Interactions of *Pseudomonas syringae* pv. *glycinea* with Host and Nonhost Plants in Relation to Temperature and Phytotoxin Synthesis

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Pseudomonas syringae pv. *glycinea* PG4180 causes bacterial blight of soybean and produces the phytotoxin coronatine (COR) in a temperature-dependent manner. COR consists of a polyketide, coronafacic acid (CFA), and an amino acid derivative, coronamic acid, and is produced optimally at 18°C whereas no detectable synthesis occurs at 28°C. We investigated the impact of temperature on PG4180 during compatible and incompatible interactions with soybean and tobacco plants, respectively. After spray inoculation, PG4180 caused typical bacterial blight symptoms on soybean plants when the bacteria were grown at 18°C prior to inoculation but not when derived from cultures grown at 28°C. The disease outcome was quantified by determination of bacterial populations in planta. The temperature effect was not observed when PG4180 was artificially infiltrated into soybean leaves, indicating that the pre-inoculation temperature and phytotoxin synthesis were important for bacterial invasion via natural plant openings. In the incompatible interaction, PG4180 elicited the hypersensitive response (HR) on tobacco plants regardless of the bacterial pre-inoculation temperature. However, the HR was significantly delayed when tobacco plants were treated with cells of the CFA-overproducing derivative, PG4180.N9, which were derived from cultures grown at 18°C, compared with parallels incubated at 28°C. CFA biosynthesis by PG4180.N9 was optimal at 18°C and negligible at 28°C. The impact of CFA synthesis on the HR was studied with different growth media, mutants, and transconjugants of PG4180, indicating that the amount of synthesized CFA but not that of COR influenced the outcome of the HR. Feeding experiments with purified coronafacoyl compounds suggested that the observed delay of the HR was mediated by CFA, shedding further light on CFA's putative role as a molecular mimic of the plant signaling molecule, jasmonic acid.

Plant-pathogenic bacteria are subject to numerous environmental stimuli during infection processes, and it has been well established that temperature can have a major impact on plant

disease outcome. However, in contrast to our understanding of thermoregulation in pathogenic bacteria of human or animal hosts, little is known about how temperature influences cellular processes in plant pathogens ultimately leading to virulence and pathogenicity. Previous reports showed that the synthesis of virulence factors in *Agrobacterium tumefaciens*, *Pseudomonas syringae* pvs. *phaseolicola* and *glycinea*, *Erwinia chrysanthemi*, and *Erwinia carotovora* was thermoresponsive (Lanham et al. 1991; Hugovieux-Cotte-Pattat et al. 1992; Jin et al. 1993; Rowley et al. 1993; Palmer and Bender 1993; Budde et al. 1998). In all of these cases, low temperature was conducive to virulence factor synthesis, which agrees with the etiology of the diseases incited by these bacteria.

In the compatible interaction, *P. syringae* pv. *glycinea* PG4180 causes bacterial blight of soybean plants and symptoms are water-soaked lesions that develop into necrotic leaf spots surrounded by chlorotic halos. Low temperature and high humidity can favor bacterial blight infection and disease outcome (Dunleavy 1988). *P. syringae* pv. *glycinea* and the closely related pathovars *atropurpurea*, *morsprunorum*, *maculicola*, and *tomato* produce the non-host-specific polyketide phytotoxin coronatine (COR; Fig. 1) (Mitchell 1982; Bender et al. 1993; Wiebe and Campbell 1993). COR is an important virulence factor in diseases caused by these bacteria. As shown by mutant analysis, COR production enhanced the virulence of *P. syringae* strains on tomato plants, *Arabidopsis thaliana*, and Chinese cabbage plants (Bender et al. 1987; Mittal and Davis 1995; Tamura et al. 1998).

The biological effects of COR include the induction of leaf chlorosis, promotion of senescence in tobacco (Kenyon and Turner 1990), hypertrophy of potato tuber tissue (Sakai et al. 1979), inhibition of root growth in wheat (Sakai 1980), stimulation of stomatal opening in ryegrass, ethylene synthesis in tobacco, and the synthesis of proteinase inhibitors in tomato (Mino et al. 1987; Ferguson and Mitchell 1985; Kenyon and Turner 1992; Palmer and Bender 1995).

In *P. syringae* pv. *glycinea* PG4180, all enzymes involved in COR biosynthesis are encoded by the plasmid-borne, 32.8-kb COR gene cluster, which can be subdivided into two biosynthetic operons and three regulatory genes (Ullrich and Bender 1994; Liyanage et al. 1995; Ullrich et al. 1995; Rangaswamy et al. 1998a, 1998b; Fig. 2). Production of COR by PG4180 is maximal at 18°C whereas no toxin formation can be detected at 28°C, the optimal growth temperature of *P. syringae*

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(Palmer and Bender 1993; Ullrich et al. 1995; Budde et al. 1998). The temperature dependence was mediated at the transcriptional level by a modified two-component regulatory system consisting of the environmental sensor kinase, CorS, and two response regulator proteins, CorR and CorP (Ullrich et al. 1995; Penalzoa-Vazquez and Bender 1998; Wang et al. 1999). PG4180 does not produce COR when grown in complex medium (Palmer and Bender 1993), a feature frequently observed for synthesis of secondary metabolites in plant pathogenic bacteria.

COR consists of two moieties, coronafacic acid (CFA; Fig. 1), which is derived from the polyketide pathway, and coronamic acid (CMA), a cyclized derivative of isoleucine. Both CFA and CMA function as intermediates in the biosynthesis of COR and are joined together by an amide bond linkage. Like the production of COR, the synthesis of CFA and CMA is temperature dependent, with maximal levels produced at 18°C. CMA structurally resembles 1-aminocyclopropane-1-carboxylic acid (ACC), which is a precursor in the ethylene biosynthetic pathway. This encouraged previous speculations that CMA might modulate plant-borne ethylene biosynthesis (Kenyon and Turner 1992).

CFA structurally resembles the global plant signaling molecule jasmonic acid (JA; Fig. 1). JA accelerates senescence and chlorophyll degradation and induces ethylene synthesis in various plants (Sembdner and Parthier 1993). At lower concentrations, JA functions as signal transducer in plant defense against herbivore or pathogen attack (Farmer and Ryan 1990; Reymond and Farmer 1998) and is involved in mechanotransduction (Weiler 1997; Bleichert et al. 1999). CFA and JA had similar biological activities when tested in JA-responsive assays (Koda et al. 1996). COR was effective in these assays with activities 100 to 10,000 times higher than those of JA or CFA. Weiler et al. (1994) pointed out that COR should not be regarded as a structural analogue of JA or methyl jasmonate (MeJA) because the position of the essential carboxyl group of COR relative to the cyclopentyl ring is different from that in JA (Fig. 1). Interestingly, COR shares significant structural and functional characteristics with 12-oxophytodienoic acid (OPDA; Fig. 1), an intermediate in JA biosynthesis that was recently associated with a number of regulatory mechanisms in plant defense (Dittrich et al. 1992; Weiler 1997; Zhang et al. 1997). This suggested that COR might mimic octadecanoid-derived signaling compounds in higher plants (Feys et al. 1994; Weiler et al. 1994; Boland et al. 1995). Putative plant receptors for JA, OPDA, or COR are still unknown but are the subject of ongoing investigations (Schüler et al. 1999).

During the incompatible interaction (e.g., with tobacco plants), *P. syringae* pv. *glycinea* elicits the hypersensitive response (HR), a rapid localized necrosis of plant tissue that is associated with programmed cell death and active plant defense at the site of pathogen infection. The ability to elicit the HR on nonhost plants and to be pathogenic on host plants is controlled by HR and pathogenicity (*hrp*) genes, which are universal among phytopathogenic bacteria of the genera *Pseudomonas*, *Erwinia*, *Xanthomonas*, and *Ralstonia* (Alfano and Collmer 1997). The *hrp* genes are clustered and many encode components of type III protein secretion systems dedicated to the secretion of virulence proteins into plant cells (He 1998). Nucleotide sequencing of the *hrp* gene clusters from plant pathogens revealed homologies with components of the *yop*

secretion system of *Yersinia* spp. and other protein translocation systems of pathogenic bacteria, suggesting the conservation of type III protein secretion pathways in gram-negative pathogens of both plants and animals (He 1998). Numerous studies have dissected this secretion system and mutants were obtained that were impaired at various steps of the respective secretion mechanisms (Lindgren 1997; He 1998). The characteristic phenotype associated with *hrp* mutants is the inability to cause disease on susceptible host plants, as well as the inability to elicit the HR on resistant cultivars of susceptible plants and on nonhost plants.

To study the influence of temperature and phytotoxin synthesis on virulence of *P. syringae* pv. *glycinea* during the compatible interaction with soybean plants, we monitored disease outcome by visual estimation of symptom development and by enumeration of bacteria in planta at different temperature and inoculation conditions. Furthermore, we studied the impact of temperature and coronafacyl compounds during the incompatible interaction of *P. syringae* pv. *glycinea* with tobacco plants. Here we demonstrate that a CFA-overproducing derivative of *P. syringae* pv. *glycinea*, PG4180.N9, elicits the HR in a temperature-dependent manner and that the amount of synthesized CFA delayed the outcome of the HR. This effect could be reproduced by the addition of purified CFA to bacterial inocula not producing any coronafacyl compounds and could be abolished by overproduction of HrpL, the alternative sigma factor involved in transcriptional activation of *hrp* genes.

RESULTS

Symptom development and multiplication of *P. syringae* pv. *glycinea* in soybean plants is dependent on the bacterial pre-inoculation temperature.

Symptom development and in planta bacterial multiplication were monitored in soybean plants inoculated with PG4180 and its COR⁻ mutant PG4180.D4 grown at 18 and

Fig. 1. Structural relatedness of coronatine, coronafacic acid, 12-oxo-phytodienoic acid, and jasmonic acid.

28°C, respectively. Mutant PG4180.D4 harbors a transposon insertion in the regulatory *corR* gene that is required for transcriptional activation of the COR biosynthetic genes (Ullrich et al. 1995; Fig. 2) and is completely defective in COR biosynthesis (Fig. 3A). Following inoculation by either spraying or infiltrating the bacterial suspensions to the plant tissue, replica plants were placed in growth chambers set to 18 or 28°C. When sprayed on soybean plants, PG4180 cells grown at 18°C caused severe disease symptoms initially characterized by water-soaked lesions that developed into necrotic spots surrounded by chlorotic halos after 5 to 7 days (Fig. 3B). In contrast, when PG4180 cells grown at 28°C were inoculated by spraying, plants exhibited significantly fewer symptoms and were characterized by smaller, water-soaked lesions and reduced necrosis and chlorosis, respectively (Fig. 3C). Soybean plants treated with cell suspensions of the *COR*⁻ mutant PG4180.D4 also developed fewer symptoms that were characterized by less water soaking and smaller, necrotic lesions lacking significant chlorotic halos (data not shown). The pre-inoculation temperature of the PG4180.D4 cultures did not influence this result. Furthermore, the temperature at which the plants were kept post inoculation did not significantly alter the described symptom development. Thus, all subsequent experiments were standardized to a post-inoculation temperature of 28°C.

To substantiate the results of symptom development, in planta bacterial multiplication was monitored by reisolating bacterial cells from infected plant tissue in a time-dependent manner (Fig. 3D). Results showed that bacterial multiplication was relatively low (100 to 1,000-fold over 10 days) in plants inoculated with PG4180 grown at 28°C. In comparison, bacterial growth was higher (10,000-fold over 10 days) in plants inoculated with bacteria derived from 18°C cultures. Measurement of bacterial growth in soybean plants exposed to the *COR*⁻ mutant PG4180.D4 showed low multiplication rates regardless of the pre-inoculation temperature of the bacterial cultures (Fig. 3D). Moreover, the number of viable bacterial cells in those plants appeared to decrease after 6 to 10 days, indicat-

ing that the infection process was not successful. These results demonstrated that symptom development was directly correlated with bacterial multiplication in planta and that the disease outcome in the compatible interaction depended on the pre-inoculation temperature and subsequent phytotoxin production.

To investigate whether this result depended on the inoculation method, soybean plants were also infected by artificial infiltration of PG4180 cells that were grown at either 18 or 28°C. Typical bacterial blight symptoms were observed at the infiltration sites after 3 to 5 days (data not shown). Interestingly, the pre-inoculation temperature of the bacterial cultures had no effect on symptom development under these inoculation conditions. Additionally, estimation of bacterial growth in planta showed no significant difference between the multiplication rates of PG4180 derived from cultures grown at 18 or 28°C (Fig. 3E). Similar results were obtained when PG4180.D4 was infiltrated into soybean plant tissue. In general, multiplication of PG4180.D4 was approximately five- to 10-fold lower than PG4180 and was not influenced by the pre-inoculation temperature of the bacteria (Fig. 3E). In summary, the results of these experiments indicated that pre-inoculation temperature and phytotoxin synthesis dramatically influenced the bacterial invasion efficiency via natural plant openings but did not significantly affect virulence when the cells were artificially introduced into the plant tissue.

Effect of temperature on the HR.

Initially, the outcome of the HR was monitored in tobacco plants inoculated with PG4180 grown at either 18 or 28°C prior to inoculation. Bacteria were grown in HSC medium to an OD₆₀₀ of 1.5 to 1.8 (1.7 to 2.2 × 10⁹ CFU per ml) and then injected into 8-week-old tobacco plants that were subsequently maintained at 18 or 28°C. After 24, 48, and 72 h the development of the HR was monitored visually. All inoculated plants exhibited a typical HR at 24 h post inoculation (hpi) regardless of the bacterial pre-inoculation growth temperature (Fig. 4, top). As shown for the compatible interaction, the

Fig. 2. Genetic and functional organization of the coronatine (COR) biosynthetic gene cluster of *Pseudomonas syringae* pv. *glycinea* PG4180. Genes for coronafacic acid (CFA) biosynthesis (*cfl*, *cfa1-9*), for coronamic acid (CMA) biosynthesis (*cmaA*, *cmaB*, *cmaT*, and *cmaU*), and regulatory genes (*corP*, *corS*, and *corR*) are outlined. Vertical lines with closed circles indicate position of mutational insertions in PG4180.N9 (N9), PG4180.C0 (C0), PG4180.P1 (P1), and PG4180.D4 (D4). Position of the insert DNA of pMUH34 is outlined with respect to the *SstI* restriction map of the COR gene cluster. *tnp* is a transposase gene naturally occurring within the gene cluster.

temperature under which the treated plants were kept did not affect the outcome of the HR. Experiments were repeated with the COR⁻ mutant PG4180.D4 and similar results were obtained. Furthermore, no influence of temperature on the out-

come of the HR was observed when the *P. syringae* pv. *syringae* strain Pss61, a COR⁻ strain, and the COR⁺ *P. syringae* pv. *tomato* strain DC3000 were used for the inoculation procedure. This demonstrated that neither bacterial growth tem-

Fig. 3. Compatible interaction of *Pseudomonas syringae* pv. *glycinea* PG4180 and the COR⁻ mutant PG4180.D4 with soybean plants. **A**, Coronatine (COR) and coronafacic acid (CFA) synthesis in PG4180 at 18°C and lack of synthesis at 28°C and in mutant PG4180.D4 at 18°C as determined by high-performance liquid chromatography (HPLC) analysis. **B**, Symptom development at 5 days after spray inoculation with PG4180 pre-incubated at 18°C. **C**, Symptoms at 5 days after spray inoculation with PG4180 pre-incubated at 28°C. **D**, In planta bacterial multiplication of PG4180 (filled diamonds) and the COR⁻ mutant PG4180.D4 (open circles) after spray inoculation with bacteria derived from either 18°C (solid lines) or 28°C (dashed lines) cultures. **E**, In planta bacterial multiplication of PG4180 (filled diamonds) and the COR⁻ mutant PG4180.D4 (open circles) after infiltration of bacteria derived from either 18°C (solid lines) or 28°C (dashed lines) cultures. Results represent data from three experiments, each with two replicates.

perature nor COR synthesis remarkably influenced the outcome of the HR of tobacco plants.

Temperature-mediated suppression of the HR.

Next, we tested whether an altered synthesis of COR or CFA could influence the outcome of the HR in tobacco plants. As quantified by HPLC analysis, PG4180 grown to an OD_{600} of 1.5 to 1.8 produced 2.3 ± 1 mg of CFA and 7.6 ± 2 mg of COR per liter at 18°C . No coronafacoyl compounds could be isolated from PG4180 cultures grown at 28°C (Fig. 5). In contrast, the PG4180 derivative PG4180.N9 (Ullrich et al. 1994) overproduced CFA, one of the COR precursors, with 10.1 ± 3 mg of CFA per liter and 7.3 ± 1 mg of COR per liter at 18°C (CFA and COR synthesis were negligible at 28°C) (Figs. 4 and 5). This strain carries a Tn5 insertion approximately 1.5 kb downstream of *cmaU* in one of the insertion sequence (IS) elements flanking the CMA gene cluster (Fig. 2; Alarcon-Chaidez et al. 1999). The reason for the overproduction of CFA by PG4180.N9 is unknown but the genetic organization of the COR gene cluster in this strain was not altered when investigated by Southern blot hybridization (data

not shown). Interestingly, PG4180.N9 caused a remarkably different, temperature-dependent reaction when injected into tobacco plants. Tobacco plants inoculated with PG4180.N9 cells produced a typical HR at 24 hpi only when the bacterial inoculum was derived from cultures grown at 28°C (Fig. 4, bottom). In 70% of 50 independent experiments, PG4180.N9 elicited no HR at 24 hpi when the bacterial inoculum was derived from cultures grown at 18°C and produced 11.4 ± 2 mg of CFA per liter and 7.9 ± 1 mg COR per liter (Fig. 4). When the treated tobacco plants were re-evaluated at 48 and 72 hpi, a HR-like necrosis of the plant tissue could occasionally be observed. These results suggested that PG4180.N9 might produce a factor(s) at 18°C that significantly delayed or suppressed the onset of the HR in tobacco plants.

Subsequently, we investigated whether the putative HR-delaying factor(s) were associated with cells or the cell-free supernatant of PG4180.N9. Interestingly, when washed or filtered cells of PG4180.N9 grown at 18°C were used as inoculum, the HR was suppressed in only 45% of 50 independent experiments, suggesting that factors from the original supernatant contributed to the suppression of the HR. As expected,

Fig. 4. Hypersensitive response phenotypes on tobacco plants caused by PG4180 and PG4180.N9 pre-incubated at 18 and 28°C , respectively. Level of coronatine (COR) and coronafacic acid (CFA) production by both strains at 18°C is outlined as determined by high-performance liquid chromatography analysis.

no HR was induced 24 to 72 hpi when tobacco plants were inoculated with cell-free culture supernatants or heat-killed cells grown at 18°C. This confirmed the established fact that living bacterial cells are essential for the induction of the HR. Our results demonstrated that the HR-suppressing phenotype of PG4180.N9 was only detectable when the cells were grown at 18°C and that factors excreted from the cell were required to efficiently suppress the HR.

Comparison of the *hrp* gene clusters of PG4180 and PG4180.N9.

To determine whether PG4180.N9 showed abnormalities in the structure of its *hrp* gene cluster, Southern blot hybridizations were carried out with the insert of cosmid pHIR11 as a nonradioactively labeled probe. Cosmid pHIR11 contains the entire *hrp* gene cluster of the *P. syringae* strain Pss61 (Huang et al. 1988). Genomic DNA of PG4180 and PG4180.N9 was cleaved with restriction enzymes *EcoRI*, *EcoRI/HindIII*, and *EcoRI/BamHI*, separated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with the probe. The results showed that there was no visible restriction fragment length polymorphism (RFLP) among the two strains (data not shown). Although we cannot rule out point mutations, our results indicate that the *hrp* gene clusters in these two strains are essentially identical and unlikely cause the temperature-mediated suppression of the HR.

The suppression of the HR caused by PG4180.N9 is growth-medium dependent.

To determine the effects of medium composition on the phenotype of PG4180.N9, this strain was grown in HSC minimal medium, KB complex medium, and *hrp*-inducing IM medium to an OD₆₀₀ of 1.5 to 1.8 at 18 and 28°C. Tobacco

plants were inoculated with the bacterial culture suspensions and the HR was monitored 24, 48, and 72 hpi. Results for PG4180.N9 grown in HSC medium or in IM medium were identical to those described above. Plants inoculated with PG4180.N9 derived from complex KB medium showed a typical HR after 24 h regardless of the pre-inoculation temperature. Neither CFA nor COR were produced by PG4180.N9 grown in KB medium (Fig. 5). In IM medium, this strain produced CFA and COR at 18°C in amounts similar to those synthesized in HSC medium (10.4 ± 3.7 mg of CFA per liter and 6.35 ± 1.1 mg of COR per liter) but did not produce any coronafacoyl compounds at 28°C (Fig. 5). These results indicated that the HR phenotype of PG4180.N9 was nutrient dependent and correlated with the overproduction of CFA in minimal media.

Influence of thermoresponsive CFA production by various PG4180 derivatives on elicitation of the HR.

Strains PG4180 and PG4180.N9 differed remarkably with respect to CFA production (Fig. 5) and their respective HR phenotypes (Fig. 4), but not with respect to COR synthesis. This prompted us to investigate whether the amount of CFA had a role in the outcome of the HR. Initially, another mutant, PG4180.C0, that produces CFA but no COR (Bender et al. 1993) was considered. This mutant consistently produced only about one-fifth of the CFA amount synthesized by PG4180.N9 (Fig. 5). When tested in HR experiments, no effect of pre-inoculation temperature was observed for PG4180.C0, possibly because the amount of CFA produced (1.88 ± 0.5 mg/liter at 18°C and 1.77 ± 0.8 mg/liter at 28°C) was not sufficient to suppress the HR. We then utilized the CFA⁻ COR⁻ mutant PG4180.P1, which is a PG4180.N9 derivative harboring a Gm^r cassette in *corS*, the gene encoding the histidine protein kinase CorS (Ullrich et al. 1995; Fig. 2). Cell suspensions of PG4180.P1 elicited a typical HR at 24 hpi when derived from either 18 or 28°C cultures. Since PG4180.P1 is a derivative of PG4180.N9, this result suggested that the temperature-responsive HR phenotype of PG4180.N9 was not linked to any additional chromosomal alteration in this strain.

Simultaneous lack of CFA production and HR suppressiveness by PG4180.P1 furthermore strengthened our hypothesis that the amount of CFA might be critical for suppression of the HR. To test this hypothesis, plasmid pMUH34 (Ullrich et al. 1995), which harbors the COR regulatory genes *corR*, *corS*, and *corP* (Fig. 2), was introduced into PG4180.N9. In addition to the high level of produced COR (8.8 ± 2 mg/liter) and CFA (12.3 ± 3 mg/liter) at 18°C, PG4180.N9 (pMUH34) synthesized considerable amounts of CFA (4.5 ± 2 mg/liter) and COR (1.6 ± 1 mg/liter) at 28°C (Fig. 5), possibly because of copy number effects of the regulatory genes in trans. The transconjugants were grown at 18 and 28°C and subsequently tested in HR assays. Interestingly, PG4180.N9 (pMUH34) grown at 18 or 28°C did not induce a HR in tobacco plants at 24 hpi (Fig. 5). When re-evaluated after 48 and 72 h, a HR-like necrosis occasionally developed at the sites of infiltration. These results indicated that bacterial strains that produced no or low amounts of CFA caused a typical HR at 24 hpi. Furthermore, our results suggested that cells that produced CFA above the threshold value of approximately 4.0 mg/liter (19 µM concentration of CFA) caused a significant delay or a suppression of the HR.

Fig. 5. Quantitative high-performance liquid chromatography (HPLC) analysis showing amounts of coronafacic acid (CFA) and coronatine (COR) (mg/liter) in different *Pseudomonas syringae* strains as well as evaluation of the hypersensitive response (HR) caused by these bacterial strains on tobacco plants at 24 h post inoculation. All strains were grown at 18 and 28°C in HSC medium or otherwise indicated media to an OD₆₀₀ of 1.5 to 1.8. CFA and COR were extracted from 1.5 ml of cell-free culture supernatants and quantified by HPLC analysis. (+) development of HR; (-) no visible HR. Dashed line = threshold level of CFA synthesis (approx. 4 mg of CFA per liter) required for delay of HR.

Addition of purified CFA alters the outcome of the HR.

To further substantiate the potential role of CFA in HR suppression, feeding experiments with purified coronafacoyl compounds were carried out. Cultures of PG4180.N9 were grown at 28°C to an OD₆₀₀ of 1.5 to 1.8 and injected into tobacco leaf tissue. Subsequently, purified CFA (100 µM), COR (100 µM), or sterile HSC medium were individually infiltrated at 0, 3, or 6 hpi or successively at 3 and 6 hpi into the same area of the leaf, which was pre-inoculated with PG4180.N9 grown at 28°C (Fig. 6). The infiltration of CFA, COR, and HSC medium at 0, 3, or 6 hpi had no impact on the development of HR (Fig. 6); all leaf areas inoculated with bacteria exhibited a clear HR at 24 hpi. In contrast, the HR in response to cells grown at 28°C was abolished in 65% of 25 independent experiments when CFA (100 µM) was added successively 3 and 6 hpi (Fig. 6). When COR (100 µM) was infiltrated at 3 and 6 hpi a delay in the HR occurred in only 18% out of 25 independent experiments. Sterile HSC medium had no impact on the outcome of the HR when injected after 3 and 6 hpi (Fig. 6). To confirm these results, various concentrations of CFA (0, 25, 50, 100, and 200 µM) were added at 3.5 and 6.5 hpi to tobacco leaves that were pre-infiltrated with washed PG4180.N9 cells grown at 28°C (Fig. 7). Although addition of 200 µM CFA to the inoculated leaf areas often caused an atypical, grayish, HR-unlike necrosis of the tissue (data were therefore omitted from Figure 7), the lower CFA treatments showed a rather clear concentration-dependent suppression of the HR in at least 15 independent experiments. At this point, it remains obscure why addition of 50 µM CFA had a less suppressive effect on the outcome of the HR than addition of 25 µM CFA. In summary, our results clearly showed that purified CFA had the potential to suppress the HR in tobacco plants. Why COR had a negative impact on the outcome of the HR in some experiments remains unclear at the moment.

Since CFA is a structural analogue of JA, we investigated JA for a similar effect on the HR in tobacco tissue. Like CFA, JA (100 µM) was infiltrated 3 and 6 hpi into tobacco leaves pre-inoculated with PG4180.N9 cells grown at 28°C. Evaluation of the HR at 24 hpi demonstrated that JA had no inhibitory effect on the outcome of the HR (Fig. 6). Based on these results it could be speculated that exogenously added CFA but not the addition of JA might influence the signal transduction pathway leading to the HR.

Overexpression of *hrpL* results in the elicitation of the HR at low temperature.

To fully restore the ability of PG4180.N9 to cause an HR on tobacco plants when pre-incubated in minimal medium at 18°C, plasmid pDRL3DSp was conjugated into PG4180.N9. This plasmid carries a transcriptional fusion of *hrpL* to the inducible P_{lac} promoter (S. W. Hutcheson, *personal communications*). The gene product of *hrpL* represents an alternative sigma factor governing the expression of the *hrp* gene cluster and thus functioning as a key regulator for HR induction (He 1998). PG4180.N9 (pDRL3DSp) produced amounts of CFA (11.04 ± 2.3 mg/liter) and COR (5.85 ± 2.9 mg/liter) similar to that of the parental strain (Fig. 5). Upon induction with 5 mM IPTG at an OD₆₀₀ of 0.5, bacterial cultures were injected into tobacco leaves after they reached an OD₆₀₀ of 1.5 to 1.8 and the resulting HR was evaluated at 24, 48, and 72 hpi. All treated plants developed a typical HR regardless of the bacte-

rial pre-inoculation temperature, indicating that *hrpL* overexpression abolished the thermosensitive delay of the HR and the suppressive effects of CFA.

DISCUSSION

In the present study, we investigated the impact of temperature and synthesis of coronafacoyl compounds on *P. syringae* during compatible and incompatible interactions with soybean and tobacco plants, respectively. Special emphasis was put on the bacterial component because changes in the temperature at which the plants were maintained did not significantly influence the outcome of the interaction. Plants like soybeans or tobacco obviously do not depend on small temperature changes when responding to this pathogen.

P. syringae PG4180 preferentially produces the phytotoxin COR and its precursor, CFA, at 18°C whereas no detectable COR or CFA is produced at 28°C. Several previous reports demonstrated that COR⁻ mutants of *P. syringae* caused smaller lesions and exhibited lower levels of bacterial multiplication in plants than COR⁺ strains (Bender et al. 1987; Mittal and Davis 1995; Tamura et al. 1998). Our results indicate that the synthesis of coronafacoyl compounds by PG4180 is also important for the initial steps in the compatible plant-microbe interaction. In plants inoculated by spraying, a smaller number of bacteria enter the host via stomata, other natural openings, or wounds than in plants inoculated by infiltration. These low levels of inoculum resemble to some extent naturally occurring infections (Beattie and Lindow 1999). Consequently, our data suggest that COR synthesis at low temperature was advantageous for the bacterial multiplication in planta. In infiltrated soybean plants, the effects of pre-inoculation temperature and COR synthesis were masked by the artificial and massive introduction of bacteria into the mesophyll tissue. Interestingly, the COR⁻ mutant PG4180.D4 did not produce significant disease symptoms, regardless of the growth temperature, when spray inoculated onto soybean plants but reached considerable cell densities when artificially introduced into plants. This result indicated that COR synthesis might be important in the early steps of the infection. Similar results were reported by Mittal and Davis (1995) for the *P. syringae* pv. *tomato*-*A. thaliana* interaction.

In the incompatible interaction with tobacco plants, PG4180 elicited the HR regardless of the pre-inoculation growth temperature. This result clearly demonstrated that COR did not influence the outcome of the HR during the incompatible interaction. However, the CFA-overproducing derivative, PG4180.N9, elicited the HR 24 hpi when pre-incubated at 28°C but barely when grown at 18°C, a temperature at which this strain synthesizes large amounts of CFA. Our results also indicate that the delay of the HR depended on factors derived from the bacterial supernatant. To our knowledge, PG4180.N9 is the only bacterial strain able to secrete large amounts of CFA, and suppression of the HR required at least 4 mg of CFA per liter of culture medium.

Since the thermosensitive delay of the HR was tightly linked to strain PG4180.N9, additional chromosomal alterations within this strain eventually related to the *hrp* gene cluster had to be ruled out. According to RFLP analysis, it was shown that the *hrp* gene clusters of PG4180.N9 and its parental strain were very similar if not identical. Furthermore,

overproduction of HrpL in PG4180.N9 abolished the delay of the HR, suggesting that this strain is fully capable of eliciting the HR. From those results, taken together, it can be concluded that the interesting HR delay phenotype of this strain may not be related to defects in *hrp* genes or their regulation.

This is the first report demonstrating that the HR can be inhibited by the bacterial compound CFA, a molecular mimic of plant-borne JA. Previously, it was demonstrated that the HR of leaf tissue toward bacteria could be prevented by treatments with several compounds such as calcium, strontium, and uranium salts (Cook and Stall 1971), cytokinins (Novacky 1972), glycoproteins of the bacterial cell wall (Wacek and Sequeira 1973), the phytotoxin phaseolotoxin (Rose and Rudolph 1976), or subcellular bacterial components, such as lipopolysaccharides or protein-lipopolysaccharide complexes (Graham et al. 1977; Minardi 1995; Newman et al. 1997) as well as by extracellular polysaccharides (Mazzucchi and Rudolph, 1984). Furthermore, Robinette and Matthysse (1990) described that the HR can be delayed by injection of *Agrobacterium tumefaciens* prior to injection of *P. syringae* pv. *phaseolicola*.

When we added bacterial CFA prior to the injection of PG4180.N9 into tobacco leaves, the HR was visible at 24 hpi, resulting in complete tissue collapse. Similar results were obtained when CFA was added to PG4180.N9 cultures grown at 28°C or to infiltrated leaf areas alternatively at 0, 3, or 6 hpi. In contrast, the HR was blocked when bacterial CFA was added successively at 3 and 6 hpi to the same leaf tissue that was inoculated with PG4180.N9 grown at 28°C. With different concentrations of added CFA, these data could be confirmed. A nearly linear correlation between the amount of added CFA and the suppression of the HR could be observed. These results indicated that the timing and quantity of CFA build-up within the infiltrated leaf area were essential for CFA's mode of action as an HR-delaying compound and pos-

sible molecular mimic of JA. Kenton et al. (1999) recently demonstrated that tobacco leaves infected with *P. syringae* pv. *phaseolicola* and undergoing the HR showed a significant JA accumulation within the developing lesions between 3 and 9 hpi. Likewise, Veronesi et al. (1996) and Rickauer et al. (1997) reported that the production of JA increased within the first hours after elicitor treatment. Based on this, we conclude that the presence of CFA at 3 and 6 hpi within the treated leaf tissue is essential to suppress the proper development of a HR. However, in almost half of our experiments with supernatant-free PG4180.N9 cells grown at 18°C we could also observe a delay in HR development. We speculate that this result may be attributed to the amount of CFA synthesized by PG4180.N9 de-novo inside the plant tissue.

In summary, our results showed that synthesis of coronafacoyl compounds by *P. syringae* pv. *glycinea* is important for the initial steps in both compatible and incompatible plant-microbe interactions. COR is required for the initial establishment of bacterial populations at the infection site whereas its precursor, CFA, plays an important role in preventing pathogen recognition. Further studies of this fascinating interplay between plant defense and bacterial secondary metabolism are currently underway in our laboratory.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. *P. syringae* was routinely maintained on King's medium B (KB; King et al. 1954) or on mannitol-glutamate medium (MG; Keane et al. 1970) at 28°C. For liquid cultures at 18 or 28°C, bacteria were incubated in KB medium, HSC medium (Hoitink-Sinden minimal medium optimized for COR production; Palmer and Bender 1993) or in *hrp*-inducing minimal medium (IM; Huynh et al. 1989) as described previously (Budde et al. 1998). The *Escherichia coli* strain DH5 α was used in cloning studies and was grown in Luria-Bertani (LB) broth at 37°C. Bacterial growth was monitored by measuring the optical density at 600 nm

Fig. 6. Addition of coronafac acid (CFA) to leaf areas initially infiltrated with PG4180.N9 cells derived from cultures pre-incubated at 18 or 28°C. Applied samples: 1, PG4180.N9 28°C; 2, PG4180.N9 28°C + 100 μ M CFA at 3 h post inoculation (hpi) (entire area); 3, PG4180.N9 28°C + 100 μ M CFA at 6 hpi (entire area); 4, PG4180.N9 18°C; 5, PG4180.N9 28°C + 100 μ M CFA at 3 and 6 hpi (entire area); 6, PG4180.N9 18°C; 7, PG4180.N9 28°C; 8, PG4180.N9 28°C + HSC medium at 3 and 6 hpi (entire area); 9, PG4180.N9 28°C + 100 μ M coronatine (COR) at 3 and 6 hpi (entire area); 10, PG4180.N9 28°C + 100 μ M jasmonic acid (JA) at 3 and 6 hpi (entire area).

Fig. 7. Development of hypersensitive response (HR) following addition of various amounts of purified coronafac acid (CFA) to tobacco leaves pre-infiltrated with washed PG4180.N9 cells grown at 28°C. Bars = outcome of HR (%) when CFA was added at 3.5 and 6.5 h post inoculation for at least 15 independent experiments.

(OD₆₀₀). The following antibiotics were added to media when needed: kanamycin, 25 µg/ml; spectinomycin, 25 µg/ml; streptomycin, 25 µg/ml; tetracycline, 25 µg/ml.

Standard genetic procedures.

Isolation of genomic DNA from *P. syringae*, agarose gel electrophoresis, restriction enzyme digestions, purification of DNA fragments from agarose gels, triparental matings, and electroporations were performed by standard techniques (Sambrook et al. 1989). Preparations of large-scale plasmid DNA were carried out with the Plasmid Midi kit (Qiagen, Hilden, Germany). Southern hybridizations were carried out according to a nonradioactive DNA labeling and detection kit (Boehringer-Mannheim, Mannheim, Germany) following the manufacturer's recommendation.

Isolation and quantitation of coronafacoyl compounds.

Organic acids were extracted from cell-free bacterial supernatants (1.5 ml), analyzed and quantified for the presence of CFA and COR by an established high pressure liquid chromatography (HPLC) method (Palmer and Bender 1993). For feeding experiments, large amounts of CFA and COR (approximately 30 to 60 mg/liter) were isolated by extraction with ethyl acetate from 5-day-old cultures of *P. syringae* pv. *glycinea* grown at 18°C in HSC medium. Both compounds were subsequently purified by thin-layer-chromatography (TLC) as described by Mitchell (1984). TLC-purified coronafacoyl compounds were dissolved in water, quantified by HPLC analysis, and subsequently added to cell suspensions, cell-free media, or leaf tissue as described below.

Plant material, inoculation procedures, and HR assays.

Soybean plants (*Glycine max* (L.) Merr. 'Maple Arrow') and tobacco plants (*Nicotiana tabacum* cv. Petit Havana SR1)

were grown in a greenhouse at 20 to 25°C, 60% humidity, with a 12-h photoperiod (15,000 lux). Four-week-old soybean plants were inoculated by two different methods. Plants were either sprayed with bacterial cultures grown at 18 and 28°C to an OD₆₀₀ = 1.0 (approximately 1×10^9 CFU per ml) or the bacteria were infiltrated into leaves by means of a needleless syringe at an OD₆₀₀ = 0.001 (1×10^6 CFU per ml). Following inoculation, soybean plants were transferred to a growth chamber (Controlled Environments, Pembina, ND) at 18 or 28°C, 80% humidity, and a 12-h photoperiod (15,000 lux; cold white fluorescent light). For determination of the in planta growth of *P. syringae*, three leaf disks (0.5 cm²) from different plants were removed, surface sterilized with 70% ethanol, pooled, and homogenized in sterile 0.9% NaCl. Serial dilutions were subsequently plated onto KB agar plates for determination of the number of CFU per milliliter. To acclimatize tobacco plants for bacterial inoculations, 8-week-old plants were placed in growth chambers set to 18 or 28°C 24 h before inoculation. Bacterial cultures grown at either 18 or 28°C to an OD₆₀₀ of 1.5 to 1.8 (1.7 to 2.2×10^9 CFU/ml) were infiltrated into the third and fourth tobacco leaf with a syringe. For generation of washed cells and cell-free supernatants, culture suspensions of an OD₆₀₀ of 1.5 to 1.8 were centrifuged at $5,000 \times g$ for 10 min and filtered through membranes with 0.2-µm-diameter pores. The sterile bacterial supernatants were then directly injected into the leaf tissue. To obtain supernatant-free cells without centrifugation, cell suspensions were directly filtered through membranes (0.2-µm-diameter pores) and cells were subsequently washed from the surface of the membranes. Bacterial cells were resuspended in fresh HSC medium and their density was adjusted to an OD₆₀₀ of 1.5 to 1.8. Subsequently, the cells were infiltrated into tobacco plant tissue. Purified coronafacoyl compounds (100 µM COR and 25, 50, 100, or 200 µM CFA suspended in HSC medium) were added to the bacterial inoculum or into pre-infiltrated or untreated tobacco leaf tissue. As negative controls, tobacco plants were infiltrated with sterile, distilled water, HSC, KB, or IM medium. Infiltrated areas were monitored for development of the HR in form of necrosis covering the entire infiltrated leaf area at 24, 48, and 72 hpi.

hrpL overexpression with the P_{lac} promoter.

Plasmid pDRL3D contains a transcriptional fusion of the *P. syringae* Pss61 *hrpL* gene to the P_{lac} promoter (S. W. Hutcheson, personal communication). To guarantee proper selection, a spectinomycin/streptomycin resistance gene cassette was inserted into the *EcoRI* recognition site of plasmid pDRL3D to yield pDRL3DSp. Plasmid pDRL3DSp was mobilized into *P. syringae* pv. *glycinea* PG4180.N9 and transconjugants were selected on MG medium supplemented with kanamycin and spectinomycin. The presence of pDRL3DSp in PG4180.N9 was confirmed by agarose gel electrophoresis of plasmid DNA preparations. To express the *hrpL* gene, PG4180.N9 containing pDRL3DSp was grown at 18 and 28°C to an OD₆₀₀ of 0.5, induced with 5 mM IPTG, and subsequently incubated until the cultures reached an OD₆₀₀ of 1.5 to 1.8. Aliquots of cell suspensions (1.5 ml) were taken prior to and after induction, pelleted by centrifugation, and resuspended in protein extraction buffer (50 mM Tris, 200 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, pH 7.4). Samples were then sonicated and centrifuged at $14,000 \times g$ for 20 min at 4°C. Pellets were dis-

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
<i>Escherichia coli</i> DH5α		Sambrook et al. 1989
<i>Pseudomonas syringae</i> pv. <i>glycinea</i>		
PG4180 wild type	COR ⁺ CMA ⁺ CFA ⁺	Bender et al. 1993
PG4180.N9	COR ⁺ CMA ⁺ CFA ⁺ Km ^r	Ullrich et al. 1994
PG4180.P1	COR ⁺ CMA ⁺ CFA ⁺ Km ^r Gm ^r	Ullrich et al. 1995
	<i>corS</i> mutant	
PG4180.C0	COR ⁺ CMA ⁺ CFA ⁺ Km ^r	Bender et al. 1993
PG4180.D4	COR ⁺ CMA ⁺ CFA ⁺ Km ^r	Bender et al. 1993
<i>P. syringae</i> pv. <i>syringae</i>		
Pss61	COR ⁺ CMA ⁺ CFA ⁺	A. Collmer
<i>P. syringae</i> pv. <i>tomato</i>		
DC3000	COR ⁺ CMA ⁺ CFA ⁺	D. A. Cuppels
Plasmids		
pMUH34	Tc ^r ; contains a 3.4-kb <i>HindIII</i> / <i>EcoRI</i> insert derived from p4180A in pRK415; required for complementation of COR ⁺ CMA ⁺ CFA ⁺ PG4180 mutants	Ullrich et al. 1995
pHIR11	Tc ^r ; 25-kb cosmid containing the <i>P. syringae</i> pv. <i>syringae</i> 61 <i>hrp</i> gene cluster	Huang et al. 1988
pDRL3D	Km ^r ; 8.71-kb plasmid containing <i>hrpL</i> under the control of the P _{lac} promoter	S.W. Hutcheson
pDRL3DSp	Km ^r Sm ^r Sp ^r ; 10.8-kb plasmid containing <i>hrpL</i> under the control of the P _{lac} promoter	This study

carded, and the supernatants were subsequently analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for production of HrpL.

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