Identification and Characterization of a Well-Defined Series of Coronatine Biosynthetic Mutants of *Pseudomonas syringae* pv. *tomato* DC3000

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To identify *Pseudomonas syringae* pv. *tomato* genes involved in pathogenesis, we carried out a screen for Tn5 mutants of *P. syringae* pv. *tomato* DC3000 with reduced virulence on *Arabidopsis thaliana*. Several mutants defining both known and novel virulence loci were identified. Six mutants contained insertions in biosynthetic genes for the phytotoxin coronatine (COR). The *P. syringae* pv. *tomato* DC3000 COR genes are chromosomally encoded and are arranged in two separate clusters, which encode enzymes responsible for the synthesis of coronafacic acid (CFA) or coronamic acid (CMA), the two defined intermediates in COR biosynthesis. High-performance liquid chromatography fractionation and exogenous feeding studies confirmed that Tn5 insertions in the *cfa* and *cma* genes disrupt CFA and CMA biosynthesis, respectively. All six COR biosynthetic mutants were significantly impaired in their ability to multiply to high levels and to elicit disease symptoms on *A. thaliana* plants. To assess the relative contributions of CFA, CMA, and COR in virulence, we constructed and characterized *cfa6 cmaA* double mutant strains. These exhibited virulence phenotypes on *A. thaliana* identical to those observed for the *cmaA* or *cfa6* single mutants, suggesting that reduced virulence of these mutants on *A. thaliana* is caused by the absence of the intact COR toxin. This is the first study to use biochemically and genetically defined COR mutants to address the role of COR in pathogenesis.

Additional keywords: disease lesions.

Pseudomonas syringae is a gram-negative, necrogenic pathogen that elicits a wide variety of symptoms on plants, including blights, cankers, leaf spots, and galls. The infection of host plants by *P. syringae* is a multistep process involving epiphytic colonization, entry, and establishment of infection sites in the

intercellular spaces (the apoplast) of the host tissue. *P. syringae* is an extracellular pathogen that grows to high levels in the apoplast and generally causes damage to host tissue that results in production of disease symptoms. The pathogen virulence factors involved in these processes are largely unknown, and how they function to modify host cell signaling and physiology is not well understood.

To identify genes involved in *P. syringae* pathogenesis, several groups have conducted screens for mutants with reduced virulence. These studies have resulted in the identification of several pathogenicity and virulence determinants, including *hrp/hrc* genes (Cuppels 1986; Somylai et al. 1986), global regulators governing the production of secreted virulence factors (Kitten et al. 1998), and phytotoxins (Bender et al. 1999). The *hrp/hrc* genes encode a specialized (type III) protein secretion system that mediates transfer of bacterial proteins (referred to as “effectors”) into the cytosol of eukaryotic host cells (Alfano and Collmer 1997; Collmer et al. 2002; Galan and Collmer 1999; Greenberg and Vinatzer 2003). The *hrp/hrc* secretion system is required for *P. syringae* to grow and cause disease on susceptible host plants and to elicit plant defense responses (often characterized by the hypersensitive response [HR]) in resistant plants (Alfano and Collmer 1997; Hirano et al. 1999; Lindgren et al. 1986). These host responses are elicited because the *hrp/hrc*-encoded type III secretion system delivers multiple effectors (e.g., virulence or host range determinants) to the host cell.

In addition to the *hrp/hrc* type III secretion system and its effectors, many *P. syringae* strains produce phytotoxins that induce chlorosis (e.g., coronatine, phaseolotoxin, tabtoxin, tagetitoxin) or necrosis (e.g., syringomycin, syringopeptin) (Bender et al. 1999). Coronatine (COR) is a chlorosis-inducing phytotoxin produced by several *P. syringae* pathovars including pvs. *atropurpurea*, *glycinea*, *maculicola*, and *tomato* (Bender et al. 1987; Moore et al. 1989; Sato et al. 1983; Ullrich et al. 1993; Zhao et al. 2000). COR contributes to *P. syringae* growth and lesion formation or expansion in several host plants, including ryegrass, soybean, tomato, and several crucifers (Bender et al. 1987; Budde and Ullrich 2000; Mittal and Davis 1995; Sato et al. 1983). COR consists of two distinct moieties that are joined by an amide linkage (Fig. 1): i) the polyketide coronafacic acid (CFA), and ii) coronamic acid (CMA), an ethylcyclopropyl amino acid (Ichihara et al. 1977). COR, especially the CFA moiety, shares structural and functional relatedness with methyl jasmonate (Fig. 1), a plant growth regulator and defense

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signaling molecule that is produced under conditions of biological stress (Wasternack and Parthier 1997). The CMA moiety of COR also has biological activity (Toshima et al. 1993) and resembles aminocyclopropyl carboxylic acid, the immediate precursor to ethylene (Fig. 1). The relative contributions and modes of action of CMA, CFA, and COR in the pathogenesis of *P. syringae* are not well understood.

We are interested in the molecular mechanisms governing susceptible interactions between *P. syringae* pv. *tomato* DC3000 and its hosts. *P. syringae* pv. *tomato* DC3000 has become an important model organism for studying the molecular basis of pathogenesis, as this strain infects several plant species, including tomato, several crucifers, and the model plant *Arabidopsis thaliana* (Moore et al. 1989; Whalen et al. 1991; Zhao et al. 2000). Further, the recent completion of the *P. syringae* pv. *tomato* DC3000 genome sequence (Buell et al. 2003) has greatly facilitated the identification of potential type III effector molecules (Collmer et al. 2002; Greenberg and Vinatzer 2003). In order to identify additional factors involved in pathogenesis, we initiated a screen for Tn5 insertion mutants of *P. syringae* pv. *tomato* DC3000 with reduced or altered virulence on *A. thaliana*. This has resulted in the identification of several insertion mutants defining both known and novel virulence loci. Here, we present the characterization of several mutants that comprise the first biochemically defined series of *P. syringae* pv. *tomato* DC3000 mutants disrupted for COR biosynthesis. These mutants will facilitate studies designed to elucidate the mechanism of action of CFA, CMA, and COR in pathogenic interactions with *A. thaliana*, a well-established model plant host that is widely used for molecular genetic studies of plant-pathogen interactions.

RESULTS

Isolation of *P. syringae* pv. *tomato* DC3000 mutants with reduced virulence.

We initiated a screen for mutants with reduced or altered virulence to identify additional genes that contribute to the pathogenesis of *P. syringae* pv. *tomato* DC3000. Given that we are interested in the overall process of infection and disease production, we chose to inoculate the plants using the Silwet L-77 dipping procedure, a surface inoculation method that requires the pathogen to enter the leaf, colonize and grow to high levels within the apoplast, and elicit disease symptoms. Thus, this approach should allow the identification of mutants with defects in any one of the above steps in pathogenesis. Preliminary results of this screen are reported elsewhere (Kloek et al. 2000).

In the current study, 947 individual Tn5 mutants of *P. syringae* pv. *tomato* DC3000 were used to dip-inoculate *A. thaliana* plants, which were then examined for disease symptoms 4 days after inoculation. Disease symptoms caused by wild-type *P. syringae* pv. *tomato* DC3000 consist of small water-soaked lesions surrounded by regions of chlorosis (Fig. 2A and D). Mutant strains that caused few or no water-soaked lesions, or little or no chlorosis, or both were chosen for further characterization. A total of 37 mutants consistently exhibited reduced virulence in multiple infection experiments and were confirmed to possess a single Tn5 insertion in DNA hybridization experiments (data not shown). To identify mutants potentially defective in type III secretion, the mutants were assayed for their ability to elicit macroscopic tissue collapse indicative of the HR on the nonhost plant, tobacco. When infiltrated at high densities (1×10^8 CFU/ml) into tobacco leaves, six mutants failed to cause a HR, suggesting that these strains were impaired for type III secretion. Further genetic and molecular analyses confirmed that the Tn5 insertions in these six mutants mapped to various genes in the *hrp/hrc* locus (data not shown). As the

P. syringae *hrp/hrc* genes have been well characterized (Alfano and Collmer 1997), these mutants were not analyzed further. The Tn5 mutants were also tested for their ability to grow on minimal media. Eighteen mutants were determined to be auxotrophs and grew on minimal media only when supplemented with the appropriate nutrients (discussed below).

Identification of genes disrupted by Tn5 insertions.

The insertion sites for 18 HR⁺ mutants, including five of the auxotrophs, were determined using two approaches. The first involved cloning the Tn5 and flanking *P. syringae* pv. *tomato* DC3000 genomic DNA into pBluescript II KS(+) and using an oligonucleotide primer complementary to the *uidA* gene (encoded within the transposon) to sequence the adjacent *P. syringae* pv. *tomato* DC3000 genomic DNA. The resulting nucleotide sequence data was then used to search for nucleotide and amino acid sequence similarities in existing databases. Using this approach, we mapped five of the Tn5 insertions, three to the following known genes (Table 1): i) *avrE* (AK6G11) (Lorang and Keen 1995; Lorang et al. 1994); ii) *dsbA* (AK3A1) (Kloek et al. 2000); and iii) the presumed regulatory region of the CMA biosynthetic operon (AK6F3) (Table 1 and Fig. 3, discussed below), and two insertions to novel genes that will be described elsewhere. The insertion sites in the 13 remaining HR⁺ mutants were determined using the second approach, which involved direct sequencing of genomic DNA flanking the Tn5 element. This resulted in the identification of six independent mutants with Tn5 insertions in genes encoding biosynthetic enzymes for the *P. syringae* phytotoxin COR (Table 1 and Fig. 3) (Bender et al. 1999). These include insertions in *cfa6* (DB4G3), *cfa7* (DB3E8, DB5A6, and DB3A5), *cfa8* (DB1G7), and *cmaA* (AK7E2). The *cma* and *cfa* loci encode enzymes involved in the synthesis of CMA and CFA, respectively, the two compounds that are ligated together to form COR (Fig. 1) (Bender et al. 1999). Two additional novel mutants were identified using this approach and will be described elsewhere.

The five auxotrophs analyzed in this manner carry insertions in *purE* (purine biosynthesis; mutant DB5B9), *his4* (histidine biosynthesis; DB3D2), *pyrB* and *carA* (pyrimidine biosynthesis; DB4C6 and DB1F4), and *ilvC* (branched chain amino

Fig. 1. Structures of the *Pseudomonas syringae* phytotoxin coronatine (COR), the coronafacic acid (CFA) and coronamic acid (CMA) moieties, and the endogenous plant compounds methyl jasmonate (MeJA) and aminocyclopropyl carboxylic acid (ACC), the precursor to ethylene.

acid biosynthesis; DB4G12). The auxotrophic requirements of these mutants were confirmed by their ability to grow on minimal media supplemented with the appropriate nutrients (data not shown). The insertion sites of the remaining 13 auxotrophs were not determined. However, by growing these strains on minimal media supplemented with different pools of nutrients (Maloy et al. 1994), we determined that they were deficient in the production of histidine, tryptophan, proline, branched chain amino acids (isoleucine, leucine, and valine), purines, uracil, or pyrimidines. The inability of these mutants to multiply to high levels in *A. thaliana* leaves, even when infiltrated at relatively high doses into leaf tissue (data not shown), suggests that the nutrients required for multiplication by these auxotrophs are not readily available in the apoplast of *A. thaliana* leaves.

The coronatine biosynthetic cluster of *P. syringae* pv. *tomato* DC3000.

Although several *P. syringae* pv. *tomato* DC3000 mutants defective in COR production have been previously described (Ma et al. 1991; Moore et al. 1989), the precise transposon insertion sites in these mutants have not been identified, nor have these mutants been characterized for the production of additional coronafacoyl compounds. Further, the COR biosynthetic genes from *P. syringae* pv. *tomato* DC3000 have not been well characterized. To further investigate these genes, we isolated and characterized a series of cosmid clones carrying the COR genes (Fig. 3A; discussed below). We also initiated the annotation of the *P. syringae* pv. *tomato* DC3000 COR biosynthetic genes, using a draft copy (release date 11/26/02) of the genomic sequence obtained from the TIGR genome project (The Institute for Genomic Research, *personal communication*). These analyses revealed that the COR biosynthetic genes of *P. syringae* pv. *tomato* DC3000 are arranged in two major clusters that are separated by 26 kb of intervening DNA (Fig. 3A). The CMA biosynthetic genes (e.g., *cmaABT* and *U*) and *corR*, *corS*, and *corP*, which regulate COR production in *P. syringae* pv. *glycinea*, are located adjacent to each other in a

cluster that spans 9.0 kb (Fig. 3A and B). This region also contains several open reading frames (ORF) predicted to encode additional proteins that may be involved in CMA and COR synthesis (Buell et al. 2003). The 22-kb operon required for CFA synthesis constitutes the second gene cluster (Fig. 3C). The finding that the *P. syringae* pv. *tomato* DC3000 COR biosynthetic genes map to two distinct clusters on the chromosome is of interest, as the *cfa* and *cma* genes of *P. syringae* pv. *glycinea* PG4180 map to a contiguous region that is located on the endogenous plasmid p4180A (Bender et al. 1993).

Quantification of COR and CFA production in the COR insertion mutants.

To determine whether the mutants carrying Tn5 insertions in the COR biosynthetic genes resulted in impaired COR production, these strains were analyzed for production of CFA and COR in culture, using a high-performance liquid chromatography (HPLC) fractionation method developed specifically for *P. syringae* pv. *tomato* DC3000 (discussed below). Using this method, we found that *P. syringae* pv. *tomato* DC3000 produced very little free CFA (48 ng per ml of culture), presumably because most of it was converted to COR. The *cmaA* (AK7E2) mutant did not synthesize significant amounts of COR (Fig. 4A) but produced higher amounts of CFA (611 ng/ml) relative to the wild-type *P. syringae* pv. *tomato* DC3000. This is consistent with a biosynthetic block in the production of CMA, which results in the increased accumulation of CFA, coronafacoyl amides (Ullrich et al. 1994), or both. None of the five mutants containing Tn5 insertions in *cfa* genes (DB4G3, DB3E8, DB5A6, DB3A5, and DB1G7) produced detectable levels of either CFA or COR. Mutant AK6F3, which contains an insertion a few nucleotides upstream of the start codon of PSTO4707, the first ORF in the CMA biosynthetic gene cluster (Fig. 3B), produced negligible amounts of CFA and no COR. A precise role for this gene in COR biosynthesis has not been defined (Buell et al. 2003).

To further define the biosynthetic defects in these mutants, we carried out exogenous feeding studies of two representative

Fig. 2. Disease symptoms caused by *Pseudomonas syringae* pv. *tomato* DC3000 or the indicated mutants on whole *Arabidopsis thaliana* plants (A, B, and C) and individual *A. thaliana* leaves (D, E, and F). **A, and D,** wild-type *P. syringae* pv. *tomato* DC3000; **B, and E,** *cfa6* (DB4G3); **C, and F,** *hrcC* (CB200). Plants were inoculated by dipping in a suspension of approximately 4×10^8 CFU of the indicated mutant per ml of solution containing 0.02% Silwet L77 and 10 mM $MgCl_2$. The arrowhead in E indicates the flecking and pitting symptoms observed on plants inoculated with COR⁻ strains. Plants were photographed 4 days postinoculation. Similar results were observed in a minimum of three additional independent experiments.

mutants, *cfa6* (DB4G3) and *cmaA* (AK7E2). Supplementation of the *cmaA* mutant (AK7E2) with exogenous CMA but not CFA restored COR production to this mutant, confirming that AK7E2 is defective in CMA production but not in CFA production or the ability to form COR (Fig. 4A). Similarly, when the *cfa6* mutant (DB4G3) was supplied with exogenous CFA (but not CMA), COR was produced (Fig. 4A), indicating that DB4G3 is impaired only in CFA production. The cocultivation of AK7E2 and DB4G3 also resulted in COR production (Fig. 4B), which is consistent with the secretion and uptake of CFA and CMA by *P. syringae* cells in the fermentation. Thus, the mutants that carry Tn5 insertions in the *cfa* and *cma* genes are COR biosynthetic mutants that are disrupted for CFA and CMA synthesis, respectively.

Complementation of COR production in culture.

To confirm that impaired COR production of the COR biosynthetic mutants is due to the gene disruptions caused by the Tn5 insertions, we sought to complement the mutants with wild-type genomic clones corresponding to the *P. syringae* pv. *tomato* DC3000 *cma* and *cfa* gene clusters. Since we obtained several *cfa* mutants, we selected mutant DB4G3 as a representative of this group for these studies, as this strain contains an insertion in *cfa6* that encodes a large multifunctional

polyketide synthase that is essential for CFA synthesis (Fig. 3; Rangaswamy et al. 1998a). It also carries the insertion closest to the 5' end of the *cfa* operon obtained in our screen and, thus, is most likely to represent the strongest mutation in our series of *cfa* mutants.

Cosmid pL5B4, which carries the genes involved in CMA synthesis (Fig. 3A), was introduced into mutant AK7E2, and cosmid pLB4, which contains the entire *cfa* operon (Fig. 3A), was introduced into DB4G3. The resulting strains were then analyzed for COR production in vitro. As is summarized in Table 2, introduction of the wild-type *cma* clone restored COR production to approximately 60% of the level observed in *P. syringae* pv. *tomato* DC3000. Thus, the COR biosynthetic defect in mutant AK7E2 was partially complemented by pL5B4. In contrast, strain DB4G3 carrying the CFA biosynthetic operon on cosmid pLB4 did not produce detectable levels of COR (Table 2). However, this strain consistently synthesized 80 ng of CFA per ml, which is comparable to the 48 ng of CFA per ml synthesized by *P. syringae* pv. *tomato* 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.

Table 1. Bacterial strains and vectors used in this study

Strain or plasmid	Relevant characteristics ^y	Reference or source
<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	Derivative of NCPPB1106; Rif ^r	Cuppels 1986
<i>P. syringae</i> pv. <i>tomato</i> DC3000 transposon mutants ^z		
DB4G3	<i>cfa6</i> ::Tn5 <i>uidA</i> Km ^r ; CFA ⁻ CMA ⁺ COR ⁻	This study
DB3E8	<i>cfa7</i> ::Tn5 <i>uidA</i> Km ^r ; CFA ⁻ COR ⁻	This study
DB5A6	<i>cfa7</i> ::Tn5 <i>uidA</i> Km ^r ; CFA ⁻ COR ⁻	This study
DB3A5	<i>cfa7</i> ::Tn5 <i>uidA</i> Km ^r ; CFA ⁻ COR ⁻	This study
DB1G7	<i>cfa8</i> ::Tn5 <i>uidA</i> Km ^r ; CFA ⁻ COR ⁻	This study
AK6F3	<i>cma</i> ::Tn5 <i>uidA</i> Sm/Sp ^r	This study
AK7E2	<i>cmaA</i> ::Tn5 <i>uidA</i> Sm/Sp ^r ; CFA ⁺ CMA ⁻ COR ⁻	This study
DB29	<i>cmaA</i> ::Tn5 <i>uidA</i> Sm/Sp ^r ; <i>cfa6</i> ::Tn5 <i>uidA</i> Km ^r CFA ⁻ CMA ⁻ COR ⁻	This study
DC81	<i>cfa6</i> :: <i>nptII</i> Km ^r , CFA ⁻ COR ⁻	This study
AK87	<i>cmaA</i> ::Tn5 <i>uidA</i> Sm/Sp ^r , <i>cfa6</i> :: <i>nptII</i> Km ^r	This study
	CFA ⁻ CMA ⁻ COR ⁻	
CB200	<i>hrcC</i> ::Tn3 <i>uidA</i> Km ^r	C. Boucher, D. Dahlbeck, B. Staskawicz
<i>P. syringae</i> pv. <i>glycinea</i> strains		
PG4180	CFA ⁺ CMA ⁺ COR ⁺	Young et al. 1992
PG4180.G70	CFA ⁻ CMA ⁺ COR ⁻ Km ^r ; <i>cfl</i> ::Tn5	Liyanage et al. 1995b
PG4180.G87	CFA ⁻ CMA ⁺ COR ⁻ Km ^r ; <i>cfa2</i> ::Tn5	Penfold et al. 1996
PG4180.B21	CFA ⁻ CMA ⁺ COR ⁻ Km ^r ; <i>cfa7</i> ::Tn5	Rangaswamy et al. 1998b
<i>Escherichia coli</i> strains		
DH5α	<i>recA lacZ</i> ΔM15	Invitrogen, Carlsbad, CA, U.S.A.
SM10	<i>E. coli</i> λ- <i>pir</i>	Miller and Mekalanos 1988
S17-1	<i>E. coli</i> λ- <i>pir</i>	Simon et al. 1983
pRK7813-based plasmids		
pRK7813	Broad host range cosmid; RK2 replicon; Tc ^r	Jones and Gutterson 1987
pL5B4	DC3000 genomic clone; complements AK6F3 and AK7E2; Tc ^r	This study
pLB4	DC3000 genomic clone; complements DB4G3; Tc ^r	This study
Other plasmids		
pBluescript KS or SK	Cloning vectors; Ap ^r	Stratagene, La Jolla, CA, U.S.A.
pBSL14	Contains 1.2-kb <i>nptII</i> cassette Ap ^r , Km ^r	Alexeyev 1995
pFA1R	7.0-kb EcoRI fragment from pEC18 in pBluescript SK+; contains part of <i>cfa7</i> and <i>cfa8</i> ; Ap ^r	Wang et al. 2002
pCAM140	Mob ⁺ ; delivery vector for mini-Tn5 Sm/Sp <i>uidA</i> ; Ap ^r	D. Bauer
pUT Km	<i>tnp*</i> ; <i>mob</i> RP4; R6K replicon; delivery vector for mini-Tn5 <i>uidA</i> Km; Km ^r Ap ^r	S. Y. He
pRK2013	Tra ⁺ , ColE1 replicon; Km ^r	Figurski and Helinski 1979
pME6031	Replicating plasmid, stable in <i>P. syringae</i> ; Tc ^r	Heeb et al. 2000
pDB628	pME6031 containing the 6.5-kb <i>cma</i> operon; Tc ^r	This study
pRK415	Replicating plasmid, unstable in <i>P. syringae</i> ; Tc ^r	Keen et al. 1988
pDB29	pRK415 containing the DB4G3 <i>cfa6</i> mutation; Tc ^r	This study
pHLC4	pLAFR3 cosmid containing <i>cfa</i> operon from <i>P. syringae</i> pv. <i>glycinea</i> PG4180; Tc ^r	Liyanage et al. 1995a

^y Rif^r, Km^r, Sm^r, Ap^r, Tc^r, and Sp^r = resistance to rifampicin, kanamycin, streptomycin, ampicillin, tetracycline, and spectinomycin, respectively.

^z The location of each Tn5 insertion was determined by sequencing from the 3' end of the *uidA* gene internal to the Tn5 element into flanking *P. syringae* pv. *tomato* DC3000 genomic DNA.

To confirm that the entire CFA biosynthetic cluster was contained on pLB4, we also tested whether pLB4 could restore COR production to derivatives of *P. syringae* pv. *glycinea* PG4180 that are defective in CFA production (Liyanage et al. 1995b; Penfold et al. 1996; Rangaswamy et al. 1998b). In this experiment, we utilized mutants PG4180.G70, PG4180.G87, and PG4180.B21, which contain Tn5 insertions in *cfl*, *cfa2*, and *cfa7*, respectively; these three genes are essential for CFA and COR production in PG4180 (Liyanage et al. 1995b; Penfold et al. 1996; Rangaswamy et al. 1998b). When pLB4 was introduced into these three mutants, COR production was approximately 50% of that produced by the wild-type PG4180 (Table 2). The ability of pLB4 to restore COR production to *cfa* mutants of PG4180 supports our hypothesis that this cosmid contains all genes necessary for production of CFA. The partial complementation of COR production in PG4180 may explain why pLB4 did not restore production of COR to levels above the threshold of detection in DB4G3; this is particularly relevant given the fact that PG4180 synthesizes over 40-fold more COR in vitro as compared with *P. syringae* pv. *tomato* DC3000 (Table 2).

P. syringae pv. *tomato* DC3000 COR mutants exhibit reduced symptom production and growth in *A. thaliana*.

Although several well-characterized COR biosynthetic mutants of *P. syringae* pv. *glycinea* are available, they have not been used to study the contribution of COR to pathogen virulence. We took advantage of our biochemically and genetically defined COR biosynthetic mutants to investigate this question in the *P. syringae*–*A. thaliana* interaction. To more thoroughly characterize the reduced virulence of the *P. syringae* pv. *tomato* DC3000 COR biosynthetic mutants, we determined the percentage of leaves exhibiting disease symptoms after dip-inoculation into each mutant strain (Ton et al. 1999). When assessed in this manner, the majority of leaves (70%) on plants inoculated with wild-type *P. syringae* pv. *tomato* DC3000 developed disease symptoms consisting of water-soaked lesions and chlorosis (Fig. 2A and D). In contrast, plants inoculated with *hrp/hrc* mutants (e.g., CB200, a *hrcC* mutant) or mock-inoculated with buffer alone did not develop disease symptoms (Fig. 2C and F). All seven of the COR biosynthetic mutants isolated in this screen were dramatically reduced in their ability to elicit disease symptoms

Fig. 3. The phytotoxin coronatine (COR) biosynthetic region of *Pseudomonas syringae* pv. *tomato* 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. 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 region. Several new predicted genes (loci designated 4707, 4708, 4711, and 4713) map within the CMA region containing *cmaA*, *cmaB*, *cmaT*, and *cmaU* (Buell et al. 2003). The genomic DNA cloned in pDB628 is indicated. **C**, The CFA biosynthetic region. A predicted new gene (designated 4688) maps within the CFA region. Plasmid pDB29 contains the *cfa6*::Tn5 insertion from DB4G3 used for generation of the DB29 *cfa6 cmaA* double mutant. Vertical arrows (in B and C) indicate the transposon insertions described in this study, and the strain designation used for each insertion mutant is indicated above the arrow. Abbreviations: K = *KpnI* and B = *BamHI*.

and caused limited chlorosis and lesions on few (<5%) to no leaves (Fig. 2B and E). Interestingly, an additional phenomenon consisting of the development of small chlorotic flecks and occasional gray, necrotic pits was also repeatedly observed on leaves of plants inoculated with these mutants (Fig. 2E).

To determine whether the reduction in disease symptom production was correlated with reduced multiplication in plant tissue, the *cmaA* (AK7E2) and *cfa6* (DB4G3) mutants

were analyzed for their ability to grow in *A. thaliana* leaves after dip inoculation. As shown in Figure 5A, wild-type *P. syringae* pv. *tomato* DC3000 multiplied four to five orders of magnitude over 4 days. In contrast, the *cfa6* and *cmaA* mutants reproducibly exhibited a significant reduction (approximately 100- to 1000-fold) in growth relative to *P. syringae* pv. *tomato* DC3000 at 4 days postinoculation. Interestingly, the reduction in growth of the *cfa* and *cma* mutants was less pronounced when the strains were inocu-

Fig. 4. Biochemical characterization of *Pseudomonas syringae* pv. *tomato* DC3000 mutants lacking coronatine (COR⁻). **A**, Supplementation of mutants *cfa6* (DB4G3), *cmaA* (AK7E2), *cfa6 cmaA* (AK87), and *cfa6 cmaA* (DB29) with exogenous coronamic acid (CMA) and coronafacic acid (CFA). Cultures were incubated for 48 h at 18°C, and equimolar amounts (7 µM) of CFA, CMA, or both were added. Cultures were incubated an additional five days and were analyzed for COR production. **B**, Cocultivation experiments. Pairs of mutants were grown in Hoitink-Sinden medium optimized for COR production broth for seven days and then were analyzed for COR production. Values represent the means from experiments containing three replicate cultures. Vertical lines represent the standard error.

lated by vacuum infiltration (Fig. 5B). Similar results were obtained by Mittal and Davis (1995), who examined growth of strain DC3661, a *P. syringae* pv. *tomato* DC3000 COR-mutant with undefined biochemical and molecular phenotypes. These findings are consistent with the hypothesis that COR is important for either tissue colonization, persistence within host tissue, or both.

To verify that the reduced virulence of the COR mutants is due to the disruption of COR biosynthesis, we assayed virulence of the mutant strains carrying the wild-type genomic clones described above. For the *cma* (AK6F3 and AK7E2) and *cfa6* (DB4G3) mutants, the presence of the corresponding genomic clones (pLB4 or pL5B4; Fig. 3 and Table 1) increased production of disease symptoms but not to the extent observed for *P. syringae* pv. *tomato* DC3000 containing these clones. The inability to fully complement these mutants was correlated with the instability of the pRK7813-based cosmid clones (data not shown). When monitoring in planta growth of both wild-type and mutant strains carrying cosmids pLB4, pL5B4, or the parental pRK7813 vector (Table 1), we observed that less than 25% of the bacteria recovered from plant tissue 4 days after inoculation retained the tetracycline resistance conferred by the cosmid vector. The instability of RK2-based cosmids in the absence of selection (e.g., during growth in plant tissue) has been previously reported (Lorang et al. 1994).

To overcome this problem, we generated a smaller subclone carrying the *P. syringae* pv. *tomato* DC3000 CMA biosynthetic genes in pME6031, a plasmid vector that is very stable in *P. syringae* (Heeb et al. 2000). This clone, pDB628, restored the ability of the *cma* (AK6F3 and AK7E2) mutants to grow and cause disease on *A. thaliana* (Figs. 3 and 5C). Thus, the reduction in virulence of the AK6F3 and AK7E2 mutants is due to the disruption of the *cma* genes. Unfortunately, we have not been able to obtain a stable cosmid clone carrying the entire 22-kb *cfa* operon. However, the fact that we identified five independent insertion mutants in the *cfa* operon that exhibit the same combination of impaired CFA production (discussed above) and reduced virulence strongly suggests that the virulence defect of these mutants is due to the disruption of CFA synthesis. Furthermore, the generation of a second, independent mutant carrying an insertion in *cfa6* (DC81; Table 1) that exhibits reduced virulence phenotypes indistinguishable from DB4G3 further supports the causal relationship between the disruption of CFA synthesis and reduced virulence.

Characterization of a CMA⁻ CFA⁻ double mutant of *P. syringae* pv. *tomato* DC3000.

The reduced virulence phenotypes of the *P. syringae* pv. *tomato* DC3000 *cfa* and *cma* mutants indicate that both CFA and CMA are required for full virulence on *A. thaliana*. However, it is not clear from these results whether the CFA and CMA moieties contribute individually to virulence or, alternatively, whether they are required only for synthesis of COR and it is the intact COR molecule that is required for full virulence. To assess the relative contributions of CFA, CMA, and COR in virulence, we constructed two independent CFA⁻ CMA⁻ double mutants of *P. syringae* pv. *tomato* DC3000. The first of these were generated by introducing the *cfa6::Tn5* mutant allele from DB4G3 into the *cmaA* (AK7E2) mutant by marker replacement (Table 1). A second *cfa6 cmaA* double mutant, AK87, was obtained by introducing a *cfa6::nptII* insertion mutation derived from strain PG4180 into AK7E2 (Table 1). The *cfa6::nptII* mutation from PG4180 was also recombined into wild-type *P. syringae* pv. *tomato* DC3000 to generate an independent *cfa6* mutant, DC81. Both *cmaA cfa6* double mutants were deficient in COR and CFA synthesis but synthesized COR when both CFA and CMA were supplied exogenously (Fig. 4A).

We also investigated the ability of pairs of mutants to synthesize COR when cocultivated. Cocultivation of the *cfa6* (either DB4G3 or DC81) and *cmaA* (AK7E2) mutants resulted in COR production (Fig. 4B). These results are consistent with the secretion and uptake of CFA and CMA during fermentation. However, when either DB4G3 or AK7E2 were cocultivated with the *cfa6 cmaA* double mutants (DB29 and AK87), no COR was synthesized, further confirming the phenotype of the two double mutants.

The *cfa6 cmaA* double mutant strains exhibited reduced virulence phenotypes on *A. thaliana* that were essentially identical to those observed for the *cmaA* or *cfa6* single mutants. This was evident both in the reduction in growth of the double mutants on *A. thaliana* plants (Fig. 5A) and in the symptoms on plants infected with the double mutants, which were indistinguishable from plants infected with the *cfa6* and *cmaA* single mutants (data not shown). Thus, using the above infection assays, we were not able to detect a significant role for the individual CFA and CMA compounds in virulence of *P. syringae* pv. *tomato* DC3000 on *A. thaliana*. These observations suggest that the reduction in virulence of these mutants on *A. thaliana* is caused by the absence of the intact COR toxin.

Table 2. Coronatine (COR) production in vitro for complemented strains^u

Strain	Cosmid ^v		
	None	pL5B4 (CMA-REG)	pLB4 (CFA)
<i>P. syringae</i> pv. <i>tomato</i> DC3000 ^w	549 a	NT ^x	NT
AK7E2 (<i>cmaA</i>)	0 c	345 b	NT
DB4G3 (<i>cfa6</i>)	0 c	NT	0 ^y c
PG4180 ^z	25,000 a	NT	NT
PG4180.G70 (<i>cfl</i>)	0 c	NT	11,286 b
PG4180.G87 (<i>cfa2</i>)	0 c	NT	11,023 b
PG4180.B21 (<i>cfa7</i>)	0 c	NT	12,220 b

^u Values represent the means of three or more replicate cultures (10-ml volumes) in Hoitink-Sinden medium optimized for COR production. All experiments were repeated twice with similar results. COR is expressed in ng per mg of protein. The detection level for COR using this method was 40 ng.

^v pL5B4 contains the *cma* genes (e.g., *cmaABT* and *cmaU*) and the *cor* regulatory region (*corRPS*); pLB4 contains the genes involved in coronafacic acid (CFA) biosynthesis (*cfl*, *cfa1-9*).

^w Levels of COR produced by *P. syringae* pv. *tomato* DC3000, AK7E2, and DB4G3 were subjected to statistical analysis using the Student-Newman-Keul's test; values within this data set that are followed by the same lower case letter are not significantly different at $P = 0.01$.

^x NT = not tested.

^y DB4G3 (pLB4) produced 80 ng of CFA per ml, compared with 0 ng per ml detected for DB4G3.

^z PG4180, PG4180.G70, PG4180.G87, and PG4180.B21 were analyzed together as described above; values within this data set that are followed by the same lower case letter are not significantly different at $P = 0.01$.

DISCUSSION

To identify *P. syringae* genes that play a role in pathogenesis, we isolated mutants of *P. syringae* pv. *tomato* DC3000 that exhibit reduced virulence on *A. thaliana*. Our screening strategy has proven very successful. We have isolated mutants with distinctly different virulence phenotypes, ranging from complete impairment of pathogenesis (e.g., *hrp/hrc* and auxotrophic mutants) to those exhibiting a quantitative reduction in virulence (e.g., COR biosynthetic mutants). The fact that we isolated this latter class of mutants can be attributed to the fact that the dip-inoculation method used in this screen allows for detection of mutants that exhibit a partial reduction in virulence or only subtle changes in disease symptom production. Virulence has been found to be multifactorial (Alfano and Collmer 1996; Lamb et al. 1989), and the disruption of individual virulence factors that contribute in a quantitative manner to tissue colonization, persistence in host tissue, or symptom formation may not be detectable unless a sensitive assay is employed. Likewise, dip inoculation allows for the isolation of mutants that affect epiphytic growth, entry, or colonization of the apoplast. Such mutants are likely to be overlooked in screens relying on pressure (e.g., syringe) or vacuum-infiltration, as these methods often involve introduction of large numbers of bacteria and, thus, may bypass critical steps needed during early colonization of the apoplast by small numbers of bacteria. Our success with the preliminary analysis of mutants from this screen suggests that continued use of this approach should result in identification of additional virulence factors in the future.

As expected, we isolated several auxotrophic mutants. These included strains requiring histidine, tryptophan, proline, branched chain amino acids, purines, uracil, and pyrimidines. The fact that these mutants are impaired for growth in *A. thaliana* leaves (data not shown) suggests that the concentrations of these nutrients are limiting in the intercellular space. Interestingly, growth of an *ilvC* mutant (DB4G12) was not significantly impaired in tomato plants (D. Brooks and B. Kunkel, unpublished data), suggesting that tomato and *A. thaliana* leaves may differ in the nutrient pools available in the apoplast.

Several of the reduced virulence mutants isolated in our study carry Tn5 insertions in COR biosynthetic genes (Table 1; Fig. 3), including five mutations mapping to *cfa* genes and one to the *cmaA* gene. A seventh mutation, AK6F3, maps a few nucleotides upstream of the locus designated PSTO4707, the first ORF in the CMA biosynthetic gene cluster of *P. syringae* pv. *tomato* DC3000 (Fig. 3B) (Buell et al. 2003). Given that the *P. syringae* pv. *tomato* DC3000 *cfa* locus is very large (spanning approximately 22 kb; Fig. 3) and thus defines a large target for mutagenesis, it is not surprising that we and others have isolated multiple insertions mapping to this region (Cuppels and Ainsworth 1995; Ma et al. 1991; Moore et al. 1989). However, AK7E2 and AK6F3 are the first mutants known to disrupt CMA biosynthesis in *P. syringae* pv. *tomato* DC3000. Supplementation of the *cfa6* (DB4G3) and *cmaA* (AK7E2) mutants with exogenous CFA or CMA and subsequent HPLC analyses of organic acids demonstrated that these mutants are defective in CFA and CMA production, respectively (Fig. 4A). Thus, these strains represent the first genetically and biochemically-defined *P. syringae* pv. *tomato* DC3000 COR⁻ mutants. Further, the availability of these mutants facilitated the generation of CFA⁻ CMA⁻ double mutants. Collectively, this set of mutants will be extremely valuable in future efforts to dissect the roles of COR, CFA, and CMA in interactions between *P. syringae* pv. *tomato* DC3000 and its plant hosts, which include tomato, multiple

Fig. 5. Growth of representative *Pseudomonas syringae* pv. *tomato* DC3000 Tn5 mutants in *Arabidopsis thaliana*. **A**, Plants were inoculated by dipping in a suspension of approximately 4×10^8 CFU of the indicated mutant per ml containing 0.02% Silwet L77 and 10 mM $MgCl_2$. Leaves were surface-sterilized prior to homogenization; thus, the data reflect internal bacterial populations. **B**, Plants were infiltrated with approximately 5×10^4 CFU of each strain per ml. **C**, Growth of *cmaA* (AK7E2) carrying pDB628, which contains the wild-type *P. syringae* pv. *tomato* DC3000 coronamic acid biosynthetic operon cloned into plasmid pME6031. Plants were dip-inoculated and sampled as described for A. For all experiments, bacterial growth was quantified by removing leaf tissue at the indicated times (days postinoculation). Data points represent the average of three or four replicates $\pm$ SEM. Similar results were obtained in three additional independent experiments for A and B, and in a second independent experiment for C.

Brassica spp., and *A. thaliana* (Moore et al. 1989; Whalen et al. 1991; Zhao et al. 2000).

Our preliminary annotation of the *P. syringae* pv. *tomato* DC3000 genomic sequence made available by TIGR has revealed both notable similarities and differences with the PG4180 COR gene cluster. In both PG4180 and *P. syringae* pv. *tomato* DC3000, the CFA and CMA moieties of COR are encoded by large gene clusters (Fig. 3; Bender et al. 1999; Buell et al. 2003). However, in contrast to PG4180, the *P. syringae* pv. *tomato* DC3000 CFA and CMA biosynthetic genes are not contiguous but, instead, map to two discrete regions that are separated by a large (approximately 26 kb) intervening region (Fig. 3). Interestingly, the *corP*, *corR*, and *corS* genes, which regulate COR gene expression in PG4180 (Ullrich et al. 1995), map with the CMA biosynthetic genes in *P. syringae* pv. *tomato* DC3000 but are physically separated from the CFA gene cluster (Fig. 3A).

This difference in the organization of the COR biosynthetic genes in *P. syringae* pv. *tomato* DC3000 and PG4180 suggests that the regulation of COR differs in the two strains, and previous observations support this hypothesis. For example, the amount of COR produced by *P. syringae* pv. *tomato* DC3000 when grown in culture is significantly lower than that produced by PG4180 (Table 2; Peñaloza-Vázquez et al. 2000; Wang et al. 2002). Furthermore, the signals that regulate COR production in the two strains also differ. Unlike PG4180 (Palmer and Bender 1993), COR production by *P. syringae* pv. *tomato* DC3000 is induced in response to plant-derived signals (Boch et al. 2002; Li et al. 1998; Wang et al. 2002). The importance of a "plant factor" in stimulating *cor* gene expression may explain why *P. syringae* pv. *tomato* DC3000 produces 25- to 40-fold less COR than PG4180 in vitro.

Several mechanisms have been proposed for the role of COR in virulence, including the suppression of host defense responses (Budde and Ullrich 2000; Mittal and Davis 1995), the promotion of lesion formation and expansion (Bender et al. 1999), and the triggering of programmed cell death (Yao et al. 2002). However, the mechanisms underlying these activities are not well understood. To learn more about the role of CFA, CMA, and COR in interactions between *P. syringae* pv. *tomato* DC3000 and *A. thaliana*, we characterized the virulence phenotypes of several representative COR⁻ mutants, *cfa6* (DB4G3), *cmaA* (AK7E2), and DB29 and AK87, which are *cfa6 cmaA* double mutants.

A. thaliana plants inoculated with the COR⁻ mutants did not develop typical disease lesions (Fig. 2B and E), and growth of these mutants in plant tissue was compromised (Fig. 5). However, the degree of impairment of growth in planta was dependent on the inoculation method used. When bacteria were introduced at relatively high concentrations into leaves by vacuum infiltration, the mutants initially grew to wild-type levels but then exhibited a small, but reproducible reduction in growth 4 days postinoculation (Fig. 5B). These results indicate that the basic ability of the mutants to grow in *A. thaliana* leaves is not impaired, and that COR promotes either growth, persistence, or both of *P. syringae* pv. *tomato* DC3000 at later stages of infection. However, when *A. thaliana* plants were dip-inoculated with these strains, growth of the mutants was 2.5 to 3 logs lower than observed for wild-type *P. syringae* pv. *tomato* DC3000, and in most experiments, a small impairment in in planta growth was evident by the second day after inoculation (Fig. 5A). These results are essentially consistent with the findings of Mittal and Davis (1995), who reported that a COR⁻ mutant of *P. syringae* pv. *tomato* DC3000 (DC3661) did not grow when dip-inoculated onto *A. thaliana* plants. However, as the nature of the biochemical and genetic defects in DC3661 has not been reported, it is unclear whether

the impaired colonization of DC3661 was due to the absence of COR. Collectively, our results and those reported by Mittal and Davis (1995) suggest that COR has a critical role in the establishment of a successful infection when plants are dip-inoculated, possibly by facilitating either tissue colonization, persistence, or both in host tissue.

The fact that seven independent COR biosynthetic mutants were identified in this screen clearly demonstrates that COR plays a significant role in the virulence of *P. syringae* pv. *tomato* DC3000 on *A. thaliana*. Interestingly, the importance and role of COR in pathogenesis varies among host-pathogen interactions. Both CFA- and CMA-defective mutants of *P. syringae* pv. *glycinea* PG4180 were previously isolated (Bender et al. 1993; Young et al. 1992). These mutants were primarily used to study the biosynthesis and regulation of COR production, and thus, their virulence phenotypes have not been carefully investigated. However, in general, PG4180 COR mutants elicit less chlorosis than the wild-type strain but induce otherwise normal disease lesions on susceptible soybean plants (Bender et al. 1993). Thus, although the growth of these mutants was not monitored in planta, it appears that COR is not required for lesion formation by PG4180. Several coronatine-defective mutants of *P. syringae* pv. *tomato* PT23 have also been identified but were not mapped to specific biosynthetic genes (Bender et al. 1987, 1989). When inoculated onto susceptible tomato plants, growth of the PT23 COR⁻ mutants was impaired and disease lesions were significantly smaller than those elicited by wild-type PT23, indicating that COR is required both for pathogen growth and lesion expansion in tomato. Thus, although COR is required for growth or persistence, or both, in host tissue in both the PT23-tomato and *P. syringae* pv. *tomato* DC3000-*A. thaliana* interactions, the absence of COR in the *P. syringae* pv. *tomato* DC3000-*A. thaliana* interaction results in a much more severe reduction in disease symptom production.

An additional symptom, consisting of small chlorotic flecks and occasional gray, necrotic pits reminiscent of a localized (or "punctate") HR, was repeatedly observed on leaves of *A. thaliana* plants inoculated with the COR biosynthetic mutants (Fig. 2E, arrowhead). These observations suggest that inoculation of *A. thaliana* plants with COR⁻ strains may elicit a unique host response, perhaps similar to defense responses observed upon inoculation with avirulent strains. These findings are consistent with the hypothesis that COR may promote the virulence of *P. syringae* by inhibiting the HR (Budde and Ullrich 2000) or by interfering with other aspects of host defense. Mittal and Davis (1995) reported that the expression of several defense-related genes (*PAL*, *GST*, and *ELI3*) was elevated in *A. thaliana* leaves infiltrated with the DC3661 COR⁻ mutant, suggesting that COR may function to suppress host defenses. This hypothesis is compelling, as it provides an explanation for the observation that COR insensitive (*coi1*) *A. thaliana* mutants are less susceptible to virulent strains of *P. syringae* (Feys et al. 1994; Kloeck et al. 2001). It is also consistent with the finding that reduced disease susceptibility in *coi1* plants is correlated with sensitization of the SA-dependent defense response pathway (Kloeck et al. 2001). Taken together, these findings suggest that COR promotes virulence of *P. syringae* pv. *tomato* DC3000 by inhibiting induction of host defense responses that normally deter growth of microorganisms. Production of COR would thus provide *P. syringae* pv. *tomato* DC3000 with a "window of opportunity" during which it can colonize host tissue.

To assess the relative contributions of CFA, CMA, and COR in virulence of *P. syringae* pv. *tomato* DC3000 on *A. thaliana*, we generated two *cfa6 cmaA* double mutants (DB29 and AK87) and then compared the virulence phenotypes of these mutants with *cfa6* and *cmaA* single mutants. Hypothetically, if each

moiety contributes individually to virulence, then the single mutants would be less severely impaired in virulence than the double mutants. Our finding that the double mutants are not more impaired in growth or disease production on *A. thaliana* than the single mutants indicates that neither CFA nor CMA makes a detectable contribution to virulence on this host. Thus, it appears that the intact COR molecule must be produced for maximal virulence on *A. thaliana*. Alternatively, the CFA and CMA moieties could function as separate compounds that act synergistically to promote virulence. We are not presently able to investigate this hypothesis, as we do not have mutants that synthesize the individual CFA and CMA moieties but are blocked in formation of the final COR product. However, once one or more enzymes responsible for ligating CFA and CMA are identified in *P. syringae* pv. *tomato* DC3000, we will generate mutants that will allow us to investigate this possibility. It is also possible that the individual CFA and CMA moieties have subtle effects on *A. thaliana* biology that we are unable to detect using the infection assays employed in this study. Future analysis of the CFA, CMA, and COR biosynthetic mutants using more sensitive assays, including global analysis of host gene expression and infection assays using *A. thaliana* defense and signaling mutants, may reveal roles for these molecules in the *A. thaliana*–*P. syringae* pv. *tomato* DC3000 interaction.

MATERIALS AND METHODS

Bacterial strains, plasmids, and mutagenesis.

The bacterial strains and plasmids used in this study are described in Table 1. The *P. syringae* pv. *tomato* DC3000 *hrcC::Tn3 uidA* mutant (CB200) was constructed by C. Boucher, D. Dahlbeck, and B. J. Staskawicz. A genomic library of *P. syringae* pv. *tomato* DC3000 was generated in pRK7813 as described (Boch et al. 2002). Unless otherwise specified, *P. syringae* strains were grown on King's B (KB) (King et al. 1954), NYG (Daniels et al. 1988), or minimal media containing 0.2% glucose (Huynh et al. 1989) at 28°C, and *E. coli* strains were maintained on Luria-Bertani medium (Sambrook et al. 1989) at 37°C. Antibiotics used for selection of *P. syringae* strains included (in µg/ml): rifampicin, 100; kanamycin, 25; spectinomycin, 100; and tetracycline, 16. Antibiotics used for selection of *E. coli* included (in µg/ml): ampicillin, 100; tetracycline, 10; kanamycin, 25; rifampicin, 100; and spectinomycin, 80. Tn5::*uidA* mini-transposons were introduced into *P. syringae* pv. *tomato* DC3000 by triparental matings with either *E. coli* SM10 carrying the suicide vector pUT (Tn5::*uidA* Km) or *E. coli* S17-1 containing pCAM140 (Tn5::*uidA* Sm/Sp), as described previously (Kloek et al. 2000). Plasmids were introduced into *P. syringae* recipients by triparental mating using the helper plasmid pRK2013 (Figurski and Helinski 1979).

DNA manipulation and sequencing.

Routine DNA manipulations were performed as described (Sambrook et al. 1989). Plasmid mini-preps were performed using the Wizard Plus SV kit (Promega, Madison, WI, U.S.A.). Automated DNA sequencing was performed using Big Dye terminator premix, as recommended by the supplier (Perkin-Elmer, Boston, MA, U.S.A.). Direct genomic sequencing was performed using *P. syringae* pv. *tomato* DC3000 genomic DNA purified with the Genomic-tip system (Qiagen, Valencia, CA, U.S.A.) as template. The "GUS5REV" primer (5'-TGGGGTTTCTACAGGACG-3') was used to sequence genomic DNA flanking the *uidA* gene of the Tn5::*uidA* insertions. Reactions for genomic sequencing were prepared in a total volume of 40 µl with the following components: 16 µl of Big Dye terminator premix, 3 µg of genomic DNA template,

and 13 pmol of primer. Amplification was carried out using the following program: 95°C for 5 min, followed by 99 cycles of the following: 95°C for 30 s, 55°C for 20 s, then 60°C for 4 min. Unincorporated nucleotides were removed by ethanol precipitation prior to automated sequencing.

P. syringae pv. *tomato* DC3000 genomic DNA for cloning and DNA blot analysis was prepared using the Wizard genomic DNA purification kit (Promega). Digoxigenin-labeled and radiolabeled DNA probes were prepared using the Genius system (Boehringer Mannheim, Indianapolis, IN, U.S.A.) and the Redi-Prime kit (Amersham North America, Piscataway, NJ, U.S.A.), respectively. Imaging of hybridization patterns was performed with X-ray film (Eastman Kodak, Rochester, NY, U.S.A.) or with a Personal FX phosphorimager (Bio-Rad, Hercules, CA, U.S.A.).

Bioinformatics.

Database searches were performed with the BLAST 2.0 service of the National Center for Biotechnology Information. Amino acid sequences were aligned with MegAlign (DNA Star, Madison, WI, U.S.A.). Sequence data for *P. syringae* pv. *tomato* DC3000 was obtained from the TIGR website.

Isolation and characterization of wild-type genomic clones.

To obtain *P. syringae* pv. *tomato* DC3000 genomic clones spanning the Tn5 insertions, Southern blots of digested genomic DNA from selected mutants were hybridized with a labeled Tn5 fragment to determine the fragment sizes containing the Tn5 element. Digested genomic DNA fragments containing the appropriate size range were ligated into pBluescript II KS(+) (Stratagene, La Jolla, CA, U.S.A.) and transformed into *E. coli*. After plating the transformants onto selective media, resistant colonies were purified and the transposon-containing inserts were isolated and labeled. This DNA was used as a probe to screen the wild-type *P. syringae* pv. *tomato* DC3000 genomic DNA library in pRK7813.

To obtain cosmid clones containing the *P. syringae* pv. *tomato* DC3000 *cfa* operon, the 7.0-kb insert in pFA1R, which contains *cfa7* and a portion of *cfa8* (Rangaswamy et al. 1998a and b) from *P. syringae* pv. *glycinea* PG4180 was used as a probe in colony hybridizations to the *P. syringae* pv. *tomato* DC3000 genomic library. Out of 1,440 clones screened, five cosmids hybridized to the probe, and one of them, pLB4, was selected for further study.

To generate pDB628, which contains the CMA biosynthetic region, a genomic fragment carrying the *cmaABT* and *cmaU* genes as well as newly identified ORF in the CMA region (Fig. 3B) was obtained by PCR amplification using the following primers: CMAU.1REV (5'-GCGCGCTCGAGTTTA TGAATCTCAGGCCACGAGGG-3'; *XhoI* site underlined) and CMAABTFOR4 (5'-GCGCGCATTTAAATCTACGTATA CCTCCAGTAAAGC-3'; *SwaI* site underlined). The resulting 6.5-kb fragment was subcloned into the pCR Blunt II vector supplied with the Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). The resulting plasmid was digested with *XhoI* and *SwaI* to liberate the insert, which was then cloned into pME6031 (Heeb et al. 2000). Prior to ligation with the *cma* insert, pME6031 was digested with *EcoRI*, treated with Klenow to generate blunt ends and, then, digested with *XhoI*.

Molecular characterization of cosmid clones carrying the CFA and CMA biosynthetic operons.

Since cosmid pLB4 was identified based on hybridization to a probe containing only *cfa7* and *cfa8*, we used PCR analysis to determine whether this clone contained the entire COR biosynthetic region. A primer set specific for the *cfl* gene

(Wang et al. 2002), which is located at the 5' end of the CFA region, amplified the 0.65-kb predicted product, confirming the presence of the *cfl* gene on this cosmid. However, primers from the regulatory and CMA biosynthetic region (Wang et al. 2002) did not amplify products from pLB4 (data not shown). Further analysis indicated that pLB4 contains all genes necessary for CFA production but does not contain the *cma* gene cluster or the COR regulatory genes (data not shown). Cosmid pL5B4 was also subjected to PCR analysis to map the extent of this clone. Primer sets specific to the *corR* and the *cmaT-cmaU* genes (Wang et al. 2002) amplified fragments of 342 and 890 bp, respectively, indicating that pL5B4 contains the regulatory and CMA biosynthetic regions. Primers corresponding to the *cfl* gene did not amplify a product from pL5B4, suggesting that the CFA biosynthetic genes are not present on this clone. Portions of pLB4 and pL5B4 were sequenced to confirm that they contained the entire *cfa* and *cma* gene clusters, respectively. Primers were designed based on preliminary genomic data (TIGR), and their sequences are available upon request.

Generation of *cfa6-cmaA* double mutants.

A 7.8-kb *Bam*HI-*Kpn*I genomic fragment containing the 3.2-kb Tn5 insertion in *cfa6* and 4.5 kb of flanking DNA from DB4G3 (*cfa6::Tn5 uidA* Km^r) was cloned into pRK415 to generate pDB29 (Table 1; Fig. 3). This construct was transferred into mutant AK7E2 (*cmaA::Tn5 uidA* Sm/Sp^r) by triparental mating. Several tetracycline-resistant transconjugants were then subcultured in media containing kanamycin, but lacking tetracycline, to allow for marker replacement of the wild-type *cfa6* gene with the Tn5 insertion allele. The final bacterial culture was diluted and plated onto KB containing rifampicin, kanamycin, and spectinomycin. Colonies were screened for tetracycline sensitivity and were subjected to Southern blot analysis. A single strain, designated DB29, was identified, in which the wild-type 4.5-kb *Bam*HI-*Kpn*I fragment carrying *cfa6* was replaced with a 7.8-kb fragment corresponding to the disrupted allele. The presence of the Tn5 insertion in the *cmaA* gene in this mutant was also verified by DNA hybridization.

A second *cfa6 cmaA* mutant, AK87, was generated by introducing the disrupted *cfa6* gene from pCFA6:*nptII* into *cmaA* (AK7E2). The construct pCFA6:*nptII* was constructed by cloning the 8.7-kb *Sst*I fragment containing *cfa6* from cosmid pHLC4 (Liyana et al. 1995a) into pBluescript II SK(+). The *cfa6* allele was then disrupted by inserting the 1.2-kb *Bam*HI-derived *nptII* cassette from pBSL14 (Alexeyev 1995) into a unique *Bgl*II site located in the middle of *cfa6*. pCFA6:*nptII* was introduced into both *cmaA* (AK7E2) and *P. syringae* pv. *tomato* DC3000 by electroporation, followed by selection for recombinants that were ampicillin sensitive. Mutants in which *cfa6* was replaced with the *cfa6::nptII* insertion were confirmed by DNA blot analysis (data not shown).

Plant material and inoculation procedures.

A. thaliana ecotype Colombia (Col-0) and tobacco cultivar Xanthi N/N were used in this study. *A. thaliana* plants were grown from seed in growth chambers with an 8-h photoperiod at 22°C and 75% relative humidity. All *A. thaliana* plants used for virulence studies were four to five weeks old. Tobacco plants were grown in a greenhouse. To assay for disease, dip inoculation of *A. thaliana* was conducted by immersing whole rosettes into bacterial suspensions (approximately 5×10^8 CFU/ml) containing 0.02% Silwet L-77 (OSi Specialties Inc., Danbury, CT, U.S.A.) and 10 mM MgCl₂, as described previously (Kunkel et al. 1993). To monitor bacterial popu-

lations inside the leaf, plants were vacuum-infiltrated, and bacterial growth within plant tissue was monitored as previously described (Whalen et al. 1991). Pipette infiltration to assay for the HR on tobacco was performed using bacterial suspensions of 1×10^8 CFU/ml in 10 mM MgCl₂ (Whalen et al. 1991). Plants were scored 20 h postinoculation for the macroscopic tissue collapse indicative of the HR.

In some experiments, bacterial growth was monitored after dip inoculation. Individual rosette leaves were removed 0, 2, and 4 days postinoculation, were weighed and surface-sterilized in 15% H₂O₂ for 5 min, and then, rinsed twice with sterile water. For the day 0 timepoint, leaf tissue was sampled approximately 1 h after inoculation. The leaves were then homogenized, and appropriate dilutions were plated on media containing the appropriate antibiotics and either amphotericin B (2.5 µg/ml) or cycloheximide (50 µg/ml) to inhibit fungal growth.

Detection of the phytotoxin coronatine.

P. syringae derivatives were incubated in Hoitink-Sinden medium optimized for COR production (HSC) at 18°C, and organic acids were extracted from culture supernatants (10-ml volumes, three- to four-replicate cultures), as described previously (Palmer and Bender 1993). However, it was necessary to develop a new HPLC fractionation method to analyze COR production by *P. syringae* pv. *tomato* DC3000 derivatives, because our prior fractionation method did not accurately separate COR and CFA from unrelated compounds synthesized by *P. syringae* pv. *tomato* DC3000. Organic acids were analyzed on an Ultrasphere C-8 reverse phase column (Beckman Coulter, Fullerton, CA, U.S.A.) at 208 nm. The initial conditions for gradient elution of organic acids were 10% acetonitrile in 0.05% trifluoroacetic acid (pH 3.0). Organic acids were fractionated with a flow rate of 2 ml min⁻¹, using linear gradients of 10 to 30% acetonitrile (6 min) and 30 to 40% acetonitrile (9 min). The system was then returned to initial conditions (10% acetonitrile) in 1 min. Calibration curves for COR and related compounds were constructed with authentic standards and Gold Noveau chromatography software, as described previously (Rangaswamy et al. 1998b). 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 (Peñaloza-Vázquez and Bender 1998). The protein content in bacterial cell lysates was determined with the Bio-Rad protein assay kit (Bio-Rad).

Exogenous feeding 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, DC81, AK87, and DB29 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 5 days at 18°C, and organic acids were extracted and analyzed for COR, as described above.

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