h after inoculation but remained significantly lower than the wild-type throughout the remainder of the experiment (Fig. 9).

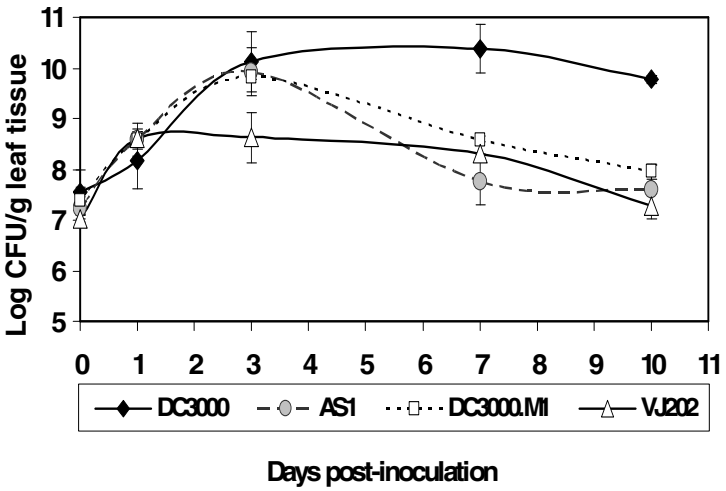

Fig. 9. Total populations of wild-type *Pst* DC3000, AS1 (*corR* mutant), DC3000.M1 (*corS*) and VJ202 (*hrpL*) on tomato leaves. Bacterial inoculum (10^6 cfu ml^{-1}) was sprayed on tomato leaves until surfaces were uniformly wet, and plants were incubated in a growth chamber as described in Fig. 8. Total populations were determined by homogenizing the leaves followed by dilution plating as described under experimental procedures. All experiments were performed twice with similar results and vertical bars indicate the standard error.

The complemented mutants, AS1 (pAS2.1), DC3000.M1 (pBL1.1) and VJ202 containing *hrpL in trans*, were restored for their ability to cause chlorosis (Fig. 8E, F, G), although the size of the necrotic lesions was less than that observed for *Pst* DC3000 (Fig. 8A). Furthermore, in leaves selected from plants inoculated with the complemented mutants, the percentage area affected was 50-75%, which was similar to that exhibited by *Pst* DC3000. The complemented mutants

were also restored for growth *in planta* (Fig. 10). At the end of the sampling period, the total population of the complemented mutants and the wild-type were not significantly different (Fig. 10).

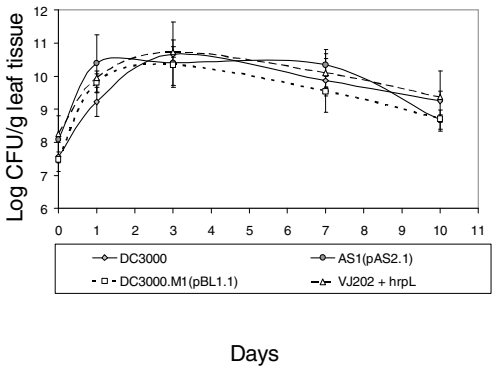

Fig. 10. Total population of wild-type *Pst* DC3000, AS1(pAS2.1), DC3000.M1(pBL1.1) and KP430 (VJ202 containing *hrpL in trans*) on tomato leaves. Bacterial inoculum (10^6 cfu ml-1) was sprayed onto tomato leaves until surfaces were uniformly wet, and plants were incubated in a growth chamber as described in Fig. 8. Bacterial populations were determined by homogenizing the leaves followed by dilution plating as described under Experimental procedures. All experiments were performed twice with similar results, and vertical bars indicate the standard error.

Glucuronidase assays

According to the COR quantification studies, *corR*, *corS* and *hrpL* are required for the regulation of COR biosynthesis. To determine if this regulation occurs at the level of expression/transcription of the *cor* biosynthetic genes, we monitored the expression of a *cfl*::uidA fusion in the *hrpL* mutant backgrounds. Expression of *cfl*::uidA in wild-type *Pst* DC3000 was first observed 12 h after inoculation to HSS medium (Fig. 11). GUS activity in this strain continued to increase until 72 h, when expression was highest (847 U GUS ml^{-1} min^{-1}) (Fig. 11). In contrast, *cfl-uidA* expression in the *hrpL* mutant (strain PRE42) was not significantly higher than the negative control (DC3000 containing a promoterless *uidA* gene in pBBR.Gus) (Fig. 11). These results indicate that *hrpL* is essential for expression of *cfl* transcription in *Pst* DC3000.

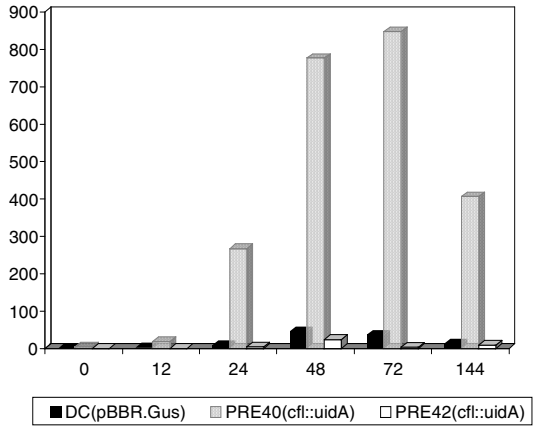

Fig. 11. Expression of the *cfl*::*uidA* transcriptional fusion in *Pst* DC3000 (PRE40) and VJ202 (PRE42). *Pst* DC3000 containing a promoterless *uidA* gene was included as a negative control (pBBR.Gus). Values represent the means from one experiment of three replicates per strain. The experiment was repeated with similar results.

Expression of *cor* genes in DC3000, *corR* and *corS* mutants using real-time PCR

The results described above indicated that both *corR* and *corS* are required for the regulation of COR biosynthesis in *Pst* DC3000. This relationship was further investigated by monitoring the expression of *cfl* and *cmaB*, which indicate transcriptional activity in the CFA and CMA gene clusters, respectively, in *Pst* DC3000, AS1 (*corR* mutant) and DC3000.M1 (*corS* mutant) using real-time quantitative PCR. Before evaluating gene expression, preliminary studies were conducted to determine the expression profile for selected *cor* genes in *Pst* DC3000 (Fig. 12) and the mutants (data not shown). Gene expression was evaluated 0, 2, 4, 6 and 12 h after transfer from KB to HSS medium, and the sampling time at which gene expression was highest in *Pst* DC3000 was selected for further analysis. For example, maximal expression of *cfl* and *cmaB* in *Pst* 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 3, Fig. 12A). *cfl* and *cmaB* transcriptional activity was significantly impaired (~100-fold

lower) in the *corR* mutant as compared to *Pst* DC3000, indicating that *corR* is absolutely required for expression of CFA and CMA genes, respectively (Table 3). Expression of *cfl* and *cmaB* was at least tenfold lower in the *corS* mutant DC3000.M1 (Table 3), confirming that *corS* is also required for transcription of the CFA and CMA gene clusters.

In addition to analyzing the expression of structural genes in the CFA (*cfl*) and CMA (*cmaB*) gene clusters, transcriptional activity of *corR* and *corS* was also analyzed in *Pst* DC3000, AS1 (*corR*) and DC3000.M1 (*corS*). Maximal expression of *corR* and *corS* in *Pst* DC3000 occurred at 4 h (Fig. 12B) and this time point was chosen for further analyses. Interestingly, *corR* expression was approximately four-fold higher in the *corS* mutant than in *Pst* DC3000, suggesting that *corS* has a negative regulatory effect on *corR* (Table 3).

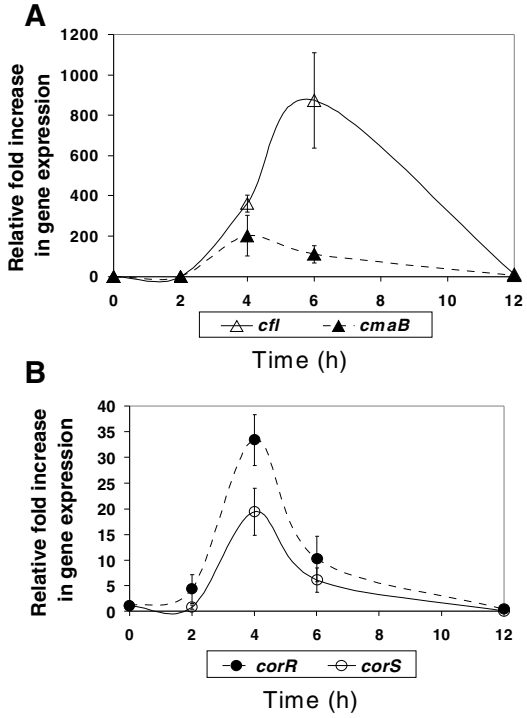

Fig. 12. Expression of *cor* genes in *Pst* DC3000 0, 2, 4, 6 and 12 h after transfer from KB to HSS media. Total RNA was isolated from *Pst* DC3000 and used to synthesize cDNA for real-time PCR as described in Experimental Procedures. Real-time PCR was conducted using the *cfl* and *cmaB* (panel A) and *corR* and *corS* (panel B) primers listed in Table 2.

Table 4. Real-time PCR experiments to evaluate expression of selected genes in *Pst* DC3000, AS1 (*corR* mutant), DC3000.M1 (*corS*) and VJ202 (*hrpL*). For all genes except *cfl* the values shown indicate expression at 4 h after shifting cells from KB medium to HSS medium. For *cfl*, the values shown indicate expression 6 h after transfer to HSS medium.

	Strains and relative fold increase in target gene expression[a]			
Target Gene	DC3000	*corR* mutant (AS1)	*corS* mutant (DC3000.M1)	*hrpL* mutant (VJ202)
corR	33 ± 5	4.3 ± 0.6	122 ± 13.5	5.9 ± 0.9
corS	19 ± 4.6	3.0 ± 1.3	ND[b]	2.1 ± 0.15
cmaB	202 ± 100	2.1 ± 1	18.7 ± 15.3	4.1 ± 2.1
cfl	874.1 ± 237.1	0.6 ± 0.00	7.15 ± 4.04	1.2 ± 1.3
hrpA	1842 ± 177*	687 ± 149	5121 ± 1676	3.5 ± 1.7

a. All values indicate relative fold increase in target gene expression as described in Table 3. Values represent the highest levels of gene expression and are the mean of three replicates; the number following ± indicates the standard deviation. All experiments were performed twice.

b. ND, gene expression not detected.

Expression of *cor* genes in the *hrpL* mutant

The expression of *cfl*, *cmaB*, *corR*, and *corS*, was evaluated in the *hrpL* mutant (VJ202) to determine whether the *hrpL*-dependence of COR synthesis was due to impaired expression of the *cor* regulatory and/or structural genes. Expression of *cfl* and *cmaB* in VJ202 was 728- and 49-fold lower than *Pst* DC3000 (Table 4), indicating that *hrpL* modulates transcription in the CFA and CMA gene clusters. Although *corR* and *corS* expression in *Pst* DC3000 was fairly low; expression in the *hrpL* mutant was approximately 5.5- and 9-fold lower than the wild-type, respectively (Table 4). These results indicate that *hrpL* modulates expression of *corR* and *corS*, although the results do not show

whether this regulation is direct or indirect (e.g. via another *hrpL*-regulated gene that modulates *corRS*).

Expression of selected *hrp* genes in the *corR* and *corS* mutants

To gain further insight into regulatory interactions between the *cor* and *hrp* genes, the expression of *hrpA*, a *hrpL*-dependent gene, was evaluated in *hrpL, corR*, and *corS* mutants. As predicted (Xiao and Hutcheson, 1994), *hrpA* transcriptional activity was negligible in the *hrpL* mutant (Table 4). In the *corR* mutant, there was a significant reduction in *hrpA* expression, suggesting that *corR* regulates transcription of *hrpA* (Table 4). To determine whether *corR* modulates *hrpA* expression further upstream in the *hrp* signal transduction cascade, the expression of *hrpL* was evaluated in *Pst* DC3000, AS1, DC3000.M1 and VJ202. There was negligible *hrpL* transcription in the *hrpL* mutant (data not shown). In the *corR* (AS1) and *corS* (DC3000.M1) mutants, *hrpL* was expressed, but there was a 2 - 4 h delay in the activation of *hrpL* transcription when compared to *Pst* DC3000 (Fig. 13), suggesting that both *corR* and *corS* modulate expression of *hrpL*.

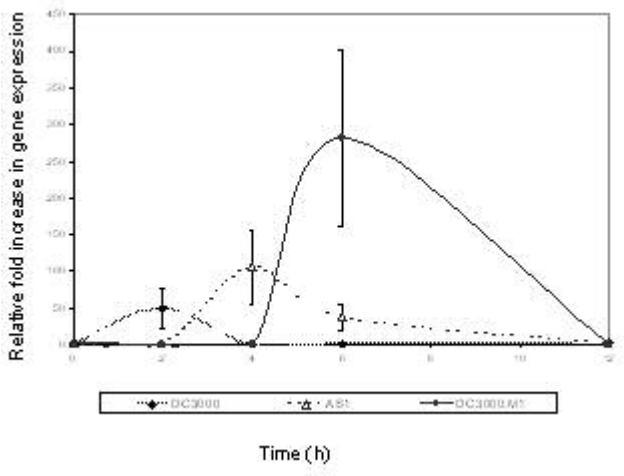

Fig. 13. Expression of *hrpL* in *Pst* DC3000, AS1 (*corR* mutant), and DC3000.M1 (*corS* mutant). Total RNA was isolated from *Pst* DC3000 and used to synthesize cDNA for real-time PCR as described in Experimental procedures. Real-time PCR was conducted using the *hrpL* primers listed in Table 2.

The delay in *hrpL* expression in the *corR* and *corS* mutants (Fig. 13) and the increased expression of *hrpL* and *hrpA* genes in the *corS* mutant (Table 4, Fig. 13) suggest that mutations in the *cor* regulatory genes can impact expression of the *hrp* genes. We have previously demonstrated that mutations in the *hrp* genes can impact *cor* gene expression, but to our knowledge, this is this is the first evidence that that *cor* regulatory mutations can impact transcriptional activity in the *hrp* gene cluster.

Gel retardation assays

The potential regulation of the *hrp* genes by CorR was further investigated using gel retardation assays to determine if CorR binds to the promoter region of *hrpL*. When a 0.8 kb *Hin*dIII-*Xba*I fragment containing the *hrpL* promoter was incubated with 16 ng MBP-CorR (see Experimental procedures), migration of the labeled fragment was markedly reduced (Fig. 14, lanes 2, 3, 4) as compared to the labeled fragment alone (Fig. 14, lanes 1, 8). The specificity of complex formation between MBP-CorR and the fragment containing *hrpL* promoter was investigated by adding increasing amounts of the unlabeled 0.8-kb *Hin*dIII-*Xba*I 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. 14, lanes 5-7). However, when poly(dI-dC) was added to the reaction mixture, binding was not altered (Fig. 14, lanes 2-4). These results indicate that CorR specifically binds to the *hrpL* promoter region, which is consistent with the presence of putative CorR-binding sites in this fragment (discussed below).

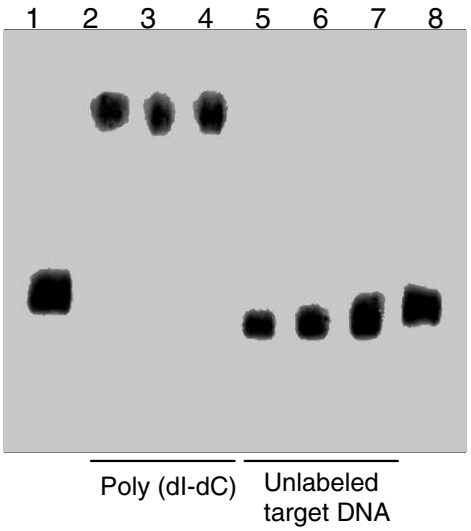

Fig. 14. Competition assays with the MBP-CorR fusion and the 800-bp HindIII-XbaI fragment contanining the hrpL promoter region of *Pst* DC3000. Lanes 1 and 8 show approximately 20ng of end-labeled target DNA (800-bp HindIII-XbaI fragment) and 0ng of MBP-CorR. The addition of the nonspecific competitor 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 16ng MBP-CorR. Specific inhibition of binding was investigated by adding the following amounts of unlabeled target fragment: lane 5, 200ng; lane 6, 400ng and lane 7, 600ng.

Discussion

COR is regulated by *corR* and *corS* in DC3000 and PG4180

The regulation and biosynthesis of COR production has been thoroughly investigated in *P. syringae* pv. *glycinea* PG4180, where the CFA and CMA biosynthetic gene clusters map together on a plasmid and are separated by a 2.4-kb region containing the regulatory genes *corR*, *corP* and *corS* (Bender and Scholz-Schroeder, 2004; Couch *et al.*, 2004). A notable difference in the COR regions of PG4180 and *Pst* DC3000 is the physical separation of the CFA and CMA biosynthetic gene clusters in *Pst* DC3000 (Fig. 6B). Although *corR* and *corS* were shown to control the expression of the CFA and CMA gene clusters in PG4180 (Liyanage *et al.*, 1995; Ullrich *et al.*, 1995), this assumption could not be made in *Pst* DC3000 because of the apparent differences in regulation of COR synthesis (Bender and Scholz-Schroeder, 2004). In the present study, *corR* and *corS* mutants of *Pst* DC3000 were shown to be defective in COR biosynthesis and expression of structural genes in the CFA (*cfl*) and CMA (*cmaB*) biosynthetic

regions (Table 4). Exogenous feeding and co-cultivation studies supported the gene expression studies and provided further evidence that *corR* and *corS* regulate both CFA and CMA biosynthesis in *Pst* DC3000 as they do in PG4180. A model showing the potential signal transduction cascade for activation of the *cor* genes and interaction with *hrpL* is shown in Fig. 18 and discussed below.

In the present study, the expression of known CorR-regulated genes was extremely low in the *corS* mutant (Table 4), indicating that CorS is required for normal expression of CorR-dependent genes in *Pst* DC3000. This is consistent with the hypothesis that CorS transphosphorylates CorR in *Pst* DC3000 (Bender and Scholz-Schroeder, 2004), and that CorR~P is important for transcriptional activation of the *cfa* and *cma* biosynthetic genes. Interestingly, we observed a four-fold increase in *corR* expression in the *corS* mutant, suggesting that *corS* functions in some manner to negatively regulate *corR*, perhaps at the posttranslational level. One possible explanation for this observation is that CorR~P, but not CorR, functions to repress transcription of the *corRS* operon, perhaps by binding to the *corRS* promoter region (discussed below). In the *corS* mutant, CorR, but not CorR~P, would be present, and repression would be relieved, resulting in higher expression of *corR*, which would be translated but not modified to the active form (CorR~P). In support of this hypothesis, a putative CorR-binding site (Penaloza-Vazquez and Bender, 1998) was identified upstream of *corR* in *Pst* DC3000 (Fig. 15). The alignment shown in Fig. 15 was constructed by comparing the upstream regions of *corR* and *hrpL* from *Pst* DC3000 with the DNaseI-protected CorR-binding site upstream of *cfl* in PG4180

(55 bp) (Fig. 15). This hypothesis was further supported in a gel shift assay where CorR was shown to bind upstream of *corR* (Fig. 16).

```
PG4180 cfl   CGAAGCCATTTCGCTGGCACAAAAAAGCGGACGAGATTCTGGGTTCCGTTGCTCG 670
DC3000 corR  TCCGTCCTCTCCACAAGCGTAAAAATGAGGACTGCAGGCCTGCTCCTGCACCGTG 428
DC3000 hrpL  CGACGCAGCTGTTTGAGCTGCTGTGTCAGGACCACATGCTCGCCCATCTCACCGT 656
CONSENSUS    CGA-GCC-CT-CTC-AGC--AAAAATGAGGAC-A-ATGCT-GCTCCTGT--CC-G
```

Fig. 15. Alignment of the CorR-binding site in P. syringae pv. glycinea PG4180 (Penaloza-Vazquez and Bender, 1998) with potential CorR-binding sites in the promoter regions of corR and hrpL in Pst DC3000. Numbers on the right represent the nucleotide position relative to the translational start (ATG). Bases shown in red font with yellow highlighting indicate nucleotides with homology to the CorR-binding site in Pg4180. The alignment was constructed using the Pretty Multiple Sequence Alignment program of SeqWeb Version 2 (Genetics Computer Group, University of Wisconsin).

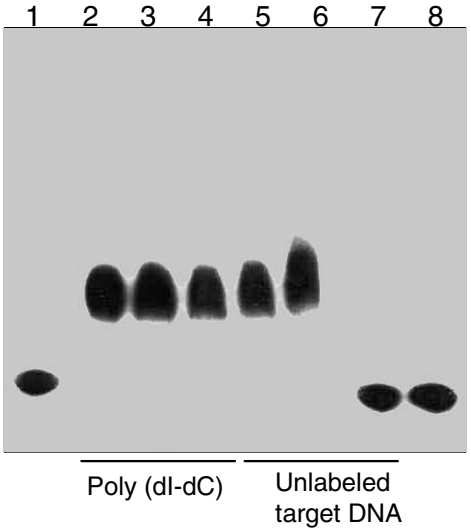

Fig. 16. Competition assays with the MBP-CorR fusion and the 800-bp HindIII-XbaI fragment contanining the *holptoAA-corRS* promoter region of *Pst* DC3000. Lanes 1 and 8 show approximately 20ng of end-labeled target DNA (800-bp HindIII-XbaI fragment) and 0ng of MBP-CorR. The addition of the nonspecific 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 8ng MBP-CorR. Specific inhibition of binding was investigated by adding the following amounts of unlabeled target fragment: lane 5, 100ng; lane 6, 200ng and lane 7, 600ng.

COR production in *Pst* DC3000 requires *hrpL*

A previous report indicated a potential role for *hrpL* in regulating COR production (Fouts *et al.*, 2002); therefore, the involvement of *hrpL* in modulating COR production was investigated using a genetic approach. When a construct that overproduced *hrpL* was introduced into *Pst* DC3000 (pCPP2385, see Table 1), COR production was 16-fold higher than that observed in the wild-type strain,

confirming that *hrpL* functions in the regulation of COR (data not shown). In multiple experiments, the *hrpL* mutant produced no detectable COR or CFA, and transcription of a *cfl*::*uidA* fusion was abrogated in the *hrpL* mutant (Fig. 11), indicating that the mutant was impaired in CFA gene expression. Furthermore, co-cultivation of the *hrpL* mutant with COR biosynthetic mutants and exogenous feeding experiments indicated that the *hrpL* mutant was also defective in CMA production. In real-time PCR experiments, expression of *cfl* and *cmaB*, which are indicative of transcriptional activity in the CFA and CMA gene clusters, was significantly lower in the *hrpL* mutant when compared to the wild-type *Pst* DC3000 (Table 4). Collectively, these results indicate a role for *hrpL* in mediating expression of COR biosynthetic genes and thus COR production.

The alternative sigma factor encoded by *hrpL* (σ^L) is believed to bind to Hrp box promoter sequences located upstream of genes in the Hrp regulon, including the effector genes that are presumably delivered via the TTSS (Collmer *et al.*, 2002). Interestingly, bioinformatic analysis revealed no candidate Hrp boxes in the regulatory regions of the CFA and CMA gene clusters. However, a putative Hrp box and its associated effector gene, *holPtoAA*, map 483 and 151 bp upstream of *corR*, respectively (Buell *et al.*, 2003), suggesting that HrpL may regulate expression of an operon containing *holPtoAA* and *corRS*. Consistent with this hypothesis, the expression of *corR* and *corS* was significantly lower in the *hrpL* mutant as compared to the wild-type *Pst* DC3000 (Table 4). Thus, the Hrp box upstream of *corR* in *Pst* DC3000 functions to regulate expression of the *corRS* operon, indicating that *holPtoAA* and *corRS* may be co-transcribed. RT-

PCR experiments revealed that the three genes are indeed co-transcribed, resulting in a *holPtoAA-corR-corS* operon (Fig. 17). Thus, *hrpL* may modulate expression of genes in the CFA (e.g. *cfl*) and CMA (e.g. *cmaB*) gene clusters indirectly by regulating expression of the *corRS* operon in *Pst* DC3000. These results indicate that HrpL, which is known to regulate the expression of effector genes and components of the TTSS, also regulates the synthesis of other virulence factors, which has been suggested previously (Boch *et al.*, 2002; Fouts *et al.*, 2002).

Mutations in *corRS* impact expression of *hrp* genes

In real-time PCR experiments, the expression of *hrpA*, a *hrpL*-dependent gene (Xiao and Hutcheson, 1994; Alfano *et al.*, 2000), was monitored in *corR*, *corS*, and *hrpL* mutants. As predicted, expression of *hrpA* was negligible in the *hrpL* mutant (Table 4). Interestingly, *hrpA* expression was significantly reduced in the *corR* mutant at 4 h after inoculation to HSS medium (Table 4), indicating that the *cor* regulatory genes impact *hrp* gene expression.

To explore whether the effect of *corR* on *hrpA* gene expression occurred further upstream in the *hrp* signal transduction cascade, real-time PCR experiments were conducted to analyze the kinetics of *hrpL* expression in wild-type *Pst* DC3000 and the *cor* regulatory mutants. Maximal expression of *hrpL* in *Pst* DC3000 occurred 2 h after shifting the cells from KB to HSS media (Fig. 13), which is consistent with the early induction of *hrp*-dependent genes observed by other investigators (Rahme *et al.*, 1992; Xiao *et al.*, 1992; Preston *et al.*, 1998; Xiao *et al.*, 2004). Interestingly, the activation of *hrpL* in the *corR* mutant AS1

was delayed by 2 h relative to *Pst* DC3000, and reached a maximum level at 4 h (Fig. 13). Thus, *corR* impacts the level and timing of *hrpA* and *hrpL* expression, respectively; e.g. when *corR* levels are low (as they are in the *corR* mutant), *hrpL* expression is delayed, and *hrpA* expression is lower.

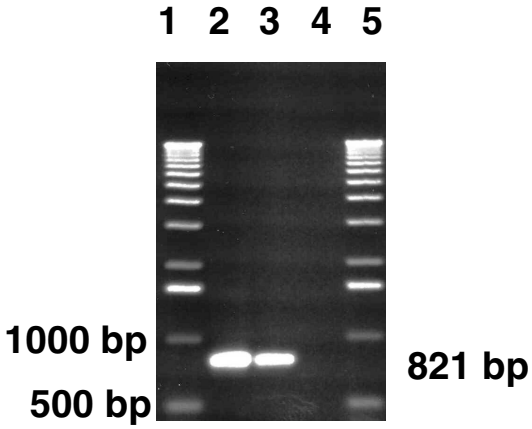

Fig. 17. RT-PCR amplification of the *holptoAAcorRS* transcript. Total RNA was isolated from *Pst* DC3000, treated with DNaseI, and used to synthesize cDNA for RT-PCR as described in Experimental procedures. RT-PCR was conducted using primers spanning from 30 bp downstream of the *holptoAA* translational start site to 52 bp upstream of the *corR* translational stop. Lanes: 1, 1 kb ladder (Invitrogen); 2, 920 bp fragment amplified from *Pst* DC3000 genomic DNA (positive control); 3, 920 bp fragment amplified from the cDNA template; and 4, *Pst* DC3000 total RNA without reverse transcriptase (negative control). The numbers on the left side indicate the size of the fragments in the 1 kb ladder; the band shown on the right (840 bp) is the predicted size of the amplified cDNA if *corR* and *corS* are co-transcribed.

It is also important to mention that mutations in *corS* impact the expression of *hrp* genes. In real-time PCR experiments, expression of *hrpA* and *hrpL* was 2.7- to five-fold higher in the *corS* mutant DC3000.M1 as compared to the wild-type DC3000 (Table 4; Fig. 13). A probable explanation for this is the increased expression of *corR* in the *corS* mutant (DC3000.M1; Table 4); in other words, when *corR* expression is elevated, there is a corresponding increase in

the expression of *hrpA* and *hrpL*. Although *hrpL* expression was increased in the *corS* mutant (Table 4), the expression of *hrpL* in the *corS* mutant was delayed relative to the DC3000, with maximal expression occurring 6 h after shifting the cells from KB to HSS media (Fig. 13). Although expression of *hrpA* and *hrpL* was high in the *corS* mutant, the lag in *hrpL* expression is quite pronounced in the *corS* mutant. Although the reason for delayed *hrpL* expression is unclear, it remains possible that CorR is phosphorylated by an alternate kinase that is less efficient than CorS. It is well-established that response regulators can be phosphorylated by non-partner sensor kinases (Parkinson and Kofoid, 1992; Wanner, 1992; Fisher *et al.*, 1995; West and Stock, 2001), and this is quite possible for CorR. These results are important in considering the impact of the *corR* and *corS* mutant on symptom manifestation (discussed below).

Symptom expression and growth of COR and *hrpL* mutants in tomato

Although the internal populations of the *corR* and *corS* mutants were similar (Fig. 9), the *corS* mutant was much more impaired in symptom expression on tomato leaves than the *corR* mutant (Fig. 8). The attenuated symptoms induced by the *corS* mutant may be at least partly explained by the 4 h delay in the expression of *hrpL* in this genetic background (Fig. 8). It has been well-established that *hrpL* is critical for symptom development (Zwiesler-Vollick *et al.*, 2002), and our results suggest that the early expression of *hrpL* is critical for symptom development. In support of this we observed that the *hrpL* mutant produced no visible symptoms on tomato leaves (data not shown).

A working model for COR-Hrp cross-talk in *Pst* DC3000

COR biosynthesis is regulated by a modified two-component regulatory system consisting of *corR*, *corS* and *corP*. CorS shows similarity to histidine protein kinases and CorR and CorP show relatedness to response regulators, although the function of CorP is not understood. In this study, we show that *corR* and *corS* are transcribed as a single operon in DC3000, and both genes are essential for the activation of structural genes encoded in the CFA and CMA biosynthetic clusters. In PG4180, it was previously shown that CorR functions as a transcriptional activator and binds to the *cfa* and *cma* promoter regions (Penaloza-Vazquez and Bender, 1998; Wang *et al.*, 1999), which may also occur in DC3000 (Fig. 18C).

In PG4180, CorS is the partner kinase for CorR (Rangaswamy and Bender, 2000) a relationship that we hypothesize holds true for DC3000 (Fig. 18A, B). Thus we predict that transphosphorylation of CorR (forming CorR~P) would be less efficient in the *corS* background. Interestingly, in the *corS* mutant, *corR* expression was elevated (Table 4), a finding that led to the working hypothesis where CorR~P, but not CorR, binds to a putative CorR-binding site (Fig. 15) upstream of *corRS*, thereby repressing the transcription of the *corRS* operon (Fig. 18G). This hypothesis was confirmed using gel-shift assays, which showed that CorR did bind upstream of *corR* (Fig 16).

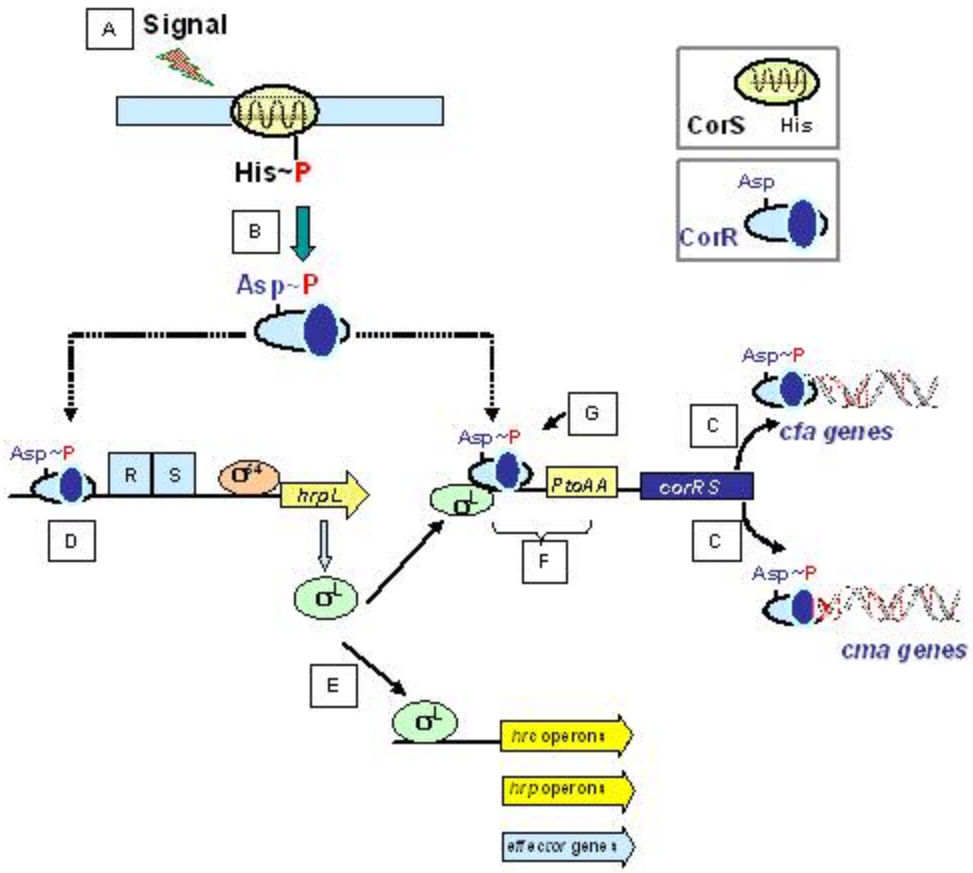

Fig. 18. Model representing a potential signal transduction cascade for activation of *cor* and *hrp* genes. **A**, CorS, a histidine protein kinase is autophosphorylated upon perception of a signal. **B,** CorR is transphospharylated by CorS, resulting in CorR~P. **C,** CorR~P activates the transcription of genes in the CFA and CMA biosynthetic gene clusters. **D**, Potential binding of CorR~P upstream of the hrpL gene, and **E,** activation of genes in the hrp operon and associated effectors by HrpL (σ^L). **F,** Location of Hrp box (for σ^L binding) upstream of *holPtoAA* (abbreviated *PtoAA*) and the *corRS* operon. **G,** Location of putative CorR binding site upstream of *corRS*.

We also found that mutations in *corR* and *corS* delayed the expression of *hrpL* (Fig. 13), and *hrpA* expression was decreased in the *corR* mutant (Table 4). These results imply that the posttranslational modification of CorR (e.g. transphosphorylation of CorS to form CorR~P) is critical for induction of *hrpL* transcription (Fig. 13). In support of this, we identified a putative CorR-binding site upstream of *hrpL* (Fig. 15), suggesting that CorR~P may bind the *hrpL*

promoter region, thereby activating transcription of σ^L along with HrpR, HrpS, and σ^{54} (Fig. 18D). This hypothesis was further tested using gel-shift assays, which confirmed that CorR bound upstream of *hrpL* (Fig. 14).

σ^L is involved in the transcription of *hrp*/*hrc* gene expression and for numerous genes that encode 'effector proteins' (Fig.18E) (Fouts *et al.*, 2002; Guttman *et al.*, 2002; Zwiesler-Vollick *et al.*, 2002; Losada *et al.*, 2004; Chang *et al.*, 2005)). Furthermore, a potential role for σ^L in modulating the *cor* genes was predicted by the identification of a putative Hrp box 483 bp upstream of *corR* in DC3000 (Fouts *et al.*, 2002); this Hrp box is adjacent to a gene encoding a putative effector protein (*holPtoAA*) (Fig. 18F). In the present study, we confirm the importance of *hrpL* in modulating COR production, since the *hrpL* mutant was severely attenuated both in COR production and *cor* gene expression. Thus, σ^L may bind to the Hrp box found upstream of *holptoAA-corR-corS*, activating the transcription of the *corR and corS*. It is important to note that the putative Hrp box and the CorR-binding site are contiguous (Fig. 18F, G), thus it is possible that the binding of CorR~P or σ^L to this region results in steric hindrance, with one protein inhibiting the binding of the other. In this scenario, when σ^L binds to the Hrp box, RNA polymerase together with σ^L initiates transcription of the *corRS* operon. If CorR~P binds to the putative CorR binding site, transcription by σ^L could be abrogated due to steric hindrance, thus resulting in decreased expression of the *corRS* operon. This model implies that the levels of σ^L and CorR~P are dynamic and influenced by signals that control both phosphorylation of CorS and activation of the Hrp regulon. Thus cross-talk between the *hrp* and

cor genes and their protein products is bidirectional, and studies to further elucidate these interactions are underway.

In summary, we have shown that *corR* and *corS* mutants of *Pst* DC3000 fail to produce COR and are also defective in the synthesis of CFA and CMA. Furthermore, studies were designed to investigate whether cross-talk exists between the type III secretion system (TTSS) and coronatine biosynthesis, which are both required for virulence in *Pst* DC3000. It is important to note that the *Pst* DC3000 *cfl* and *cmaB* genes were not strongly expressed in the HDM medium, which remains the medium of choice for most investigators working on the TTSS (Table 3). This is a critical point since studies in which HDM medium was utilized would not allow detection of the early induction of *cfa* and *cma* genes in *Pst* DC3000. In the present study, all experiments were conducted using HSS medium, which is conducive to the expression of both *cor* and *hrp* genes (Table 3). A mutation in *hrpL*, which encodes an alternate sigma factor that is required for the expression of various transcripts in the *hrp* gene cluster, abrogated production of COR in *Pst* DC3000, suggesting that mutations in the TTSS may be required for the expression of other virulence factors in DC3000, such as COR. Furthermore, we also show that mutations in *corR* and *corS* have regulatory effects on the expression of *hrpL* and *hrpA* (the latter gene encodes the major structural unit of the *hrp* pilus). These results provide compelling evidence that the *cor* regulatory system can directly impact the expression of the *hrp* regulon in *P. syringae*.

CHAPTER IV

Detection and Quantification of Coronamic Acid by Capillary Electrophoresis

Summary

The phytotoxin coronatine (COR) consists of two distinct moieties, coronafacic acid (CFA) and coronamic acid (CMA), derived from two different pathways. CFA is synthesized by a polyketide pathway, whereas CMA is an ethylcyclopropyl amino acid that originates from isoleucine. Both CMA and CFA are important in imparting biological activity to COR, and the genes involved in the biosynthesis of these two compounds are located on opposing ends of the COR gene cluster in *Pseudomonas syringae* pv. *glycinea* (*Psg*) PG4180 and pv. *tomato* (*Pst*) DC3000. Much of our knowledge regarding COR biosynthesis has been derived using mutants defective in the CMA or CFA structural gene clusters. After generation of the mutants, it is necessary to accurately detect and/or quantify the levels of COR, CFA and CMA. High-performance liquid chromatography (HPLC) can be effectively employed for the detection and quantification of both COR and CFA, although use of HPLC for CMA detection and quantification is tedious and labor intensive. In this study we used capillary

zone electrophoresis (CZE), a form of capillary electrophoresis (CE), as a fast and accurate detection method for quantification of CMA in *Psg* PG4180 and *Pst* DC3000 and selected mutants. We also showed that the kinetics of CMA production by PG4180 and DC3000 is different, and hence is dependent on the genetic background of the bacterium. Furthermore, this study revealed that although PG4180 produces 20 to 40-fold higher levels of COR than DC3000 *in vitro*, similar amounts of CMA are produced by the two strains in liquid culture. This suggests that CFA may be the limiting factor in COR biosynthesis in DC3000.

Introduction

Coronatine (COR) is a non-host specific phytotoxin produced by various pathovars of *Pseudomonas syringae* including *P. syringae* pv. *tomato* and *P. syringae* pv. *glycinea*. COR induces chlorosis and hypertrophy, inhibits root elongation and stimulates ethylene production in a wide variety of plants (Sakai *et al*, 1979; Volksch *et al*, 1989; Kenyon and Turner, 1992). COR functions as a virulence factor in some *P. syringae* pathovars (Gnanamanickam *et al*, 1982; Bender *et al*, 1987; Tamura *et al*, 1998) and has a critical role in pathogenesis (Brooks *et al*, 2004; Mittal and Davis, 1995; Peñaloza-Vázquez *et al*, 2000; Yao *et al*, 2002). COR consists of two distinct moieties, coronafacic acid (CFA) and coronamic acid (CMA), which are derived from two entirely different biosynthetic pathways. CFA is biosynthesized by a polyketide pathway, whereas CMA is an ethylcyclopropyl amino acid that originates from isoleucine (Parry *et al*, 1994).

CFA and CMA are coupled by an amide bond to form COR (Bender *et al*, 1993; Parry *et al*, 1994), and the enzyme(s) involved in this reaction lacks rigid specificity for the amino acid substrate. Hence, in addition to COR, various other CFA-amino acid complexes are biosynthesized including coronafacoylisoleucine, coronafacoylalloisoleucine, and coronafacoylvaline (Mitchell and Young, 1985; Mitchell, 1985, 1991; Mitchell and Ford, 1998). Among the analogues, COR is the most toxic coronafacoyl compound made by COR-producing organisms. Studies have shown that both CFA and CMA are important in imparting biological activity to COR (Palmer and Bender, 1995; Uppalapati *et al*, 2005).

The genes required for COR biosynthesis are plasmid-borne in *P. syringae* pv. *glycinea* PG4180. The structural genes for CFA and CMA biosynthesis are located on opposing ends of the COR gene cluster in PG4180 and are separated by a region containing the regulatory genes *corR, corS* and *corP* (Fig. 2A-C). The CFA region consists of 10 discrete ORFs, which are designated *cfl* and *cfa1-cfa9*. The CMA gene cluster was initially described as containing three genes (Ullrich and Bender, 1994), *cmaA*, *cmaT* and *cmaU*; however, later work demonstrated that the *cmaA* locus actually contained two open reading frames, which were named *cmaA* and *cmaB* (Budde *et al*, 1998). A reanalysis of the PG4180 sequence revealed the presence of three additional genes (*cmaC*, *cmaD* and *cmaE*) within the CMA gene cluster (Fig. 2A) (Couch *et al*, 2004). The role of CmaD (similar to acyl carrier proteins) and CmaE (similar to proteins with an α/β hydrolase fold) in CMA biosynthesis is not known. Sequence analysis has shown that *cmaA* encodes a protein with an amino acid

activating domain and has characteristics typical of nonribosomal peptide synthetases (Ullrich and Bender, 1994). Recently, CmaA was shown to have two domains, an adenylation domain (A domain) and a thiolation domain (T domain). Thus, CmaA catalyzes the adenylation of L-*allo*-isoleucine and the attachment of L-*allo*-isoleucine to the CmaA T domain (Couch *et al*, 2004). *cmaB* shows similarity to *syrB2*, which encodes a gene required for syringomycin synthesis (Budde *et al*, 1998). Both *cmaB* and *syrB2* show relatedness to enzymes that chlorinate amino acids, and this function suggests that CmaB may chlorinate alloisoleucine, the amino acid precursor to CMA. CmaT is related to thioesterases (Ullrich and Bender, 1994), and this activity was confirmed by overproducing CmaT in *E. coli* (Patel *et al*, 1998). Although a function for *cmaU* could not be originally deduced from sequence analysis, BLASTX has recently shown that the protein product of *cmaU* has relatedness to threonine efflux proteins.

Much of our knowledge regarding COR biosynthesis has been obtained using mutants defective in the CFA or CMA structural gene clusters (Bender *et al*, 1999). These mutants have been analyzed for CFA, CMA and COR production, and further analyzed in exogenous feeding or co-cultivation studies for restoration of COR production (Bender *et al*, 1993; Brooks *et al*, 2004; Liyanage *et al*, 1995; Ullrich *et al*, 1994; 1995; Young *et al*, 1992). It is critical, therefore, to have sensitive and accurate methods for the detection of CFA, CMA, COR, and related compounds. High-performance liquid chromatography (HPLC) (Palmer and Bender, 1993) and monoclonal antibodies have been used

to detect COR *in vitro* (Jones *et al*, 1999; 2001). A modified form of an indirect competitive ELISA, which is much more sensitive than detection by HPLC, can be used to detect very low amounts (5-40 ng/ml) of COR in plants (Zhao *et al*, 2001). However, unlike HPLC, this method cannot distinguish between COR and CFA-amide conjugates (Jones *et al*, 2001; Zhao *et al*, 2001) and is not quantitative.

In addition to COR and CFA, CMA can also be detected by reverse phase HPLC, using a C-18 reverse-phase column. However, it is important to note that the three compounds cannot be detected simultaneously, and require different extraction methods, different columns, and different wavelengths for detection. Also, detection of CMA by reverse-phase HPLC requires purification of the bacterial supernatant using an ion exchange resin column and derivatization with phenylisothiocyanate, an extra step that is time-consuming. Although HPLC can detect low levels of CMA (10 picomoles), large culture volumes of PG4180 (600 ml) were needed for accurate detection (Ullrich *et al*, 1994).

PG4180 and the model pathogen *P. syringae* pv. *tomato* (*Pst*) DC3000 differ considerably in the amount of COR produced *in vitro*, with *Pst* DC3000 producing 20 to 40-fold less COR *in vitro*. Hence, it is possible that the amount of CMA produced by DC3000 may be much lower than that produced by PG4180 and a more sensitive detection method for CMA may be required. Thus, another objective of this study was to develop a sensitive, fast and reproducible method for the detection and quantification of CMA.

Capillary electrophoresis (CE) is a highly sensitive method that can be used for the detection of various biological substances including non-derivatized amino acids (Coufal *et al*, 2003, Smith, 1999). Analysis of metabolites using CE is much faster than HPLC, since gradient elution is not required in CE (Dong *et al*, 2002; Issaq *et al*, 1995; Smith, 1997; Smith, 1999). In addition, extremely small sample volumes can be injected, and the amount of solvent waste produced is negligible in CE when compared to HPLC (Dong *et al*, 2002). There are two modes of CE, capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC) (Smith, 1999). In CZE, the separation of the particles is based on the difference in the electrophoretic mobilities, which is dependent on the solute size and charge at a particular pH. In MEKC, surfactants are added to the buffer solution at concentrations that result in micelles. Separation by MEKC is based on a differential partition between the micelle and the solvent. CZE, which is the most commonly used form of capillary electrophoresis, was used in this study for the detection of non-derivatized CMA. Thus, this study was also aimed at developing a fast and efficient method for the detection and quantification of CMA and analyzing various mutants of PG4180 and DC3000 for the production of CMA using CZE.

Experimental Procedures

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are described in Table 5. *P. syringae* strains were grown on mannitol-glutamate (MG) medium at

28°C (Keane *et al*, 1970). Antibiotics used for selection of *P. syringae* strains included (in μg ml^{-1}): rifampicin, 100; kanamycin, 25; spectinomycin, 10 or 25; and chloramphenicol 25. *P. syringae* derivatives were incubated in Hoitink-Sinden medium supplemented with either 20% glucose (HSC) (Palmer and Bender, 1993), or with 3.4% sucrose (HSS) (Penaloza-Vazquez *et al*, 2000).

Table 5. Bacterial strains and vectors used in this study.

Strain or plasmid	Relevant characteristics[a]	Reference or source
P. syringae pv. glycinea		
PG4180	COR^+ CMA^+ CFA^+	Bender *et al*, 1993, Young *et al*, 1992
PG4180.D5	Km^R; *cfa8*::Tn*5*; COR^- CMA^+ CFA^-	Bender *et al*, 1993
PG4180.C0	Km^R; *cmaB*::Tn*5*; COR^- CMA^- CFA^+	Young *et al*, 1992
P. syringae pv. tomato		
DC3000	Rif^R Derivative of NCPPB1106	Cuppels, 1986
DB4G3	Rif^R Km^R; *cfa6*::Tn*5 uidA*; CFA^- CMA^+ COR^-	Brooks *et al*, 2004
AK7E2	Rif^R Sm/Sp^R; *cmaA*::Tn*5 uidA*; CFA^- CMA^+ COR^-	Brooks *et al*, 2004
AS1	Rif^R Km^R; DC3000 containing Km^R cassette in *corR*	Chapter 3
VJ202	Rif^R Sm/Sp^R; *hrpL*::Ω	Zwiesler-Vollick *et al*, 2002
DC3000.M1	Rif^R Km^R; contains 1.4-kb deletion in *corS* of DC3000	This study

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. COR, CMA, and CFA are abbreviations for coronatine, coronamic acid, and coronafacic acid, respectively.

Isolation of coronafacoyl compounds and CMA

PG4180 and DC3000 were grown in either 5 ml, 10 ml, 20 ml (2 x 10 ml) or 50 ml (5 x 10 ml) of HSC medium or HSS medium at 18^0C for 24, 48, 120 or 168 h. Mutants of PG4180 (PG4180.D5 and PG4180.C0) and DC3000 (AK7E2, DB4G3, AS1, DC3000.M1 and VJ202) were grown in 20 ml (2 x 10 ml) of HSS medium at 18^0C for 3 days. The cells were pelleted by centrifugation at 3444 g for 10 min, and the supernatant was stored at 4^0C. The fractions soluble in the organic acid phase (including COR, CFA and its analogues) were removed by extraction using ethyl acetate at pH 2.0 as described previously (Palmer and Bender, 1993). The aqueous phase was then dried by lyophilization and used for detection of CMA.

Sample preparation for CZE

Lyophilized CMA standards and samples were resuspended in 2 ml of 50% HPLC grade acetonitrile (EM Science, Gibbstown, NJ), vortexed for 20 min and allowed to stand for 5 min to enable separation of three layers, namely: precipitate, emulsion and a clear upper organic phase. A 50-100 μl aliquot from the upper phase of the resuspended samples, which contained CMA, was filtered (45 μm) prior to separation by CZE.

Optimization of buffer and pH for capillary zone electrophoresis

Celixir buffers (Microsolv Technologies) were used as running buffer at a range of pH conditions (2.5, 4.3, 6.2, 8.2 and 9.2) to determine the optimum pH for the analysis of CMA. The samples were resuspended in 50% n-propanol and 50% Celixir buffer with pH ranges indicated. The separation of the CMA peak in

the samples was best at pH 2.5. After determining the optimum pH, a phosphate buffer (pH 2.5) that included hydoxypropyl methyl cellulose (HPMC) (Sigma Aldrich, St. Louis, MO) as a sieving agent was substituted as the running buffer for subsequent experiments. Experiments performed using this running buffer, which contained 100 mM phosphate (pH 2.5), 20% (v/v) acetonitrile, 0.4% (w/v) glycine (Pharmco Science, Brookfield, CT), and 0.05% (w/v) HPMC, yielded highly reproducible results.

Capillary zone electrophoresis

The net surface charge of the standards and samples were determined on a Beckman P/ACE 2000 (San Ramon, CA) using a 30 cm fused-silica capillary column (20 cm to the detector, 50 μm i.d.) from Polymicro Technologies Inc. (Phoenix, AZ). The column for CZE was rinsed for 1 min at 20 psi pressure with 0.1 N NaOH (Malinckrodt Chemical Inc., Paris, Kentucky) and 0.5 M acetic acid (Pharmco Science, Brookfield, CT). The column was washed with nanopure water prior to rinsing with each solvent, and was then saturated with HPMC phosphate running buffer. Buffers and samples were filtered with 45 μm nylon membrane filter (SUNSri, Wilmington, NC). The samples were injected at 0.5 psi for 5 sec and analyzed using a voltage of 12kV, at 214 nm wavelength.

Calibration curve and quantification of CMA

A calibration curve for CMA was constructed by injecting a dilution series of CMA at different concentrations (0.03125, 0.3125, 0.625, 1.25 and 2.5 μg/μl) with detection at 214 nm. Quantitative information concerning CMA yields was then obtained from the calibration curve (y = 19723x). To confirm that CMA was

being accurately detected, a pure, crystalline CMA standard was co-injected with the samples. A percentage increase in the area and height of the peak of interest and similar electrophoretic mobility of the CMA standard and sample were used to positively identify CMA in the samples.

Results

Detection of CMA by CZE

A distinct peak for CMA was observed when both the CMA standard and samples were injected using HPMC phosphate buffer at pH 2.5 (214nm). The electrophoretic mobility was reproducible and the calibration curve for CMA was linear from 31.25 to 2500 ng/μl (Fig. 19). The lowest limit for CMA detection was 31.25 ng/μl.

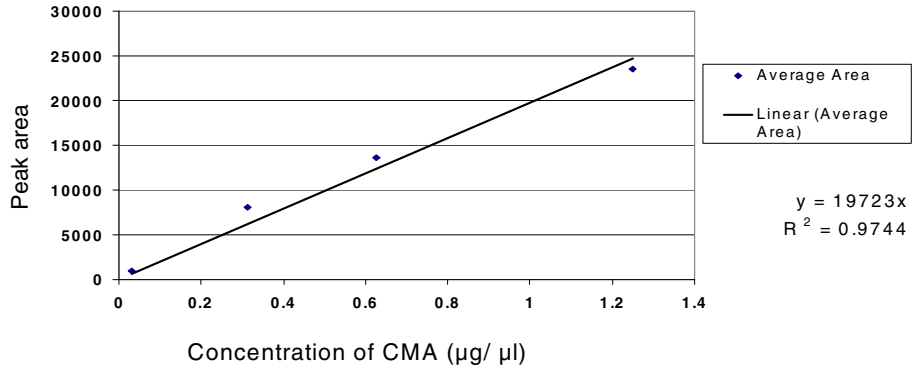

Fig. 19. Calibration curve for CMA quantification.

Isolation of CMA and optimization of culture volume for PG4180 and DC3000

As reported previously, PG4180 was grown in HSC medium, which promotes optimal COR production by this strain. HSC medium, however, contains high amounts of glucose, which affected the sample runs for CZE due to

its high viscosity. HSS medium contained lower (~5-fold) amounts of sugar and was hence used to grow both PG4180 and DC3000. The bacteria were grown in HSS media at 18^{o}C for 3 days, and different volumes (5, 10, 20 and 50 ml) of both PG4180 and DC3000 were used to determine the optimum volume of bacterial supernatant required for CMA detection. For both DC3000 and PG4180, 50 ml of culture volume was extremely viscous and hence not used for further studies. A culture volume of 20 ml was optimal for the detection of CMA in both DC3000 and PG4180 and was chosen for the remaining experiments.

Kinetics of CMA production by PG4180 and DC3000

To determine the optimum time for detection of CMA in PG4180 and DC3000, the strains were grown in HSS medium at 18^{o}C for different time periods (24, 72, 120 and 168 h), and the supernatants were used for CMA detection as described above. PG4180 and DC3000 did not produce detectable levels of CMA at 24 h. The highest level of CMA was at 72 h post-inoculation for both PG4180 (9.3 μg/ml) and DC3000 (8.3 μg/ml) (Table 6), and decreased thereafter. In PG4180, CMA was not detected at 120 and 168 h post-inoculation (data not shown). However, in DC3000, detectable levels of CMA were present at 120 h (900 ng/ml) and 168 h (555 ng/ml), although these amounts were substantially less than the amount detected at 72 h (8.3 μg/ml). Hence, 72 h was chosen as the optimal time point for detection of CMA in both PG4180 and DC3000 and derivative strains.

CMA production by the derivatives of PG4180 and DC3000

As reported previously, PG4180.C0, which contains a mutation in *cmaB*, did not produce detectable levels of CMA (Table 6). PG4180.D5 (*cfa8*) produced 12.9 μg/ml of CMA (Table 6). DB4G3, a mutant of DC3000 with a mutation in *cfa6*, produced 4 μg/ ml of CMA. As expected, a CMA structural mutant of DC3000, AK7E2 (*cmaA*), failed to produce CMA. Two COR regulatory mutants of DC3000, AS1 (*corR*) and DC3000.M1 (*corS*), also failed to produce detectable levels of CMA.

Strain	CMA (μg/ml)
PG4180	9.3
PG4180.C0	ND
PG4180.D5	12.9
DC3000	8.3
AK7E2	ND
DB4G3	4.0
AS1	ND
DC3000.M1	ND
VJ202	ND

Table 6. Production of CMA by *P. syringae* pv. *glycinea* PG4180 and *P. syringae* pv. *tomato* DC3000 and selected mutants. Production of CMA was analyzed by growing the cultures in HSS medium at 18^{o}C for 72 h and removing the organic acids using ethyl acetate extraction at pH 2.5. CMA was detected and quantified after freeze drying the remaining aqueous phase using capillary zone electrophoresis. This experiment was repeated twice with similar results.

Discussion

Detection of CMA by CZE

COR is composed of two distinct moieties, CMA and CFA, both of which impart unique biological properties to COR. To understand the biosynthesis of

COR, it is important to generate CFA and CMA mutants and characterize them by analyzing the amount of COR, CFA and CMA produced. HPLC is commonly used to detect and quantify COR and CFA. Although HPLC can also be used for CMA detection, it is tedious and time consuming since the extract needs to be purified using ion exchange resin columns and then derivatized using phenylisothocyanate. In this study, a fast, easy and sensitive method using capillary zone electrophoresis was developed for the detection and quantification of CMA. The main advantages of the CZE method was that the crude extract could be used for injection and a distinct peak representing CMA, similar to the one observed for pure CMA standard, was observed. Thus, no purification or derivatization of the extract was necessary before the samples were used for CMA detection.

Isolation of CMA and optimization of culture volume for PG4180 and DC3000

The cultures used for CMA extraction were grown either in HSC medium, which contained 20% glucose, or in HSS medium, which contained 3.4% sucrose. The high sugar content in the HSC medium affected the sample runs in CZE due to the high viscosity. Hence, HSS medium (20 ml) was used for quantification of CMA in both PG4180 and DC3000. One of the major advantages of using CZE was that the culture volume required for extraction of CMA was ~30 fold lower than that required for HPLC.

Kinetics of CMA production by PG4180 and DC3000

The CMA levels in PG4180 and DC3000 were quantified 24, 72, 120 and 168 h post-inoculation to determine the optimum time for detection of CMA in both strains. The highest level of CMA in both PG4180 and DC3000 was detected 72 h post-inoculation (9.3 and 8.3 µg/ml, respectively). The amount of CMA produced by PG4180 decreased rapidly after 72 h and was not detectable 120 h. In DC3000, detectable levels of CMA, although lower than that at 72 h, were present at both 120 h and 168 h post-inoculation.

PG4180 produces 20 to 40-fold more COR than DC3000 *in vitro*, hence it was assumed that the level of CMA produced by PG4180 would also be correspondingly higher than DC3000. Interestingly, the results from this study showed that at 72 h post-inoculation, both PG4180 and DC3000 produced similar levels of CMA. The observation that CMA levels, but not COR levels (Penaloza-Vazquez *et al*, 2000), are similar in PG4180 and DC3000 is intriguing; these data may indicate that COR production is lower in DC3000 because the amount of CFA is a limiting factor in DC3000 *in vitro*. In support of this possibility, a previous study using transcriptional fusions showed that the expression of the *cfl* promoter was approximately 4-fold lower in DC3000 than in PG4180 (Wang *et al*, 2002).

In summary, the CZE method is a valuable tool in the detection of CMA and will used in future studies to more clearly define the roles of genes in the CMA biosynthetic gene cluster.

CHAPTER V

General Conclusions

Regulation of COR biosynthesis in DC3000

The phytotoxin coronatine (COR) is produced by various strains of *Pseudomonas* including *P. syringae* pv *glycinea* PG4180 and *P. syringae* pv. *tomato* DC3000. COR consists of two moieties, derived from two different pathways, coronafacic acid (CFA) and coronamic acid (CMA). The biosynthesis and regulation of COR have been intensely studied in PG4180, but are not well understood in the model organism DC3000. Although previous studies have shown that *corR* and *corS* regulate the expression of the CFA and CMA gene clusters in PG4180, evidence suggests that there may be differential regulation of COR biosynthesis in PG4180 and DC3000 (Penaloza-Vazquez et al., 2000; Wang et al., 2002). In this study, *corR* and *corS* mutants of DC3000 were generated to address the question. Analysis of bacterial extracts by HPLC or CZE and exogenous feeding and co-cultivation studies showed that *corR* and *corS* mutants were abrogated in COR, CFA and CMA biosynthesis (Chapters 3 and 4). Furthermore, real-time PCR experiments showed that the expression of *cfl* and *cmaB*, which indicate transcriptional activity in the CFA and CMA gene

clusters, respectively, were also impaired in DC3000 *corR* and *corS* mutants (Chapter 3). These results demonstrate that COR biosynthesis in DC3000 is regulated by both *corR* and *corS*, which is also the case in PG4180. However, unlike PG4180, *corR* and *corS* are co-transcribed as a single operon in DC3000.

In the current study, real-time PCR experiments revealed that the expression of *corR* was four-fold higher in the *corS* mutant of DC3000, suggesting that *corS* functions in some manner to negatively regulate *corR*. We hypothesized that phosphorylated CorR (CorR~P), but not CorR, functions to repress transcription of the *corRS* operon, perhaps by binding to the *corRS* promoter region. In support of this hypothesis, a putative CorR-binding site was identified upstream of *corR* in *Pst* DC3000. This was further confirmed in gel shift assays, where CorR was shown to bind to the upstream region of *corR*, implying some form of autoregulation.

Comparison of CMA production in DC3000 and PG4180

As mentioned previously, various differences exist between COR biosynthesis in PG4180 and DC3000. One of the more notable differences is that DC3000 produces significantly lower amounts of COR (20-40 fold) than PG4180 *in vitro*. Previous studies have shown that the promoter activity of *cfl* (indicative of transcriptional activity in the CFA gene cluster), was four-fold lower in DC3000 than in PG4180 (Wang et al., 2002). Thus we predicted that DC3000 would produce lower levels of CMA than PG4180. The amount of CMA produced by both DC3000 and PG4180 was quantified using capillary zone electrophoresis (CZE), a fast and sensitive method developed during the course

of this study (Chapter 4). Interestingly, we observed that the level of CMA produced by both PG4180 and DC3000 was similar. Thus COR production might be lower in DC3000 because the amount of CFA is a limiting factor *in vitro*.

Wang et al. (2002) used a *cfl*::*uidA* transcriptional fusion to measure gene expression in the CFA gene cluster. Although this method is less sensitive that real-time quantitative PCR, the results are worth mentioning in the context of the present study. In PG4180, *cfl* gene expression gradually increased with time and remained high throughout the experiment (48 h) (Wang et al, 2002). However, in DC3000, *cfl* gene expression occurred much earlier (12 h) and was significantly lower at 24 h (Wang et al, 2002). These observations support the hypothesis that CFA may be a limiting factor in COR biosynthesis in DC3000. In the present study (Chapter 3), *cfl* expression was highest at 6 h, while *cmaB* expression was highest at 4 h (Chapter 3). The delay in the expression of CFA genes compared to CMA genes may also be significant, since a lower pool of available CFA may limit the supply available for conjugation with CMA to form COR

To determine the optimum time for detection of CMA in PG4180 and DC3000, the kinetics of CMA production was evaluated in both strains (Chapter 4). In DC3000, the highest level of CMA was produced 72 h post-inoculation, and this strain continued to produce detectable amounts of CMA at 120 and 168 h. In PG4180, however, CMA levels were not detectable at 120 or 168 h.

Bi directional regulation exists between *hrp* and COR systems in DC3000

A previous report indicated a potential role for *hrpL* in regulating COR production (Fouts *et al.*, 2002). Hence, we used a genetic approach to further

investigate the possible regulatory connection between the type III secretory system (TTSS) and the COR biosynthetic system. A *hrpL* mutant of DC3000 was found to be defective in COR, CFA and CMA biosynthesis using HPLC and CZE and by exogenous feeding and co-cultivation studies (Chapter 3). The expression of representative genes in the CFA (*cfl*) and CMA (*cmaB*) gene clusters was also significantly lower in the *hrpL* mutant than in DC3000. These results indicate that *hrpL* is required for expression of structural genes in the COR gene cluster and for COR biosynthesis. However, these results did not indicate whether *hrpL* modulated COR directly or indirectly, e.g. via another gene regulated by *hrpL*. The alternative sigma factor encoded by *hrpL* (σ^L) is believed to bind to hrp box promoter sequences located upstream of genes in the Hrp regulon, including the effector genes that are presumably delivered via the TTSS (Collmer *et al.*, 2002). Bioinformatic analysis revealed a putative hrp box associated with an effector gene (*holPtoAA*) upstream of *corR*. RT-PCR experiment showed that *holPtoAA* was co-transcribed with *corRS* resulting in an operon consisting of *holPtoAA*, *corR, and corS*. This suggested that σ^L binds to the hrp box upstream of *holPtoAA-corR-corS*, activating the transcription of both *corR* and *corS*, which in turn regulate the transcription of genes in the CFA and CMA gene clusters. Thus, Chapter 3 revealed that the cross-talk between the TTSS and COR biosynthetic system is direct and occurs through the *holPtoAA-corR-corS* operon.

The data presented in Chapter 3 indicated that the regulatory interaction between the TTSS and the COR systems is bi-directional. Real-time PCR

experiments were used to evaluate the expression of *hrpL* and *hrpA* (a *hrpL*-dependent gene) in the *corR* and *corS* mutants. The expression of *hrpL* was delayed by 2 and 4 h in the *corR* and *corS* mutants, respectively, when compared to DC3000. The expression of *hrpA* genes was also reduced in the *corR* mutant, indicating that *corR* impacts the expression of genes in the TTSS. In support of this finding, a putative CorR-binding site was identified upstream of *hrpL*. A gel shift assay using a CorR-MBP translational fusion confirmed that CorR binds upstream of *hrpL*. Taken together, these results demonstrate that bi-directional cross-talk exists between the COR biosynthetic system and the TTSS.

The interaction between the TTSS and COR systems may also be important in the manifestation of symptoms by DC3000 *in planta*. Although the population of the *corS* mutant was similar to the *corR* mutant *in planta*, the *corS* mutant was much more impaired in symptom expression. This may partly be explained by the 4 h delay in the expression of *hrpL* in the *corS* mutant. Earlier studies have shown that *hrpL* is necessary for early symptom development and hence, early expression of *hrpL* may be critical for symptom manifestation.

Thus, the data presented in Chapter 3 confirm that HrpL, which is known to regulate the expression of effector genes and components of the TTSS, also regulates the synthesis of other virulence factors, which has been alluded to in previous studies (Boch *et al*., 2002; Fouts *et al*., 2002). Our results also show that the *cor* regulatory system can directly impact the expression of the *hrp* regulon in DC3000. To our knowledge, these results provide the first example that the cross-talk between the TTSS and COR production is bi-directional, a

novel finding indicating that the two virulence systems function cooperatively to promote pathogenesis.

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APPENDIX I

Characterization of Selected DC3000 Mutants

Introduction

Coronatine (COR) is an important virulence factor in diseases caused by several pathovars of *Pseudomonas syringae* including *P. syringae* pv. *tomato*. COR consists of two distinct moieties, coronafacic acid (CFA) and coronamic acid (CMA) coupled together by an amide bond, which both contributing to the unique properties of COR. The CFA biosynthetic region consists of ten discrete ORFs, *cfl* and *cfa1-cfa9*, and the CMA region consists of seven ORFs, *cmaD*, *cmaE*, *cmaA*, *cmaB*, *cmaC*, *cmaT*, and *cmaU.* Three genes, *corR*, *corS* and *corP* constitute a modified two-component regulatory system that regulates the genes in the CMA and CFA biosynthetic clusters. In DC3000, the *cma* and regulatory genes (*corRPS*) are clustered together and are physically separated from the *cfa* genes by a 26-kb region (Buell et al, 2003) (Fig. 4). DC3000 is now considered as a model organism, and hence it is important to study the biosynthesis and regulation of COR, which is an important pathogenicity and virulence factor in this organism. The characterization of well-defined *Pst* DC3000 mutants defective in specific coronatine genes is critical to understanding the contribution of various loci within the cluster to pathogenicity.

Hence, several mutants with insertions in the COR biosynthetic genes were analyzed for the production of COR, CFA and coronafacoyl analogues. A novel mutant of DC3000, which may have a potential regulatory effect on COR biosynthesis, was also characterized by determining the expression profile of the two structural genes, *cfl* and *cmaB*, using real-time PCR.

Experimental Procedures

Bacterial strains and growth conditions

The mutants used in this study were obtained from Dr. Barbara Kunkel's laboratory (Washington University, St. Louis) and included a *cfa6* mutant (DB4G3), two *cfa7* mutants (DB3E8, DB5A6), and a *cmaA* mutant (AK7E2). In addition to the COR structural mutants of DC3000, a novel mutant (PRE94), where the entire TvrR (TetR-like virulence regulator R) protein coding region was deleted, was also obtained. The bacterial strains used in this study were grown on mannitol-glutamate (MG) medium at 28^{o}C (Keane et al., 1970). Antibiotics used for selection included (in μg ml^{-1}): rifampicin, 100; kanamycin, 25; spectinomycin, 10 or 25; and tetracycline, 25. *P. syringae* derivatives were incubated in Hoitink-Sinden medium supplemented with sucrose (HSS) (Palmer and Bender, 1993; Penaloza-Vazquez et al., 2000).

Detection of COR, CFA and CMA

P. syringae derivatives were incubated in HSS at 18^{o}C, and organic acids were extracted from culture supernatants (10 ml volumes, 3-4 replicate cultures) as described previously (Penaloza-Vazquez *et al.*, 2000). An Ultrasphere C-8

reverse phase column (Beckman Coulter, Fullerton, CA) was used for detection of COR and CFA production by *Pst* DC3000 derivatives (Brooks *et al.*, 2004). In all experiments, triplicate cultures were analyzed for COR production, and all experiments were repeated two or more times. In some experiments, COR production was normalized for differences in bacterial growth by expressing the quantity as a function of protein concentration (Penaloza-Vazquez and Bender, 1998). The protein content in bacterial cell lysates was determined with the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). For PRE94, the level of CMA produced was determined by using the aqueous phase of the culture supernatant after extraction of the organic acids. The aqueous phase was freeze-dried and resuspended in 2 ml 50% acetonitrile, vortexed for 20 min and allowed to equilibrate for 5 min to facilitate phase separation. The samples were loaded into the CZE as described previously (Experimental Procedures, Chapter 4).

Exogenous feeding studies.

COR production by two of the biosynthetic mutants, DB4G3 (*cfa6*) and AK7E2 (*cmaA*), was also evaluated after exogenous feeding with CFA and CMA. Exogenous feeding studies were conducted by supplying the mutants with either CFA (7 μM) or CMA (7 μM) at 48 h after incubation at 18^0C in HSS medium (Young et al, 1992). The cells were then incubated another five days, and organic acids were extracted and analyzed for COR as described earlier. Since the mutants are deficient in either CFA (DB4G3) or CMA (AK7E2), they should produce COR when exogenously fed with CFA or CMA respectively.

Co-cultivation studies.

In addition to exogenous feeding studies, the two COR mutants, DB4G3 and AK7E2 were co-cultivated in HSS medium at 18^0C for 7 days. This experiment was performed to confirm the results of the exogenous feeding studies. Conceptually, if one mutant secretes an intermediate (e.g. CFA or CMA) that is assimilated by the second mutant, the latter should be able to synthesize COR.

Complementation studies.

To confirm that impaired COR production in the mutants was due to the disruptions caused by the Tn*5* insertions, genomic clones corresponding to the *Pst* DC3000 *cma* and *cfa* gene clusters clones were introduced into the mutants. Cosmid pL5B4, which carries the genes involved in CMA synthesis, was introduced into mutant AK7E2, and cosmid pLB4, which contains the entire *cfa* operon, was introduced into DB4G3. The transconjugants were then analyzed for COR production *in vitro*. In order to complement the *tvrR* mutant (PRE94), the wild-type complementing clone was introduced into *tvrR*::Ω (PRE94) on a plasmid (*tvrR*::Ω/ptvrR+) resulting in PRE96. In all experiments, wild-type DC3000 was used as the positive control, and triplicate cultures were analyzed. All experiments were repeated two or more times.

***cfl* and *cmaB* expression using real-time PCR**

The *tvrR* mutant was further characterized by evaluating the expression profile of two genes in the CFA and CMA gene clusters, *cfl* and *cmaB*. The expression profile of these two genes is indicative of the transcriptional activity of

the CFA and CMA biosynthetic clusters, respectively. In addition to PRE94 and the complemented mutant PRE96, two control strains, PRE7 and PRE8, containing the empty vector (without insert) in DC3000, were used for gene expression studies. PRE94 (*tvrR* mutant), PRE96 (PRE94 containing *tvrR in trans*), PRE7 and PRE8 were grown in HSS medium at 18^0C, and total RNA was isolated using a method modified from Maniatis *et al.* (1989). The expression of *cfl* and *cmaB* were evaluated using with LUX fluorogenic primers (Table 2) by real-time PCR as described previously.

Results

Quantification of COR, CFA and CMA production

Pst DC3000 produced 1000 ng/ml of COR, but very little CFA (48 ng/ml culture), presumably because most of it was converted to COR. AK7E2 (*cmaA* mutant) produced no detectable levels of COR, but comparatively higher levels of CFA (611 ng/ml). This is consistent with the fact that AK7E2 has a biosynthetic block in the production of CMA, which results in increased accumulation of CFA and/or coronafacoyl amides (Ullrich et al, 1994). DB4G3, DB3E8 and DB5A6 (*cfa* mutants), did not produce detectable levels of CFA or COR. PRE94 produced 353 ng/ml COR, which is significantly lower than the wild-type DC3000 (1000 ng/ml). Interestingly, unlike the wild-type DC3000, PRE94 produced a substantial amount of coronafacoylvaline (1857 ng/ml), and very low levels of CMA (0.5 ng/ml$_{[CLB1]}$).

Exogenous feeding and co-cultivation studies.

Since several *cfa* mutants were available, mutant DB4G3 was selected as a representative of this group. DB4G3 contains an insertion in *cfa6*, which encodes a large multifunctional polyketide synthase that is essential for CFA synthesis (Rangaswamy et al, 1998). Mutant AK7E2 (*cmaA*) was studied since this mutant contains the only defined insertion in the CMA biosynthetic gene cluster (Fig. 4B). Supplementation of AK7E2 with exogenous CMA, but not CFA, restored COR production to the mutant (Fig. 21[CLB2]). Likewise, exogenous feeding of DB4G3 with CFA, but not CMA, restored COR production, confirming that DB4G3 is impaired in CFA production. Co-cultivation of AK7E2 and DB4G3 resulted in COR production (920 ng/ml culture) at levels similar to the wild-type *Pst* DC3000. This result is consistent with the secretion and uptake of CFA and CMA by *P. syringae* cells in the fermentation (Fig. 21).

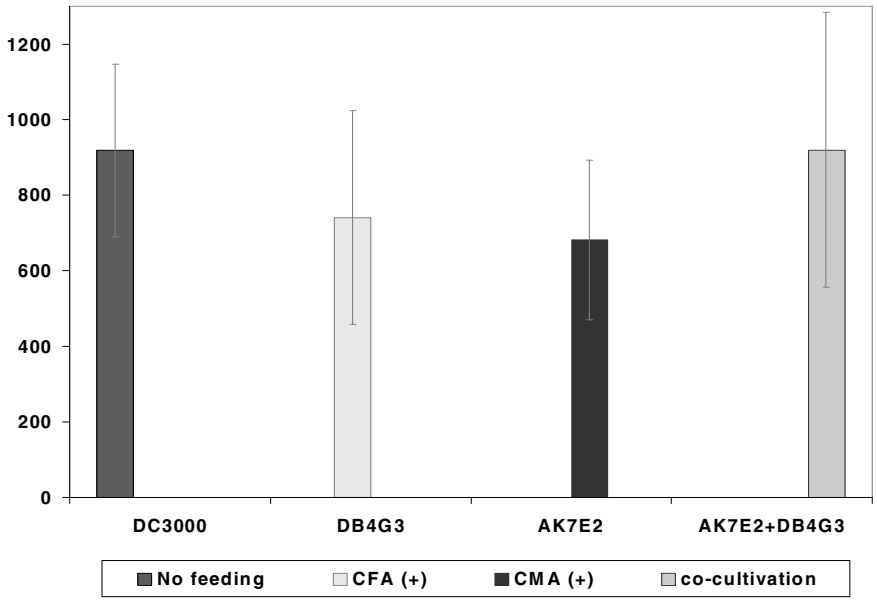

Fig. 21. Exogenous feeding and co-cultivation experiments. Exogenous feeding studies were conducted by supplying AK7E2 and DB4G3 with either CFA (7 μM) or CMA (7 μM) after 48 h of incubation in HSS medium at 18^0C. AK7E2 (CMA^-) and DB4G3 (CFA^-) were co-cultivated in HSS medium at 18^0C for 7 days. COR and other organic acids were extracted from the culture supernatant using ethyl acetate and analyzed using HPLC.

Quantification of COR production by complemented strains.

As mentioned earlier, cosmid pL5B4, which carries the genes involved in CMA biosynthesis was introduced into mutant AK7E2, and cosmid pLB4, which contains the entire *cfa* operon, was introduced into DB4G3. AK7E2 (pL5B4) produced 135 ng/ml of COR, where as DC3000 produced 888 ng/ml COR. Thus, introduction of the wild-type CMA gene cluster partially restored COR production to AK7E2. In contrast, strain DB4G3 carrying the CFA biosynthetic operon on cosmid pLB4 did not produce detectable levels of COR. However, this strain consistently synthesized 80 ng/ml CFA, which is comparable to the 48 ng/ml of CFA synthesized by *Pst* DC3000. These results indicate that CFA production was partially restored by the presence of the wild-type *cfa* genes. It is important to note that the detection level for COR using this particular HPLC method was 40 ng, and thus the DB4G3 strain carrying pLB4 may synthesize COR at levels below our detection limit.

***cfl* and *cmaB* expression using real-time PCR**

The expression of *cfl* (located at the 5' end of CFA gene cluster), *cmaB* (located within the CMA gene cluster), which are indicative of the transcriptional activity in the CFA and CMA gene clusters, respectively, were analyzed using real-time PCR. *cfl* gene expression was significantly higher in PRE94 when compared to the two controls, PRE7 and PRE8 (Fig. 22). As expected, in the complemented mutant (PRE96), the *cfl* expression levels dropped to wild-type levels. This indicates that *cfl* may be overexpressed in the *tvrR* deletion mutant, which suggests potential negative regulation of CFA biosynthesis by TvrR. *cmaB*

expression was significantly lower in PRE94 than the controls (PRE7 and PRE8), which is consistent with the low level of CMA produced by PRE94. When *tvrR* was introduced *in trans*, *cmaB* expression was partially restored to wild-type levels in the complemented mutant, PRE96 (Fig 22).

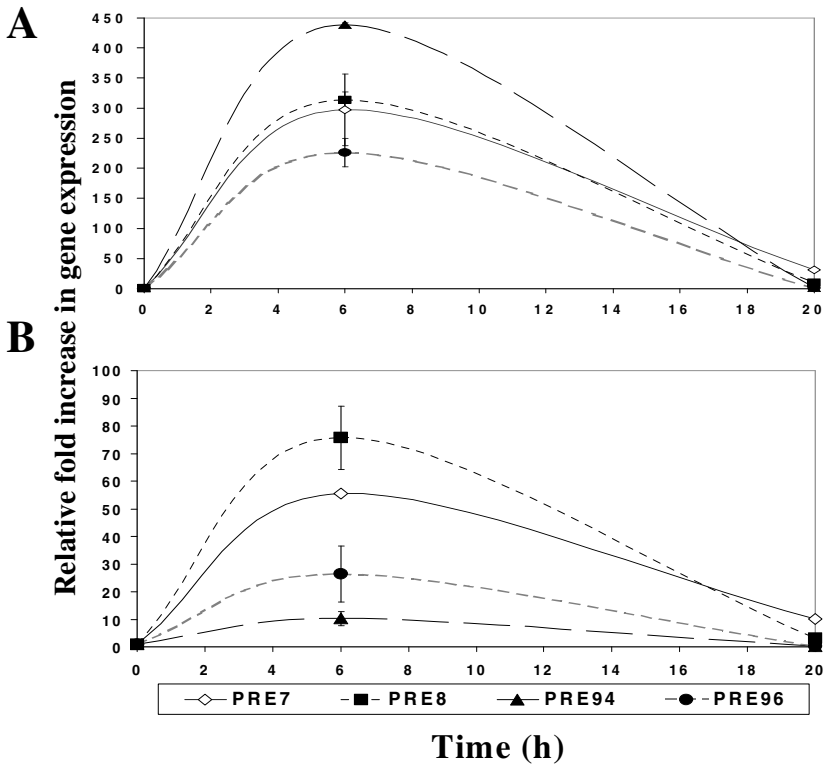

Fig. 22. Expression of *cfl*and *cmaB* genes in *Pst* DC3000 0, 6 and 12 h after transfer from KB to HSS media. Total RNA was isolated from the strains, used to synthesize cDNA for real-time PCR as described previously. Real-time PCR was conducted using the *cfl* (A) and *cmaB* (B) primers listed in Table 2.

Discussion

The mechanism of COR regulation in DC3000, which is now considered a model organism, is not fully understood. In this study, several well-defined mutants of DC3000, with insertions in the COR gene cluster, were analyzed for production of COR, CFA and CMA. HPLC analysis of DC3000 and the mutants revealed that DC3000 produced 1000 ng/ml of COR, whereas the mutants failed to produce COR. HPLC analysis along with exogenous feeding and co-cultivation studies showed that the *cma* and *cfa* mutants of DC3000 failed to produce CMA and CFA, respectively, which is consistent with the location of the transposon insertions in these mutants.

In addition to the COR biosynthetic mutants of DC3000, PRE94, a novel mutant containing a deletion in *tvrR* (TetR-like virulence regulator R), was also characterized. This mutant produced significantly lower levels of COR and CMA, but substantial amounts of coronafacoylvaline, when compared to DC3000. These results indicate that TvrR may have a role in the regulation of COR biosynthesis in DC3000. This hypothesis was further confirmed using real-time PCR, which showed that *cfl* was overexpressed in PRE94 as compared to DC3000. These results suggest potential negative regulation of CFA biosynthesis by TvrR.

APPENDIX II

Re-mapping the location of mutations in the CMA region in PG4180

The structural genes involved in the biosynthesis of the phytotoxin coronatine (COR) in *Pseudomonas syringae pv. glycinea* (*Psg*) PG4180 are plasmid-borne and are located on opposing ends of the coronatine gene cluster. The CFA region consists of ten discrete ORFs, designated as *cfl* and *cfa1-cfa9*. The CMA region was initially thought to consist of three genes, *cmaA*, *cmaT* and *cmaU* (Ullrich and Bender, 1994). A later paper demonstrated that the *cmaA* locus actually contained two open reading frames, *cmaA* and *cmaB* (Budde et al, 1998). A recent reanalysis of the CMA region revealed that additional genes, *cmaC*, *cmaD* and *cmaE* were also present (Couch et al, 2004) (Fig. 2).

Much of our knowledge regarding COR biosynthesis has been obtained using mutants defective in either the CFA or CMA structural gene clusters. Previous studies of mutants in the CMA region were based on earlier maps of the gene cluster in PG4180 (Ullrich and Bender, 1994). Since the re-sequencing of the CMA region in PG4180 (Couch *et al*, 2004) and completion of the genomic sequence of *P. syringae* pv. *tomato* DC3000 (Buell et al, 2003), the presence of previously unknown ORFs in the CMA gene cluster has made it critical to generate an updated functional map of this region. The original sequence data

for PG4180 was compared to the recently updated sequence, and the location of the mutations in PG4180.C9, PG4180.C1, PG4180.C0, PG4180.C14, PG4180.N13 and PG4180.N10 were analyzed and re-mapped. The updated map showing the location of these mutations is shown below (Fig. 22).

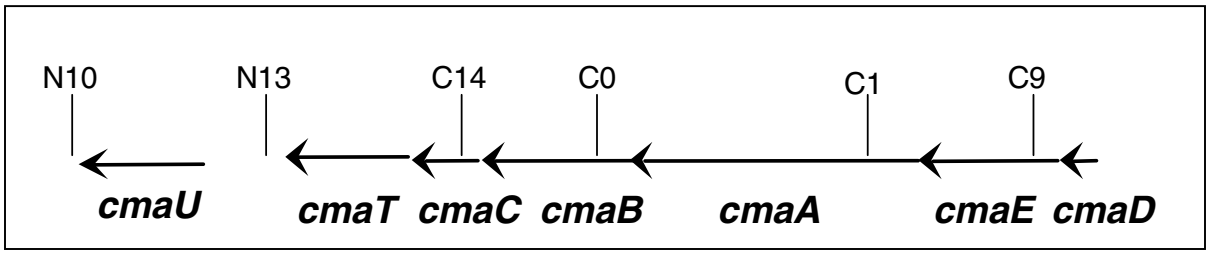

Fig. 22. Map showing the updated location of Tn*5* insertions in PG4180.C9, PG4180.C1, PG4180.C0, PG4180.C14, PG4180.N13 and PG4180.N10.

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Scope and Method of Study: 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 both function as intermediates in the COR biosynthetic pathway. In PG4180, COR biosynthesis is regulated by a modified two-component regulatory system consisting of genes encoding two response regulators, *corR* and *corP*, and the histidine protein kinase, *corS*. DC3000 also contains *corRPS*; however, it is not clear whether *corR, corP,* and *corS* regulate CFA and CMA synthesis as they do in PG4180. Furthermore, studies were designed to investigate whether cross-talk exists between the type III secretion system (TTSS) and COR biosynthesis, which are both required for virulence in DC3000.

Findings and Conclusions: We observed that similar to PG4180, *corR* and *corS* mutants of DC3000 fail to produce COR, CFA and CMA. A mutation in *hrpL*, which encodes an alternate sigma factor (σ^L), required for the expression of various transcripts in the *hrp* gene cluster, abrogated production of COR in DC3000, suggesting that mutations in the TTSS may have regulatory effects on the production of virulence factors such as COR. The presence of a potential Hrp box, the recognition site for σ^L, upstream of *corR* suggested that *corRS* might be modulated by *hrpL*. This was confirmed in RT-PCR experiments showing that the upstream effector gene *holPtoAA*, which was associated with the Hrp box, was co-transcribed with the *corRS*, constituting an operon (*holPtoAA-corR-corS*). Furthermore, we also show that mutations in *corR* and *corS* have regulatory effects on the expression of *hrpL* and *hrpA*. This finding was validated in gel shift studies showing that CorR binds to nucleotide sequences upstream of *hrpL*. To our knowledge, these results provide the first example showing that *cor* regulatory system can directly impact the expression of *hrp* regulon in *P. syringae*. We also used capillary zone electrophoresis as a fast and accurate method for quantification of CMA in PG4180, DC3000 and selected mutants.

Advisor's Approval: Dr. Carol Bender