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Regulatory interactions between the Hrp type III protein secretion system and coronatine biosynthesis in *Pseudomonas syringae* pv. *tomato* DC3000

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In *P. syringae*, the co-ordinated regulation of different systems required for pathogenicity and virulence seems logical but has not been established. This question was addressed in the present study by analysing production of the phytotoxin coronatine (COR) in defined *hrp*/*hrc* mutants of *P. syringae* pv. *tomato* DC3000. COR was produced *in vitro* by mutants of DC3000 defective in *hrcC*, which encodes an outer-membrane protein required for type III-mediated secretion. When inoculated in plants, *hrcC* mutants produced chlorotic regions indicative of COR production, but lacked the necrotic lesions produced by the wild-type DC3000. Furthermore, a DC3000 mutant containing a polar mutation in *hrcC*, which inactivates *hrcC*, *hrpT* and *hrpV*, produced significantly higher amounts of COR than the wild-type strain *in vitro*. This mutant was able to produce COR earlier and at lower cell densities than the wild-type. The results indicate that the *hrp*/*hrc* secretion system is not required for COR production, but mutations in this system may have regulatory effects on the production of virulence factors such as COR.

Keywords: type III secretion system, phytotoxin, virulence

INTRODUCTION

Pseudomonas syringae is a Gram-negative, necrogenic bacterium that elicits a wide variety of symptoms in plants, including blights (rapid death of tissue), leaf spots and galls. The species is divided into pathogenic variants (pathovars) which vary in host range. The genetic basis of pathogenicity and virulence in *P. syringae* is complex and includes global regulators (Hrabak & Willis, 1992; Kitten *et al*., 1998; Rich *et al*., 1994), the *hrp* cluster and virulence factors such as phytotoxins and exopolysaccharides (Bender *et al*., 1999; Yu *et al*., 1999). The *hrp* region (*h*ypersensitive *r*esponse and *p*athogenicity) is conserved in phytopathogenic prokaryotes and affects the ability of a bacterium to induce a hypersensitive response (HR) in non-host plants, pathogenicity on host plants and the ability to grow within or on the surface of plants (He, 1998; Hirano *et al*., 1999). A subset of the *hrp* genes was renamed *hrc* (HR and *c*onserved) because of their conservation in the type III secretion apparatus used by *Yersinia*, *Shigella* and *Salmonella* (Bogdanove *et al*., 1996; Galán & Collmer, 1999).

The *hrp*/*hrc* gene cluster has been most extensively characterized in *P. syringae* pv. *syringae* Pss61 where it is located in a 25 kb region of the chromosome and consists of 27 genes organized into eight transcriptional units (Galán & Collmer, 1999; Hutcheson, 1999). Activities in the type III secretion apparatus have been assigned to some of the Hrc proteins. For example, *hrcC* encodes an outer-membrane protein that is essential for type III protein secretion and has a primary role in protein translocation across the outer membrane (Charkowski *et al*., 1997). The *hrcC* gene in *P. syringae* pv. *syringae* Pss61 and pv. *tomato* DC3000 is flanked by four genes, *hrpF*, *hrpG*, *hrpT* and *hrpV*, which constitute an operon (*hrpFGCTV*) (Alfano *et al*., 2000; Preston, 1997; Yuan & He, 1996). *hrpF*, *hrpG* and *hrpT* are thought to encode components of the type III

Abbreviations: COR, coronatine; CFA, coronafacic acid; CMA, coronamic acid; GUS, glucuronidase; HR, hypersensitive response; HSS medium, Hoitink–Sinden medium amended with sucrose; MG medium, mannitol-glutamate medium.

secretion system, whereas HrpV functions as a negative regulator (Preston *et al*., 1998).

The *hrp/hrc* gene cluster is environmentally regulated in *P. syringae* and these genes are rapidly induced following infiltration into host tissue (Hutcheson *et al*., 1996). Several genes in the *hrp* cluster mediate environmental regulation of the *hrp/hrc* regulon. HrpR and HrpS show similarity to response regulators in two-component regulatory systems and function as transcriptional activators of *hrpL* (Xiao *et al*., 1994). *hrpL* encodes a σ factor related to the extracellular factor family of alternate σ factors and is required for expression of several transcripts in the *hrp* gene cluster (Hutcheson, 1999; Xiao *et al*., 1994). HrpV is a negative regulator of *hrp* gene expression that presumably functions upstream of HrpR and HrpS in the *hrp* regulon (Deng *et al*., 1998; Preston *et al*., 1998). *hrpA* encodes the structural protein of the Hrp pilus and is also thought to mediate the expression of *hrpR* and *hrpS* (Roine *et al*., 1997; Wei *et al*., 2000).

In addition to the *hrp/hrc* genes, many *P. syringae* strains produce low-molecular-mass, non-host specific phytotoxins that induce chlorosis or necrosis (Bender *et al*., 1999). The phytotoxin coronatine (COR) is a virulence factor produced by several *P. syringae* pathovars, including *atropurpurea*, *glycinea*, *maculicola*, *morsprunorum* and *tomato* which infect ryegrass, soybeans, crucifers, *Prunus* spp. and tomato, respectively (Bender *et al*., 1999). The primary symptom elicited by COR is a diffuse chlorosis that can be induced on a wide variety of plant species. COR also induces hypertrophy, inhibits root elongation and stimulates ethylene production in some but not all plant species (Kenyon & Turner, 1992).

COR consists of two distinct structural components that function as biosynthetic intermediates: (1) the polyketide coronafacic acid (CFA) and (2) coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine (Mitchell, 1985; Parry *et al*., 1994). The biosynthesis of COR has been intensively studied in *P. syringae* pv. *glycinea* PG4180 where the 32 kb COR gene cluster contains two distinct regions that encode the structural genes for CMA and CFA biosynthesis (Bender *et al*., 1999). The CFA and CMA gene clusters in PG4180 are separated by a 3·4 kb region that controls both CFA and CMA production, and the nucleotide sequence of this region revealed the presence of three regulatory genes, *corP*, *corS* and *corR* (Ullrich *et al*., 1995). The deduced amino acid sequences of *corP* and *corR* indicated relatedness to response regulators which function as members of two-component regulatory systems, and the translational product of *corS* showed similarity to histidine protein kinases which function as environmental sensors (Ullrich *et al*., 1995). Complementation analysis using a *corR* mutant and transcriptional fusions to a promoterless glucuronidase (GUS) gene (*uidA*) indicated that CorR functions as a positive regulator of CFA and CMA gene expression (Peñaloza-Vázquez & Bender, 1998; Wang *et al*., 1999). Although some of the signals for virulence factor production have been defined, the mechanisms used for integration of these signals remain unclear. An especially intriguing question is the potential relationship between virulence factors such as COR and the type III secretion system encoded by the *hrp/hrc* gene cluster. The coordinated regulation of the *hrp/hrc* cluster, which is required for pathogenicity, and virulence factors such as COR seems logical but has not been established. In this study, we investigated the effect of *hrp/hrc* mutations on the expression and biosynthesis of COR in *P. syringae* pv. *tomato* DC3000.

METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were maintained in Luria–Bertani medium or Terrific Broth (Sambrook *et al*., 1989). *P. syringae* pv. *tomato* strains were routinely maintained at 28 °C on King's Medium B (King *et al*., 1954), which contains the following ingredients (l^{-1}): 20 g proteose peptone no. 3 (Becton Dickinson), 1·5 g K_2HPO_4, 1·5 g $MgSO_4.7H_2O$ and 15 ml glycerol. Prior to plant experiments, *Pseudomonas* strains were cultured on mannitol-glutamate (MG) medium (Keane *et al*., 1970), a minimal medium containing the following nutrients (l^{-1}): 10 g mannitol, 2·0 g L-glutamic acid (monosodium salt), 0·5 g KH_2PO_4, 0·2 g NaCl and 0·2 g $MgSO_4.7H_2O$. The pH of MG medium was adjusted to 7·0 with 3 M NaOH prior to autoclaving. In experiments where COR production or GUS activity were quantified, derivatives of *P. syringae* pv. *tomato* DC3000 were grown in Hoitink–Sinden medium amended with sucrose (HSS), which contained the following nutrients (l^{-1}): 1·0 g NH_4Cl, 0·2 g $MgSO_4.7H_2O$, 4·1 g KH_2PO_4, 3·6 g $K_2HPO_4.3H_2O$, 3·42 g sucrose and 2 μM $FeCl_3$. The pH was adjusted to 6·5 with 10 M NaOH before autoclaving and a stock solution containing sucrose was autoclaved separately. The density of bacterial cell suspensions was determined with a Spectronic 20 spectrophotometer (Bausch & Lomb), using a cuvette with a 13 mm path length. When appropriate, antibiotics were added at the following concentrations (μg ml^{-1}): rifampicin, 100; chloramphenicol, 25; kanamycin, 25; tetracycline, 25; spectinomycin, 25.

Molecular genetic techniques. Electrophoresis and small-scale plasmid DNA preparations were performed by standard procedures (Sambrook *et al*., 1989). Plasmids were transformed into *P. syringae* by electroporation as described by Sambrook *et al*. (1989).

Quantitative analysis of COR. *P. syringae* pv. *tomato* DC3000 and derivatives were grown at 18 °C in HSS medium and supernatants were analysed for COR production by HPLC (Palmer & Bender, 1993). COR production was normalized for differences in bacterial growth by expressing the quantity as a function of protein concentration (Peñaloza-Vázquez & Bender, 1998). The protein content in bacterial cell lysates was determined with the Bio-Rad Protein Assay Kit.

Kinetics of COR production. *P. syringae* pv. *tomato* DC3000 and DC3000-*hrcC* were grown for 30 h on MG agar containing rifampicin and chloramphenicol, respectively. The bacteria were then resuspended in HSS medium to an OD_{600} of 0·1 and incubated with shaking (250 r.p.m.) at 18 °C for 10 d. Aliquots of the two strains (three replicates per sampling) were removed at 6, 12, 24, 48, 72, 120, 168 and 240 h, and

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Reference or source
E. coli		
DH5α		Sambrook *et al.* (1989)
JM109		Stratagene
P. syringae pv. *tomato*		
DC3000	Rif^r; COR^+	Moore *et al.* (1989)
D3000-*hrcC*	Cm^r; *hrcC*::Tn5Cm	Yuan & He (1996)
DC3682	Km^r; COR^- Tn5 mutant of DC3000	Ma *et al.* (1991)
CUCPB5112	Km^r; *hrc*Δ::*nptII*; 1·8 kb of *hrcC* was replaced with *nptII* from pCPP2988	W.-L. Deng, Cornell University, NY, USA
PT23.26	Km^r; COR^- Tn5 mutant of PT23.2	Bender *et al.* (1989)
Plasmids		
pCPP2371	Gm^r, contains *hrpV* expressed from *nptII* promoter in pML122	Preston *et al.* (1998)
pRGMU1	Sm^r Sp^r; contains a 2·9 kb *Pst*I insert with the *cmaABT* promoter from the COR gene cluster in pRG960sd	Ullrich & Bender (1994)

* Cm, Chloramphenicol; Gm, gentamycin; Km, kanamycin; Rif, rifampicin; Sm, streptomycin; Sp, spectinomycin.

evaluated for growth by dilution plating and for COR production by HPLC.

COR production at different inoculum densities. To evaluate the effect of inoculum density on COR production, *P. syringae* pv. *tomato* DC3000 and DC3000-*hrcC* were grown for 30 h on MG agar and resuspended in HSS broth to an OD_{600} of 2·5. Tenfold serial dilutions of bacterial suspensions were made in HSS broth, aliquots of each dilution were removed and bacterial concentrations were determined by dilution plating. Each dilution was incubated for 72 h at 18 °C (250 r.p.m.) and analysed for COR by HPLC.

GUS assays. pRGMU1, which contains the *cmaABT*::*uidA* promoter fusion, was previously shown to be transcriptionally active in DC3000 (Rohde *et al.*, 1998). In the present study, DC3000 and DC3000-*hrcC* containing pRGMU1 were grown for 24 h on MG agar containing spectinomycin, inoculated ($OD_{600} = 0·1$) in HSS medium and incubated at 18 °C (250 r.p.m.). Aliquots of cells (three replicates per sampling) were removed at 12, 24, 72 and 120 h after inoculation and analysed for GUS activity as described previously (Palmer *et al.*, 1997). GUS activity was expressed in U (mg protein)$^{-1}$ with 1 U equivalent to 1 nmol methylumbelliferone formed min^{-1}. The protein content in cell lysates was determined using the Bio-Rad Protein Assay Kit as recommended by the manufacturer.

In a second experiment, growth (OD_{600}) and GUS activity were monitored in *P. syringae* pv. *tomato* DC3000(pRGMU1) during a 5 d incubation period. DC3000(pRGMU1) was initially grown for 30 h on MG agar containing spectinomycin, resuspended in HSS medium ($OD_{600} = 0·1$) and incubated at 18 °C (250 r.p.m.). Aliquots of the fermentation (three replicates per time point) were removed at 0, 6, 12, 24, 48, 72 and 120 h after inoculation and evaluated for GUS activity as described above.

Plant inoculations. The virulence of *P. syringae* pv. *tomato* DC3000 and derivatives was evaluated on tomato (*Lycopersicum esculentum* cv. Glamour). Bacterial strains were grown for 48 h on MG agar with antibiotic selection. Bacterial cells were then resuspended in sterile distilled H_2O to an OD_{600} of 0·5 and used to inoculate 3-week-old tomato leaves by infiltration or with a spray inoculation method. In experiments where leaves were infiltrated, bacterial suspensions were adjusted to 10^5, 10^6, 10^7 and 10^8 c.f.u. ml^{-1} and the inoculum was delivered with a 1 ml syringe. When plants were inoculated by spraying, bacterial cells (10^6 c.f.u. ml^{-1}) were applied to tomato leaves with an airbrush ($\sim$ 8 p.s.i.) until leaf surfaces were uniformly wet. Inoculated plants were incubated for 7 d in a growth chamber with a 12 h photoperiod at 24 °C with 48–51% relative humidity.

In experiments designed to follow the population dynamics in tomato, six plants were inoculated with each strain and incubated as described above. Random leaf samples (one leaf per plant, six leaves in total) were removed at each sampling time (0, 1, 2, 3, 5 and 7 d after inoculation). Leaves were weighed separately and macerated in a sterile saline solution (0·85% NaCl) using a mortar and pestle. Bacterial counts were determined by plating dilutions of the leaf homogenate onto MG amended with the appropriate antibiotics. Fluorescent colonies were counted after incubating the plates for 48 h and the experiment was performed twice.

Selected strains were tested for their ability to induce an HR on *Nicotiana tabacum* cv. Petite Havana using established methods (Schaad, 1988).

Susceptibility of plant tissue to COR. The susceptibility of tomato leaf tissue to COR was evaluated using 3-week-old plants of *L. esculentum* cv. Glamour. Tomato leaves (four replicates per concentration) were inoculated with 10 µl solutions containing 0, 0·78, 1·56, 3·125, 6·25, 12·5, 25, 50 or 100 ng COR. Inoculated plants were incubated for 7 d in a growth chamber with a 12 h photoperiod at 24 °C with 48–51% relative humidity.

Complementation experiments. pCPP2371 (containing *hrpV*) was transformed into DC3000-*hrcC* by electroporation.

DC3000, DC3000-*hrcC* and DC3000-*hrcC*(pCPP2371) were incubated for 30 h on MG agar containing the appropriate antibiotics, resuspended in HSS medium to an OD_{600} of 0·1 and incubated at 18 °C (250 r.p.m.). Aliquots of each strain (0·5 ml; three replicates per sampling period) were removed 5 d after inoculation and evaluated for COR production.

RESULTS

COR production by DC3000 and DC3000-*hrcC*

Growth of both DC3000 and DC3000-*hrcC* was similar throughout the 10 d (240 h) sampling period (Fig. 1a). In this experiment, the bacteria were grown in a minimal medium (HSS) at 18 °C, conditions that are suboptimal for rapid growth. In DC3000, COR synthesis increased very slowly, reaching a maximal level of 1·1 μg COR (mg protein)$^{-1}$ at 240 h (Fig. 1b). In contrast, COR production by DC3000-*hrcC* rose dramatically from 12 to 24 h and increased from 2·4 to 9·7 μg COR (mg protein)$^{-1}$ (Fig. 1b). COR production remained high in DC3000-*hrcC* from 24 to 120 h and then decreased (Fig. 1b). These results clearly show that COR can be produced *in vitro* in the absence of a functional *hrcC* gene. Since COR production by DC3000 is normally low *in vitro*, these results suggest that COR synthesis may be derepressed in DC3000-*hrcC*, especially in the earlier phases of the sampling period.

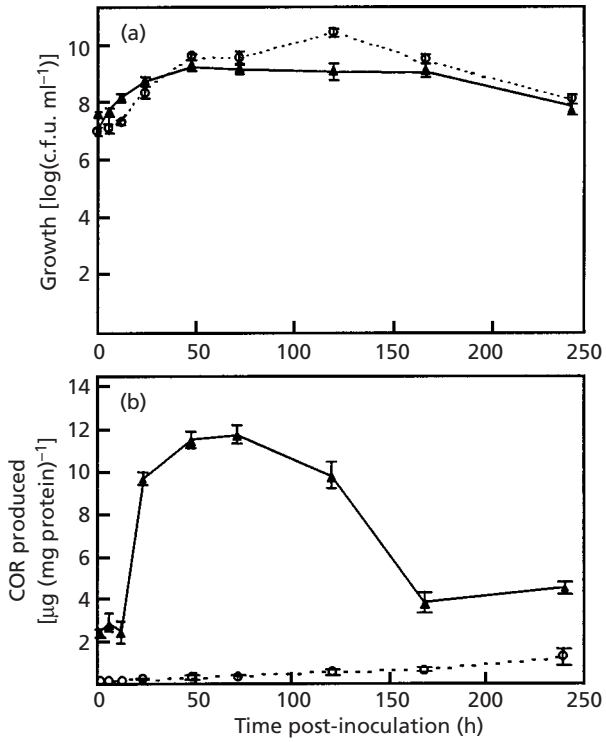

Fig. 1. Growth (a) and production of COR (b) by *P. syringae* pv. *tomato* DC3000 (○) and DC3000-*hrcC* (▲). Values represent the means from one experiment of three replicates per strain and vertical bars indicate the SEM. The experiment was repeated with similar results.

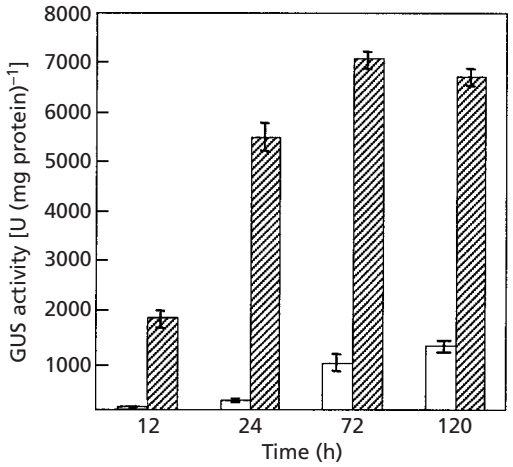

Fig. 2. Temporal expression of the *cmaABT*::*uidA* transcriptional fusion contained in pRGMU1 in *P. syringae* pv. *tomato* DC3000 (white bars) and DC3000-*hrcC* (hatched bars). Values represent the means from one experiment of three replicates per strain and vertical bars indicate the SEM. The experiment was repeated with similar results.

COR gene expression in DC3000 and DC3000-*hrcC*

To further analyse the effect of the *hrcC* mutation on COR synthesis, we used transcriptional fusions to monitor COR gene expression *in vitro*. The gene fusion used in this analysis was the *cmaABT*::*uidA* fusion cloned in pRGMU1. The *cmaABT* transcript contains the structural genes for the biosynthesis of CMA, an intermediate in the pathway to COR. *cmaABT* expression in DC3000 correlated with COR production; for example, transcriptional activity in DC3000(pRGMU1) remained low and increased very slowly (from 45 to 1308 U GUS) during the sampling period (Fig. 2). In contrast, *cmaABT* expression in DC3000-*hrcC* increased rapidly from 1847 (12 h) to 6716 U GUS (120 h) (Fig. 2). Therefore, the results obtained with the *cmaABT* transcriptional fusion correlated with the kinetics of COR production by these two strains.

Plant inoculations

Tomato plants were infiltrated with DC3000, DC3682 (COR$^-$ mutant) and DC3000-*hrcC*. When tomato plants were infiltrated with DC3000 at 10^8 c.f.u. ml^{-1}, a localized rapid, HR-like necrosis developed (data not shown). However, at inoculum concentrations of 10^7, 10^6 and 10^5 c.f.u. ml^{-1}, a necrotic, spreading lesion developed around the infiltration site and this was surrounded by a diffuse yellow chlorosis typical of COR production (Fig. 3a). In plants inoculated with the COR$^-$ mutant DC3682, necrotic lesions developed but no chlorosis was detected regardless of the inoculum level (Fig. 3b). In leaves inoculated with DC3000-*hrcC*, a diffuse chlorosis was evident when leaves were infiltrated with 10^8, 10^7 and 10^6 c.f.u. ml^{-1}, but necrotic lesions did

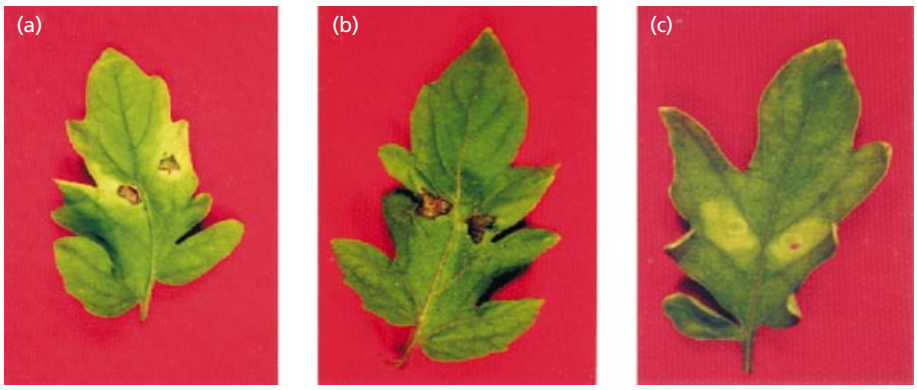

Fig. 3. Symptoms on tomato leaves infiltrated with *P. syringae* pv. *tomato* DC3000 (a), DC3682 (a COR$^-$ mutant of DC3000) (b) and DC3000-*hrcC* (c). Bacterial inoculum (10^6 c.f.u. ml^{-1}) was infiltrated into leaves using a 1 ml syringe without a needle. After inoculation, all plants were incubated in a growth chamber with a 12 h photoperiod at 24 °C with 48–51 % relative humidity. Photographs were taken 7 d after infiltration.

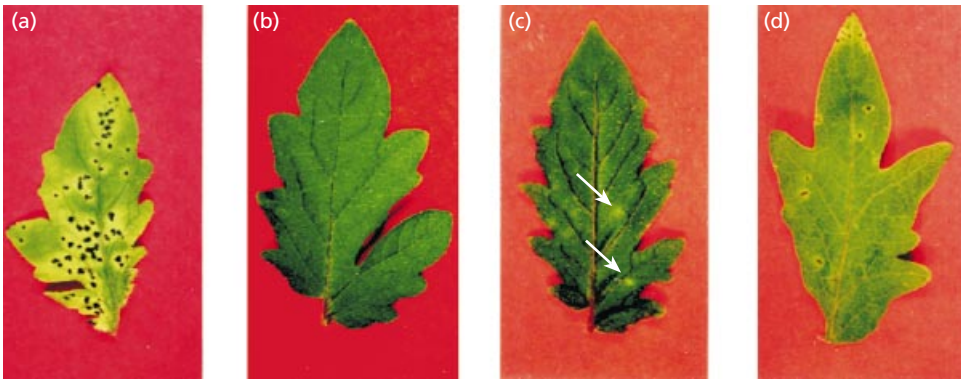

Fig. 4. Tomato leaves sprayed with *P. syringae* pv. *tomato* DC3000 (a), DC3682 (a COR$^-$ mutant of DC3000) (b), DC3000-*hrcC* (c) and PT23.26 (a COR$^-$ mutant of *P. syringae* pv. *tomato* PT23.2) (d). Bacterial inoculum was sprayed (10^6 c.f.u. ml^{-1} at ~8 p.s.i.) onto tomato leaves until surfaces were uniformly wet. After inoculation, all plants were incubated in a growth chamber as described in the legend to Fig. 3 and photographed 7 d after inoculation. The arrows in (c) show the location of chlorotic haloes on plants inoculated with DC3000-*hrcC*.

not develop (Fig. 3c). No chlorosis developed when DC3000-*hrcC* was inoculated at 10^5 c.f.u. ml^{-1} (data not shown), indicating that the inoculum density used for infiltration was a critical factor in obtaining the chlorotic reaction indicative of COR production.

Tomato plants sprayed with *P. syringae* pv. *tomato* DC3000 developed the typical symptoms of bacterial speck disease, which include necrotic lesions surrounded by a diffuse chlorotic halo (Fig. 4a). In contrast, tomato leaves inoculated with the COR$^-$ mutant DC3682 (Fig. 4b) remained symptomless for the duration of the experiment. Leaves inoculated with DC3000-*hrcC* developed small chlorotic haloes without necrosis, which suggested that COR was produced *in planta* (Fig. 4c). Tomato leaves were also spray-inoculated with *P. syringae* pv. *tomato* PT23.26, a COR$^-$ mutant characterized in previous studies (Bender *et al.*, 1989, 1991). Unlike DC3682, PT23.26 produced visible necrotic lesions on tomato leaves (Fig. 4d). Although the lesions were surrounded by a faint chlorotic region, they lacked the diffuse chlorosis typical of COR production (as shown in Fig. 4a).

Population dynamics in tomato leaves

DC3000, DC3000-*hrcC*, DC3682 and PT23.26 were inoculated into tomato leaves by spraying (see Methods) and the bacterial populations were monitored over a 7 d period (Fig. 5). The population of *P. syringae* pv. *tomato* DC3000 on day 0 was $5{\cdot}2 \times 10^5$ c.f.u. ml^{-1}; growth of this strain increased steadily over the sampling period and was approximately 1000-fold higher ($8{\cdot}6 \times 10^8$ c.f.u. ml^{-1}) at the end of the sampling period (day 7; Fig. 5). The population of DC3000-*hrcC* was approximately $5{\cdot}5 \times 10^5$ c.f.u. ml^{-1} on day 0; however, this strain failed to multiply *in planta* and the population was 1000-fold lower than the wild-type DC3000 on day 7 (Fig. 5). Interestingly, the COR$^-$ mutant, DC3682, did not multiply *in planta* and the population at day 7 was only

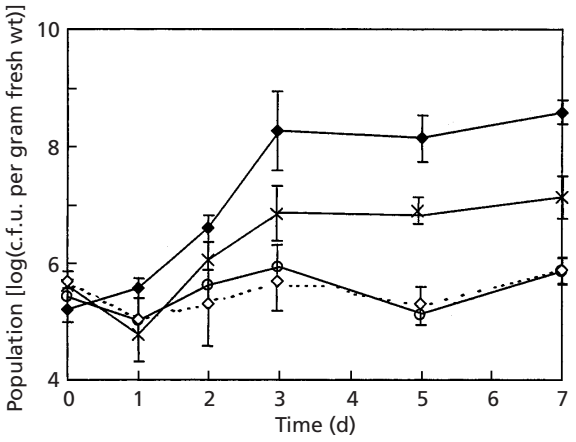

Fig. 5. Population dynamics of *P. syringae* pv. *tomato* DC3000 (◆), DC3000-*hrcC* (◇), DC3682 (COR⁻ mutant of DC3000) (○) and PT23.26 (COR⁻ mutant of PT23.2) (×) in tomato tissue. Bacterial inoculum (10^6 c.f.u. ml^{-1}) was sprayed (~8 p.s.i.) onto tomato leaves until surfaces were uniformly wet and plants were incubated in a growth chamber as described in the legend to Fig. 3. Bacterial populations were determined by homogenizing the leaves in sterile saline (0·85% NaCl) followed by dilution plating. All experiments were performed twice with similar results and vertical bars indicate the SEM.

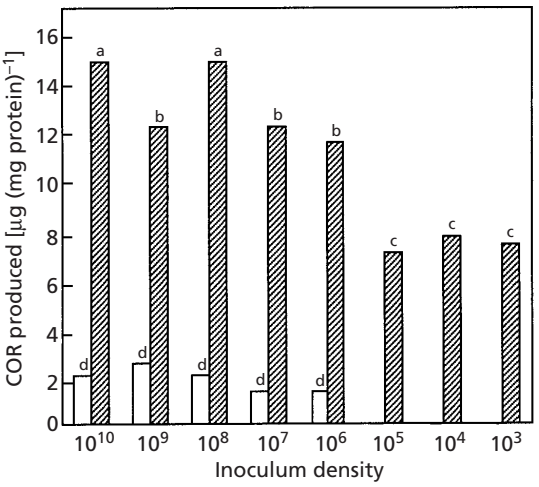

Fig. 6. Inoculum density and COR production. *P. syringae* pv. *tomato* DC3000 (white bars) and DC3000-*hrcC* (hatched bars) were inoculated into HSS broth at concentrations ranging from 10^3 to 10^{10} c.f.u. ml^{-1}, incubated for 72 h at 18 °C and analysed for COR production by HPLC fractionation of organic acid extracts (Palmer & Bender, 1993). Values represent the means from experiments containing three replicate cultures and values indicated by the same lower-case letter were not significantly different at $P = 0{\cdot}01$ as determined by Duncan's Multiple Range Test. COR was not detected in DC3000 when the initial inoculum density was 10^3–10^5 c.f.u. ml^{-1}. The detection limit for COR by HPLC was 100 ng (Rangaswamy *et al.*, 1998). The experiment was repeated with similar results.

$5{\cdot}8 \times 10^5$ c.f.u. ml^{-1}. However, PT23.26, which produced lesions in the absence of COR (Fig. 4d), did multiply *in planta* and attained a population of $7{\cdot}1 \times 10^7$ c.f.u. ml^{-1}, which is only 12-fold lower than DC3000 (Fig. 5). PT23.26 is a Tn5 insertion mutant of the COR⁺ strain, *P. syringae* pv. *tomato* PT23.2 (Bender *et al.*, 1989).

Restoration of normalized COR production to DC3000-*hrcC*

Our results clearly indicated that the expression and production of COR in DC3000-*hrcC* occurred earlier and at higher levels than DC3000. Such a phenotype could result from the inactivation of *hrcC* and/or the disruption of *hrpV*, a known regulatory gene that maps downstream of *hrcC* in the same transcriptional unit. The first possibility was examined by measuring COR production in CUCPB5112, which contains a non-polar mutation in *hrcC*. At 5 d (120 h) after inoculation, CUCPB5112 produced 1·72 μg COR (mg protein)$^{-1}$, a level similar to that obtained with DC3000 [1·0–2·5 μg COR (mg protein)$^{-1}$] and significantly lower than that obtained with DC3000-*hrcC* [9·0–10·5 μg COR (mg protein)$^{-1}$]. These results indicated that the inactivation of *hrcC* was not the reason for elevated COR production in DC3000-*hrcC* and suggested that the Tn5Cm mutation in DC3000-*hrcC* had polar effects on downstream genes. Therefore, complementation experiments with the regulatory gene *hrpV* were performed with the aim of restoring the low-level, wild-type production of COR to DC3000-*hrcC*. Construct pCPP2371 (containing *hrpV*) was introduced into DC3000-*hrcC* and COR production was evaluated at 5 d. Using this approach, the mean amount of COR produced by DC3000, DC3000-*hrcC* and DC3000-*hrcC*(pCPP2371) was 0·9, 7·1 and 0·7 μg COR (mg protein)$^{-1}$, respectively. These data were statistically analysed using Duncan's Multiple Range Test and the amounts of COR produced by DC3000 and DC3000-*hrcC*(pCPP2371) were not significantly different at $P = 0{\cdot}01$. However, COR production by DC3000-*hrcC* was significantly higher than DC3000 and DC3000-*hrcC*(pCPP2371), suggesting that *hrpV* restored the wild-type level of COR to DC3000-*hrcC* when expressed *in trans*.

COR production as a function of inoculum density

Our results suggested that COR production in DC3000 gradually increased with time but remained low throughout the sampling period (Fig. 1b). However, in DC3000-*hrcC*, COR production increased rapidly and was 75-fold higher than DC3000 when measured 24 h after inoculation (Fig. 1b). To evaluate whether the initial inoculum density had an effect on COR production, different starting concentrations of bacterial cells were inoculated in HSS broth and COR production was evaluated at 72 h. When the initial inoculum density was 10^6–10^{10} c.f.u. ml^{-1}, COR was detected in DC3000 at 72 h and the concentration ranged from 1·3 to 2·4 μg COR (mg protein)$^{-1}$ (Fig. 6). However, when the initial inoculum density was 10^5 c.f.u. ml^{-1} or lower, COR

could not be detected in DC3000 (Fig. 6). It should be noted that our detection limit for COR by HPLC was 100 ng (Rangaswamy *et al*., 1998). In contrast, COR was detected in DC3000-*hrcC* irrespective of the initial inoculum density and the amount of COR produced at 72 h ranged from 7 to 14 μg COR (mg protein)$^{-1}$ (Fig. 6).

DISCUSSION

Cross-talk between the *hrp* and *cor* gene clusters

In this study, DC3000-*hrcC* and CUCPB5112, which contain polar and non-polar mutations in *hrcC*, respectively, were evaluated for COR production. CUCPB5112 produced COR at levels comparable to the wild-type, which establishes that a functional *hrp* secretion system is not required for COR production in DC3000. However, DC3000-*hrcC* overproduced COR relative to DC3000 and the wild-type level of COR was restored to this mutant when *hrpV* was expressed *in trans*. The role of HrpV as a negative regulator of the *hrp* regulon has been established (Deng *et al*., 1998; Preston *et al*., 1998) and the results of the present study suggest that *hrpV* may have a regulatory effect on COR production in DC3000. This hypothesis will be pursued by constructing a defined mutation within the *hrpV* gene of DC3000 and analysing the mutant for COR production and *cor* gene expression. Preliminary results have indicated that mutations in the *hrpS* gene of DC3000 also have an impact on COR production. A DC3000 *hrpS* mutant (Yuan & He, 1996) produced tenfold more COR and showed elevated levels of *cor* gene expression relative to the wild-type DC3000 (Preston, 1997). The kinetics of COR production by DC3000-*hrpS* were similar to DC3000-*hrcC* in the early phases of the fermentation and high levels of COR were produced by both strains 24 h after inoculation (Fig. 1b; A. Peñaloza-Vázquez & C. L. Bender, unpublished). Therefore, in addition to *hrpV*, other genes (such as *hrpS*) may be involved in some form of cross-talk with the COR biosynthetic cluster.

It is possible that a direct regulatory connection exists between the *hrp* and *cor* gene clusters that can be analysed and dissected using molecular approaches. One gene that is required for expression of both the *hrp* and *cor* gene clusters is *rpoN*, which encodes σ^{54}. Transcriptional initiation by RNA polymerase utilizing σ^{54} requires an activator protein, and among the best-studied σ^{54}-dependent activators are NtrC and NifA (Morett & Segovia, 1993; North *et al*., 1993). Both HrpR and HrpS show sequence similarity with NtrC (Grimm *et al*., 1995; Xiao *et al*., 1994), suggesting a role for σ^{54} in transcription of the *hrp* cluster. Xiao *et al*. (1994) demonstrated that the *hrpL* gene product, which belongs to the extracellular factor subfamily of σ factors, contains a promoter region with strong homology to the consensus recognized by σ^{54}. Furthermore, Hendrickson *et al*. (2000a) showed that σ^{54} is required for the transcription of *hrpL* in *P. syringae* pv. *maculicola*. The alternative σ factor encoded by *hrpL* has a critical role in *hrp* gene expression and is required for the expression of several transcripts in the *hrp/hrc* gene cluster (Hutcheson *et al*., 1996).

rpoN mutants of *P. syringae* pv. *maculicola* ES4326 and pv. *glycinea* PG4180 were unable to synthesize COR and were defective in transcriptional activation of the CFA and *cmaABT* promoters (Hendrickson *et al*., 2000b; F. Alarcón-Chaidez & C. L. Bender, unpublished). Interestingly, the *cor* gene transcripts in PG4180 lack the consensus sequence recognized by σ^{54}, but contain enhancer-binding sequences recognized by NtrC and NifA activator proteins (Liyanage *et al*., 1995; Ullrich & Bender, 1994). Although the role of σ^{54} in the synthesis of COR by DC3000 remains unclear, it is tempting to speculate that σ^{54} may function in some way to co-ordinately regulate *hrp* and *cor* gene expression in this strain.

hrp and *cor* mutants of DC3000 fail to multiply *in planta*

In experiments where exogenous COR was added to tomato leaves, chlorosis was induced with only 0·078 ng of purified phytotoxin. When DC3000-*hrcC* was inoculated into tomato leaves by infiltration, a chlorotic zone was produced, suggesting that sufficient COR was present or produced in the plant to initiate chlorosis (Fig. 3c). Small chlorotic haloes also developed on tomato leaves spray-inoculated with DC3000-*hrcC* (Fig. 4c); however, it is important to note that COR production did not enable DC3000-*hrcC* to multiply *in planta* and cause typical disease symptoms.

When the COR$^-$ mutant DC3682 was inoculated by spraying, no symptoms were observed on tomato leaves (Fig. 4b). Furthermore, this mutant did not multiply in the host and the bacterial population was 1000-fold lower than the wild-type DC3000 at the end of the 7 d sampling period (Fig. 5). Further characterization of DC3682 showed that this mutant elicited an HR on tobacco leaves (data not shown), indicating that DC3682 contains a functional type III secretion system. DC3682 was complemented for COR production by cosmid pEC18, which contains the CFA gene cluster (Ma *et al*., 1991; A. Peñaloza-Vázquez & C. L. Bender, unpublished). The inactivation of the CFA gene cluster in DC3682 eliminated the ability of this mutant to synthesize COR, CFA and phytotoxic analogues of COR (A. Peñaloza-Vázquez & C. L. Bender, unpublished).

The results obtained with DC3682 in this study agree with an earlier report where *Arabidopsis* and tomato plants were inoculated with DC3661, another COR$^-$ mutant of DC3000 (Ma *et al*., 1991; Mittal & Davis, 1995). Symptoms did not develop with DC3661 on either host when plants were inoculated by dipping leaves into bacterial suspensions and multiplication of the COR$^-$ mutant was several logs lower than DC3000 (Mittal & Davis, 1995). In contrast, when DC3661 was infiltrated into host plants, necrotic lesions developed and the bacteria multiplied to levels approaching the wild-type DC3000. Mittal & Davis (1995) concluded that COR

was required during the early stages of infection and presented data demonstrating that COR inhibited the host defence response in *Arabidopsis*. Apparently, COR was ineffectual in suppressing host defence when bacterial cells were directly infiltrated into plant tissue (Mittal & Davis, 1995). These results are consistent with those obtained in the present study, although it remains unclear whether COR functions to inhibit defence in tomato tissue.

Interestingly, the COR$^-$ mutant PT23.26, which also contains a Tn5 insertion in the CFA gene cluster, produced visible necrotic lesions on tomato leaves (Fig. 4d) and the population was only 12-fold lower than DC3000 (Fig. 5). These results agree with a previous study where we examined the role of COR in *P. syringae* pv. *tomato* PT23.2. COR$^-$ mutants of PT23.2 produced visible necrotic lesions on tomato leaves when inoculated by spraying and the population of COR$^-$ mutants was only tenfold lower than the wild-type *in planta* (Bender *et al*., 1987). We concluded that COR functioned as a virulence factor in *P. syringae* pv. *tomato* PT23.2 by enhancing multiplication and lesion expansion *in planta*. However, the results obtained in the present study suggest that COR is required for the successful infection of tomato leaves by DC3000. Therefore, the importance and role of COR varies in *P. syringae* pv. *tomato* DC3000 and PT23.2. One possible explanation for this disparity is the location of the COR biosynthetic gene cluster in these two strains; the COR gene cluster in PT23.2 is borne on a 101 kb plasmid, designated pPT23A (Bender *et al*., 1989), whereas COR is chromosomally encoded in DC3000 (Moore *et al*., 1989). In our experience, pPT23A is inherently unstable (Bender *et al*., 1992), a phenomenon consistent with the role of COR as a dispensable virulence factor. Furthermore, pathogenic strains of *P. syringae* pv. *tomato* have been identified that do not produce COR (Mitchell *et al*., 1983). Therefore, it is possible that PT23.2 acquired the COR plasmid, pPT23A, after it became pathogenic. However, the chromosomally encoded COR gene cluster in DC3000 may have co-evolved with the *hrp/hrc* secretion system and the requirement for COR in the establishment of a successful infection may reflect this.

COR production in DC3000-*hrcC* is derepressed or constitutively up-regulated

In the wild-type strain, DC3000, COR production and COR gene expression increased with time (Figs 1b and 2) and expression of the *cmaABT* operon increased during exponential phase (data not shown). However, in DC3000-*hrcC*, COR production was not a function of growth phase (Fig. 1b) or the initial inoculum concentration (Fig. 6). These results suggest that COR production is derepressed or up-regulated in DC3000-*hrcC*, presumably because of the polar effects of the Tn5Cm mutation on *hrpV*. It is also important to note that the overproduction of COR in DC3000-*hrcC* did not increase the ability of the bacteria to colonize the host (Fig. 6), even though this would provide a biological rationale for the observed phenomenon. In conclusion, this study indicates that a regulatory connection exists between the type III Hrp secretion system and COR biosynthesis. The identification of the regulatory circuitry that connects the two systems warrants further investigation and will improve our understanding of bacterial pathogenesis.

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