Molecular characterization of field isolates of *Pseudomonas syringae* pv. *glycinea* differing in coronatine production

MATTHIAS ULLRICH,^{1,2*} STEFAN BERESWILL,¹ BEATE VÖLKSCH,² WOLFGANG FRITSCHÉ² and KLAUS GEIDER¹

¹Max-Planck-Institut für medizinische Forschung, Jahnstraße 29, D-69028 Heidelberg, Germany

²Institut für Mikrobiologie, Universität Jena, Philosophenweg 12, D-07743 Jena, Germany

(Received 17 July 1992; revised 6 April 1993; accepted 26 April 1993)

Coronatine-producing and non-producing strains of *Pseudomonas syringae* pv. *glycinea* have been examined. We found a connection between copper resistance and synthesis of coronatine. Published data implied that these properties may be encoded on different plasmids. Production of coronatine and copper resistance were also found to be correlated for pv. *glycinea* in 19 field-isolates from leaf spots of plants in a soybean field and in 28 strains of a bacterial culture collection. Genomic diversity within pv. *glycinea* was investigated by plasmid profiling, DNA hybridization studies and PCR analysis. All strains unable to produce coronatine (cor⁻) were sensitive to copper ions and showed no homology to DNA from plasmid pSAY1, which carries a gene cluster for steps in coronatine production. In addition, cor⁻ strains could be distinguished from coronatine-producing strains by a single unique band when amplified by random primer PCR. Plasmid profiles of strains isolated from field-populations during 1983, 1985 and 1990 showed that coronatine-producing and non-producing strains were present. The plasmid patterns also varied in 28 strains examined from a culture collection. No correlation between plasmid patterns and race specificity was observed. Cosmid pSAY1 proved to be an effective probe for detection of the coronatine synthesis genes and also revealed polymorphisms in coronatine producing strains of pv. *glycinea*.

Introduction

Pseudomonas syringae pv. *glycinea*, which causes bacterial blight of soybean [*Glycine max* (L.) Merrit], can produce the phytotoxin coronatine. Other pathovars of *P. syringae* producing this toxin are pv. *atropurpurea* (Nishiyama *et al.*, 1976), pv. *morsprunorum* (Mitchell, 1982), and pv. *tomato* (Bender *et al.*, 1987), which are pathogenic to ryegrass, *Prunus* spp. and tomato, respectively. Recently, analogues of coronatine were shown to be produced by *Xanthomonas campestris* pv. *phormiicola*, a pathogen of New Zealand flax (Mitchell, 1991). Coronatine induces chlorosis, hypertrophy and stunting of plant tissue. It has an unusual structure consisting of a bicyclic moiety, coronafacic acid, which is coupled to an ethylcyclopropyl amino acid, coronamic acid. The biosynthetic pathway of this phytotoxin has

been partially determined (Mitchell, 1985; Parry *et al.*, 1991; Parry & Mafoti, 1986; Young *et al.*, 1992).

Although coronatine is not a pathogenicity factor, several reports have shown that synthesis of the phytotoxin enhances the virulence of producing bacteria and therefore contributes to the biological fitness of pathogens *in planta* (Bender *et al.*, 1987; Gnanamanickam *et al.*, 1982; Sato *et al.*, 1983). Little is known about natural field-populations of *P. syringae* with respect to coronatine production. A study of coronatine synthesis *in vitro* and *in planta* revealed that 7 out of 19 pathogenic strains of *P. syringae* pv. *glycinea* did not produce coronatine (Gnanamanickam *et al.*, 1982). It was suggested that the negative strains produced another heat-stable factor which inhibits the growth of *Escherichia coli* cells. This inhibition could be reversed by arginine or citrulline, a feature characteristic of phaseolotoxin production in *P. syringae* pv. *phaseolicola* (Staskawicz & Panopoulos, 1979). Genes required for coronatine production have been linked to plasmid DNA in at least four pathovars (Sato *et al.*, 1983; Bender *et al.*, 1989, 1991). Recently, a 34 kb cloned region of

* Author for correspondence. Tel. 49 6203 106 117; fax 49 6203 106 122.

Abbreviation: HR, hypersensitive reaction.

plasmid p4180A in *P. syringae* pv. *glycinea* PG4180 was saturated with Tn5, and 27 kb were shown to be required for coronatine production (Young *et al.*, 1992). Furthermore, mutants of strain PG4180 deficient in steps of toxin biosynthesis could be complemented in feeding experiments by addition of coronatine precursors (Young *et al.*, 1992). In *P. syringae* pv. *tomato* strain DC3000, steps for coronatine production were found to be chromosomally encoded and have been cloned in cosmid pEC18 (Ma *et al.*, 1991). Interestingly, pEC18 showed homology to three *Sst*I fragments of pSAY1 (C. Bender, personal communication).

In *P. syringae* pv. *tomato*, the *cop* genes determining copper resistance are plasmid-encoded. In strain PT23.2, resistance was associated with pTC23C (67 kb) and pPT23D (35 kb). Both plasmids transferred copper resistance by forming a cointegrate which resembled in size the 100 kb plasmid involved in coronatine synthesis (Bender & Cooksey, 1986, 1987). Recently, two periplasmic proteins, CopA (72 kDa) and CopC (12 kDa), and the outer-membrane protein CopB (39 kDa) were found to mediate copper resistance in *P. syringae* pv. *tomato* by sequestration of copper ions in the periplasm, thereby preventing the entry of the toxic Cu^{2+} ions into the cytoplasm (Cha & Cooksey, 1991). It has been suggested that copper resistance and coronatine synthesis are encoded on different plasmids (Bender & Cooksey, 1986; Bender *et al.*, 1989).

In various pathovars of *P. syringae* and *X. campestris*, specific races have been established (Cross *et al.*, 1966; Fett & Sequeira, 1981; Cook & Stall, 1982). Host-compatible races of phytopathogenic bacteria are characterized by their ability to overcome plant defence mechanisms and produce disease symptoms in certain host cultivars. In resistant cultivars, early recognition causes a hypersensitive reaction (HR). The molecular basis for this taxonomic differentiation within pathovars is the gene-to-gene relationship of dominant plant disease resistance genes and dominant avirulence genes in the bacteria (Keen & Staskawicz, 1988). In *X. campestris* pv. *vesicatoria* copper resistance and avirulence loci were reported to be linked on a large plasmid (Stall *et al.*, 1986). However, in *P. syringae* pv. *glycinea* the correlation between phytotoxin production, copper resistance and race-specificity is not known.

In the current study we investigated the ecologically relevant phytotoxin production in field isolates of *P. syringae* pv. *glycinea* using a bioassay for coronatine and by Southern blot analysis using a DNA probe containing coronatine synthesis genes from *P. syringae* pv. *tomato* (Bender *et al.*, 1991). *P. syringae* pv. *glycinea* strains were also examined for differences in plasmid profiles, race affiliation, and tolerance to CuSO_4 . The correlation of coronatine synthesis and copper resistance described here

also provides a convenient tool to monitor coronatine-positive strains of *P. syringae* pv. *glycinea* by growth on media with CuSO_4 .

A PCR amplification method using a random-synthesized oligonucleotide primer has been used not only for identification of organisms (Welsh & McClelland, 1990; Williams *et al.*, 1990; Welsh *et al.*, 1991; Martin *et al.*, 1991), but also for the discrimination of pathogenic and non-pathogenic fungi (Schäfer & Wöstemeyer, 1992). The latter approach was applied here to distinguish coronatine producing and non-producing strains of pv. *glycinea*. A preliminary report on plasmid profiles and coronatine production in *P. syringae* pv. *glycinea* strains has been published elsewhere (Ullrich *et al.*, 1991).

Methods

Bacterial strains and plasmids. During the 1990 growing season *P. syringae* pv. *glycinea* cultures were isolated from field grown plants of soybean cv. Maple Arrow at Dornburg near Jena, Germany. At each isolation date, three to five leaf spots with necrotic lesions surrounded by chlorotic haloes were excised with a corkborer (7 mm diam.), surface sterilized in 70% (v/v) ethanol, washed three times in sterile water and macerated. Homogenates were dissolved in 1 ml 0.9% NaCl and serially diluted for plating on King's medium B agar (King *et al.*, 1954). After 3–4 d incubation at 28 °C, colonies typical for *P. syringae* were identified by microscopic, physiological and biochemical tests (Bradbury, 1988; B. Völksch *et al.*, unpublished data), including pigment production (King *et al.*, 1954), the LOPAT tests and nitrate reduction (Lelliott *et al.*, 1966), and the Hugh–Leifson test (Hugh & Leifson, 1953). Strains characterized as fluorescent pseudomonads were tested for pathogenicity on 20-d-old plants of soybean cv. Maple Arrow. Symptom-producing isolates were assigned as *P. syringae* pv. *glycinea* and one strain per leaf spot was stored for future experiments (Table 1). Strains from various geographic origins were obtained from the Göttinger Sammlung Phytopathogener Bakterien (GSPB; Institut für Pflanzenpathologie und Pflanzenschutz der Universität Göttingen, Germany) (Table 1). Additionally, reference strains of *P. syringae* pv. *glycinea* were obtained from the bacterial culture collection at the University of Jena (Institut für Mikrobiologie). *P. syringae* pv. *syringae* PS61 is a copper-sensitive, non-producer of coronatine which has been described by Bender & Cooksey (1986) and Bender *et al.* (1989). *P. syringae* pv. *syringae* PS61(p4180A) produces coronatine and was constructed previously by transforming the coronatine plasmid p4180A, which resides in *P. syringae* pv. *glycinea* PG4180, into PS61 by electroporation (Young, 1991).

P. syringae strains were propagated on standard I (Merck) or on King's medium B agar plates at 28 °C. *Escherichia coli* was grown on standard I agar or on Luria–Bertani (LB) solidified medium at 37 °C. If required for plasmid selection, tetracycline was added to media at 25 µg ml⁻¹. For plasmid isolation the strains were grown in LB medium. Plasmid pSAY1 (52 kb) was generously provided by Dr C. L. Bender, Stillwater, Oklahoma, USA.

Preparation and hybridization of plasmid DNA. Plasmids were isolated after alkaline lysis of the cells according to the Qiagen procedure (DIAGEN). The DNA was analysed on 0.8% agarose gels at 120 V for 2 h in the case of whole plasmids and at 25 V for 18 h for restriction fragments. Restriction digests and Southern transfers were done by standard procedures (Maniatis *et al.*, 1982). The nylon filters

Table 1. Sources and properties of *P. syringae* pv. *glycinea* strains

Coronatine phenotypes were determined by the potato hypertrophy assay. The MIC of CuSO₄ was defined as the minimal concentration which inhibited confluent growth of the bacterial culture after incubation at 28 °C for 72 h. Strains resistant to 2 mM-Cu²⁺ are indicated. GSPB strains were obtained from the Göttinger Sammlung Phytopathogener Bakterien. Plasmid pSAY1 carries genes required for coronatine synthesis (Bender *et al.*, 1991).

Strain	Year of isolation*	Coronatine phenotype	MIC of CuSO ₄	DNA homology to pSAY1	Race*	Source
GSPB 67	1943	cor ⁻	0.4 mM	—	?	Canada
GSPB 472	1951	cor ⁻	0.4 mM	—	?	USA
GSPB 1201	?	cor ⁻	0.6 mM	—	2	Race reference
GSPB 1545	1985	cor ⁺	resistant	+	4	Zaire
GSPB 1548	1985	cor ⁺	resistant	+	4	Rwanda
GSPB 1565	1984	cor ⁺	resistant	+	4	Hungary
GSPB 1834	?	cor ⁻	0.4 mM	—	0	Race reference
GSPB 1835	?	cor ⁻	0.4 mM	—	1	Race reference
GSPB 1837	?	cor ⁻	0.4 mM	—	5	Race reference
GSPB 1971 (SB1ab)	1989	cor ⁺	resistant	+	4	Race reference
GSPB 1974 (Lab-a)	1990	cor ⁺	resistant	+	6	Race reference
GSPB 1985 (SJ130-1)	1990	cor ⁻	0.4 mM	—	9	France
GSPB 1988 (SJ309-17)	1990	cor ⁺	resistant	+	4	France
GSPB 1993 (IPVBO 1479)	1983	cor ⁺	resistant	+	4	Italy
GSPB 1994 (IPVBO 1481)	1982	cor ⁺	resistant	+	4	Italy
GSPB 1995 (IPVBO 2116)	1985	cor ⁻	0.6 mM	—	4	Italy
GSPB 1996 (S-1)	1983	cor ⁻	0.6 mM	—	4	Serbia
GSPB 1997 (S-49)	1983	cor ⁻	0.6 mM	—	X	Race reference
GSPB 1999 (1B-PL)	1990	cor ⁺	resistant	+	4	Poland
GSPB 2001 (4B2b-PL)	1990	cor ⁺	resistant	+	4	Poland
GSPB 2002 (H-1a)	1990	cor ⁺	resistant	+	4	Hungary
GSPB 2003 (H-3c)	1990	cor ⁻	0.4 mM	—	9	Hungary
GSPB 2004	?	cor ⁻	0.4 mM	—	9	Race reference
GSPB 2005 (H-7a)	1990	cor ⁺	resistant	+	6	Hungary
GSPB 2006 (1d)	1980	cor ⁻	0.4 mM	—	X	Hungary
GSPB 2033 (Bar-a)	1990	cor ⁺	resistant	+	4	Germany
GSPB 2034 (1561)	?	cor ⁻	0.4 mM	—	4	France
PG 4180	1975	cor ⁺	resistant	+	4	New Zealand
Psg 7a/90	1990	cor ⁺	resistant	+	4	This study
Psg 15a/90	1990	cor ⁺	resistant	+	4	This study
Psg 16a/90	1990	cor ⁻	0.6 mM	—	9	This study
Psg 18a/90	1990	cor ⁻	0.6 mM	—	9	This study
Psg 24a/90	1990	cor ⁻	0.6 mM	—	9	This study
Psg 27a/90	1990	cor ⁻	0.6 mM	—	9	This study
Psg 28a/90	1990	cor ⁺	resistant	+	4	This study
Psg 34a/90	1990	cor ⁻	1.2 mM	—	?	This study
Psg 36a/90	1990	cor ⁻	0.6 mM	—	?	This study
Psg 43a/90	1990	cor ⁺	resistant	+	4	This study
Psg 44a/90	1990	cor ⁻	0.6 mM	—	9	This study
Psg 46a/90	1990	cor ⁺	resistant	+	4	This study
Psg 49a/90	1990	cor ⁺	resistant	+	4	This study
Psg 51a/90	1990	cor ⁻	0.6 mM	—	?	This study
Psg 55a/90	1990	cor ⁻	0.6 mM	—	?	This study
Psg 58a/90	1990	cor ⁺	resistant	+	4	This study
Psg S8/83	1983	cor ⁺	resistant	+	4	This study
Psg S30/85	1985	cor ⁺	resistant	+	4	This study
Psg S161/83	1983	cor ⁺	resistant	+	4	This study

*?, unknown isolation date or race.

were prehybridized for 2 h and hybridized in 5 × SSC (1 × SSC is 0.15 M-NaCl and 0.015 M-sodium citrate), 0.02 % SDS, 0.1 % *N*-lauroylsarcosine, and 1 % (w/v) dried skimmed milk at 68 °C for 16 h. Post-hybridization washes were twice in 2 × SSC plus 0.1 % SDS at room temperature for 5 min, and twice in 0.1 × SSC plus 0.1 % SDS at 68 °C for 15 min. DNA labelling with digoxigenin-11-dUTP and detection of blots were done according to the manufacturer's specifications (Boehringer Mannheim).

Bioassay for coronatine and copper resistance. Coronatine production was assessed by screening strains for their ability to induce hypertrophy on potato tubers using Völksch's (Völksch *et al.*, 1989) modification of Sakai's method (Sakai *et al.*, 1979). Copper resistance was assessed by incubating the cultures for 72 h at 28 °C on standard I agar plates containing CuSO₄ at concentrations of 0.4, 0.6, 1.2 and 2.0 mM. The minimal inhibitory concentration (MIC) of CuSO₄ was defined as the lowest concentration which inhibited confluent growth of the bacteria

after 72 h incubation at 28 °C. The possible linkage of copper resistance and coronatine production was investigated by assessing growth of *P. syringae* pv. *syringae* PS61 and PS61(p4180A) on standard I agar containing 2.0 mM-CuSO₄.

Determination of bacterial races. All strains of *P. syringae* pv. *glycinea* isolated in this study were tested for their ability to elicit a compatible or incompatible reaction on eight different soybean cultivars: Acme, Bicentennial, Chippewa, Flambeau, Harosoy, Merrit, Norchief and Peking (Cross *et al.*, 1966; Fett & Sequeira, 1981). Inoculum was prepared from colonies on standard I agar plates, suspended in sterile water, and adjusted to a final concentration of 1×10^8 c.f.u. ml⁻¹ (OD₅₉₀ 0.1–0.15). Trifoliolate leaves of 20-d-old soybean plants of the appropriate differential cultivars were inoculated on the underside of the leaf by means of a syringe. Treated plants were then incubated in a greenhouse with artificial neon light (for 12 h) at 20 °C and 70–90% humidity for 5–7 d. The hypersensitive reaction (incompatible reaction) was generally observed 24–48 h after inoculation, whereas the compatible reaction was observed at 5–7 d following inoculation.

Random primer-dependent PCR. The Tn5-derived 17mer sequence CAGGACGCTACTGTGT was used as primer to perform PCR-directed fingerprinting of genomic DNA of coronatine-producing and non-producing *P. syringae* pv. *glycinea* strains. The genomic DNA was prepared by lysozyme/SDS lysis of the cells followed by phenol/chloroform extraction. The primer was synthesized with a DNA synthesizer (Applied Biosystem 380B), purified by HPLC (0.1 M-triethylamine/acetic acid (pH 7.0) and acetonitrile; ODS-C₁₈ column, Beckman), and stored in aliquots at –20 °C. The amplification reactions were performed in volumes of 50 µl containing 67 mM-Tris/HCl (pH 8.8), 1.5 mM-MgCl₂, 10 mM-2-mercaptoethanol, 160 µg bovine serum albumin ml⁻¹, 10% (v/v) dimethylsulphoxide, 0.2 mM each of dATP, dCTP, dGTP and TTP, 70 ng primer (12.5 pmol), 10 ng template DNA and 0.5 U of *Tth* DNA polymerase (from *Thermus thermophilus*, Pharmacia). The assays were performed in a liquid temperature bath cycler (H.-P. Vosberg, Max-Planck-Institut, Heidelberg, Germany). The temperature cycles were controlled by a computer program with denaturation at 93 °C (for 2 min in the first cycle, then for 1 min), primer annealing to the template DNA at 32 °C for 2 min, and extension steps at 72 °C for 2 min. After 37 cycles, the amplification products were separated on a 1.5% (w/v) agarose gel.

Results

Coronatine production in *Pseudomonas syringae* pv. *glycinea* field isolates

During the growing season from June to October 1990, 76 isolates of *P. syringae* pv. *glycinea* were recovered from 69 necrotic soybean leaf spots surrounded by chlorotic haloes. Of the 76 pv. *glycinea* isolates, 23 failed to produce detectable amounts of coronatine when tested in the potato hypertrophy assay. The other 53 strains produced coronatine at various levels. Coronatine-producing (cor⁺) and non-producing (cor⁻) phenotypes were obtained throughout the growing season. From these pv. *glycinea* isolates, seven cor⁺ and nine cor⁻ strains were selected for further genetic studies. The cor⁻ strains were also able to cause formation of small haloes, which were not significantly different from haloes of cor⁺ strains. The nature of this effect is unclear, since we

eliminated the possible production of phaseolotoxin-like substances for the coronatine-negative pv. *glycinea* strains examined in this study. None of the coronatine-negative strains inhibited growth of *E. coli*, *Chlorella pyrenoidosa*, or *Geotrichum candidum* in bioassays (B. Völksch & M. Ullrich, unpublished data).

Plasmid profiles of various coronatine-producing and non-producing *P. syringae* pv. *glycinea* strains

Plasmid DNA was purified from sixteen strains isolated in 1990 and three cor⁺ pv. *glycinea* strains which were isolated from the same soybean field in 1983 and 1985 (Fig. 1a). Plasmid DNA was also isolated from cor⁺ strain PG4180. Plasmids could be grouped into four size classes ranging from 70 to 100 kb (class A), 30 to 60 kb (class B), 11 to 15 kb (class C), and 8 kb (class D) (Table 2). Classes A, B and C contained plasmids of more than one size. Subclasses were accordingly designated as A1, A2, A3, A4, B1, B2, C1 and C2. Plasmids of subclass A1 (approximately 95 kb) were found in all pv. *glycinea* strains investigated. Plasmids of classes B and D were present in most cor⁺ isolates, and class C plasmids (11–15 kb) dominated in cor⁻ strains. The small plasmid pD (8 kb) was observed in over half of the cor⁺ strains. Plasmids of classes B and D were not detected in

Fig. 1. Plasmid patterns of *P. syringae* pv. *glycinea* strains and hybridization to plasmid pSAY1 carrying genes for coronatine synthesis. Plasmids were isolated and separated on an agarose gel (a). Homology was detected with digoxigenin-labelled pSAY1 DNA (b). Lanes: 1, *Psg* 55a/90; 2, *Psg* 18a/90; 3, *Psg* 7a/90; 4, *Psg* 58a/90; 5, *Psg* S8/83; 6, *Psg* 49a/90. The size classes A (100–70 kb), B (60–30 kb), C (15–11 kb) and D (8 kb) are indicated. Known plasmids of *P. syringae* pv. *glycinea* strain PG4180 (Leary *et al.*, 1987) and pv. *tomato* strain PT23.2 (Bender & Cooksey, 1986) were used as references for sizing. The arrow indicates fragments of chromosomal and plasmid DNA. The positive signal in this area after hybridization with pSAY1 is derived from broken plasmid DNA migrating in the same size range as chromosomal fragments.

Table 2. Plasmid profiles of *P. syringae* pv. *glycinea* strains from field samples

Typical plasmids for group I: A1, A3, B1, B2, C2, D; group II: A1, A2, B2, D; group III: A1, A2, B1; group IV: A1, A3, C1; group V: A1, A3, A4, C2; group VI: A1, A2, A3, A4, D; group VII: A1, A4, C1. +, Presence of plasmids in classes A (100–70 kb), B (60–30 kb), C (15–11 kb) or D (8 kb); ++, double bands in a plasmid class.

Strain	Toxin phenotype	Plasmid classification									Plasmid profile group
		A1	A2	A3	A4	B1	B2	C1	C2	D	
PG 4180	cor ⁺	+	+	+	+					+	VI
<i>Psg</i> S8/83	cor ⁺	+	+	+	+					+	VI
<i>Psg</i> S30/85	cor ⁺	+	++			+					III
<i>Psg</i> S161/83	cor ⁺	+		+		+	+		+	+	I
<i>Psg</i> 7a/90	cor ⁺	+		+		+	+		+	+	I
<i>Psg</i> 15a/90	cor ⁺	+	++			+					III
<i>Psg</i> 28a/90	cor ⁺	+	++				+			+	II
<i>Psg</i> 43a/90	cor ⁺	+	++			+					III
<i>Psg</i> 46a/90	cor ⁺	+	++			+					III
<i>Psg</i> 49a/90	cor ⁺	+	++			+					III
<i>Psg</i> 58a/90	cor ⁺	+	++				+			+	II
<i>Psg</i> 16a/90	cor ⁻	+		++				+			IV
<i>Psg</i> 18a/90	cor ⁻	+		++				+			IV
<i>Psg</i> 24a/90	cor ⁻	+		++				+			IV
<i>Psg</i> 27a/90	cor ⁻	+		++				+			IV
<i>Psg</i> 34a/90	cor ⁻	+			+			+			VII
<i>Psg</i> 36a/90	cor ⁻	+		+	+				+		V
<i>Psg</i> 44a/90	cor ⁻	+		++				+			IV
<i>Psg</i> 51a/90	cor ⁻	+		+	+				+		V
<i>Psg</i> 55a/90	cor ⁻	+		+	+				+		V

the cor⁻ pv. *glycinea* isolates obtained from the field. Class C plasmids were present in both cor⁺ and cor⁻ strains. The pv. *glycinea* strains were divided into five groups. The cor⁺ strain Psg S8/83 (isolated in 1983) and the cor⁻ isolate Psg 34a/90 (isolated in 1990) did not fit into any of the other groups. However, the plasmid pattern of Psg S8/83 was similar to that of strain PG4180, which was isolated in New Zealand in 1975 (Mitchell, 1982). These and strain Psg 34a/90 may be ordered into additional groups VI and VII. All other coronatine-negative strains belonged to two groups distinguishable by their plasmid profiles. The cor⁺ pv. *glycinea* isolates were more diverse. Group III contained plasmids of size classes A and B and was dominant in the 1990 isolation (Table 2). When strains isolated from the same field but from different seasons were compared, a conservation of plasmid pattern was found for the cor⁺ strains Psg S161/83 and Psg 7a/90, which were isolated in 1983 and 1990, respectively, and for cor⁺ Psg S30/85 and Psg 15a/90 isolated in 1985 and 1990, respectively. Thus, members of groups I and III were found in more than one growing season.

Copper resistance of the isolated *P. syringae* pv. *glycinea* isolates

All 19 pv. *glycinea* isolates were tested for growth on solid medium containing CuSO₄ (Table 1). Both cor⁺

and cor⁻ isolates of pv. *glycinea* grew well on plates containing 0.4 mM-CuSO₄. CuSO₄ at 0.6 mM inhibited all cor⁻ strains, except the cor⁻ isolate 34a/90, which grew on plates with 0.6 mM but not with 1.2 mM-CuSO₄. No spontaneous change in copper sensitivity of cor⁻ strains was observed. All cor⁺ pv. *glycinea* strains grew well on agar containing 2 mM-CuSO₄. No attempts have yet been made to produce copper-sensitive mutants in order to correlate them to coronatine production. When plasmid p4180A was transferred into *P. syringae* pv. *syringae* PS61 (Young, 1991), neither PS61 nor PS61(p4180A) grew on standard I agar containing 2.0 mM-CuSO₄ but PS61(p4180A) produced coronatine while strain PS61 did not. This result indicates that copper resistance and coronatine production are not linked on p4180A in *P. syringae* pv. *glycinea* PG4180.

Race determination

The race affiliation of the isolated *P. syringae* pv. *glycinea* strains was examined by investigating their interaction with eight different soybean cultivars (Table 3). This differential series of soybean cultivars was also inoculated with known pv. *glycinea* races from the bacterial culture collection in Göttingen (GSPB). All cor⁺ strains produced disease symptoms on eight cultivars and were therefore classified as race 4. These strains could be separated from reference strain GSPB 1201 (race 2),

Table 3. Race typing of field isolates of *P. syringae* pv. *glycinea*

C, Susceptible (compatible) reaction; I, incompatible reaction (HR); GSPB, strains used as race specific reference strains; ud, strains undefined in race affiliation.

Strains	Reaction of soybean cultivars								Race
	Acme	Bicentennial	Chippewa	Flambeau	Harosoy	Merrit	Norchief	Peking	
GSPB 1835	C	C	I	C	I	I	I	C	1
GSPB 1201	C	C	C	C	C	C	C	C	2*
GSPB 1971	C	C	C	C	C	C	C	C	4
GSPB 1837	I	I	I	I	C	C	I	C	5
GSPB 1974	I	I	I	C	I	I	C	I	6
GSPB 2004	I	I	C	I	C	C	I	I	9
GSPB 1834	I	C	I	I	I	C	I	I	0*
GSPB 1997	I	I	I	I	C	C	I	C	X
All cor ⁺ pv. <i>glycinea</i> isolates	C	C	C	C	C	C	C	C	4
cor ⁻ pv. <i>glycinea</i> isolates: 16a/90, 18a/90, 24a/90, 27a/90, 44a/90	I	I	C	I	C	C	I	I	9
cor ⁻ pv. <i>glycinea</i> isolates: 36a/90, 51a/90, 55a/90	I	C	I	I	I	C	C	I	ud
cor ⁻ isolate Psg 34a/90	I	I	C	I	C	I	C	I	ud

* Causes browning on agar media.

which shows the same incompatibility/susceptibility scheme as race 4, by the absence of browning on standard I nutrient agar or on King's medium B agar. This browning effect is typical for members of races 2 and 0. The reaction of the soybean cultivars to the cor⁻ pv. *glycinea* strains corresponded to the classification scheme obtained by plasmid profiles. Group IV consisted of strains Psg 16a/90, Psg 18a/90, Psg 24a/90, Psg 27a/90 and Psg 44a/90, and induced a hypersensitive reaction on cultivars Acme, Bicentennial, Flambeau and Norchief, a reaction pattern which is typical for race 9. These strains originated from soybean cv. Maple Arrow, which is susceptible to race 4 and race 9, but not to the other races. Three cor⁻ strains (GSPB 1995, 1996, 2034) were ordered into race 4 by their reaction to the soybean cultivars used for race differentiation (Table 1).

Coronatine-negative isolates Psg 36a/90, Psg 51a/90, and Psg 55a/90 were characterized by HR induction on plants of cvs Acme, Chippewa, Flambeau and Harosoy. These strains could not be classified as members of any known races of pv. *glycinea*.

Homology of P. glycinea pv. *glycinea* plasmid DNA to genes involved in coronatine synthesis

Recently, Bender *et al.* (1991) cloned a DNA fragment which contains genes involved in coronatine synthesis. In the present study, the cosmid clone pSAY1 was used as a probe to find possible plasmid DNA homology among the isolated pv. *glycinea* strains. When intact plasmid

DNA was probed with digoxigenin-labelled pSAY1, the largest plasmid band in each cor⁺ strain hybridized to the probe, whereas no hybridization signal was detected for plasmids contained in cor⁻ strains (Fig. 1b). pSAY1 did not hybridize to pPG1, an 8 kb plasmid of size class D which was previously suggested to play a role in coronatine production (Leary *et al.*, 1987), nor to plasmids in classes other than A1. When pSAY1 was probed to *Sst*I-digested plasmid DNA of strains isolated in the present study, hybridization occurred to six *Sst*I fragments in the cor⁺ isolates which were similar in size to six *Sst*I fragments contained in pSAY1 (8.3, 5.4, 4.6, 3.7, 3.3 and 2.5 kb). In contrast, no homology to DNA fragments from plasmid DNA of cor⁻ strains was observed.

Furthermore, plasmid DNA of several pv. *glycinea* isolates was digested with *Bam*HI and probed with pSAY1. For strain Psg 7a/90 six fragments of 13.0, 10.0, 7.9, 6.0, 4.7 and 3.3 kb hybridized with the probe (Fig. 2). Since the construction of pSAY1 resulted in loss of the *Bam*HI sites of the vector's polycloning site (C. Bender, personal communication), it was not possible to resolve the bordering insertion in pSAY1 by digestion with this enzyme (Fig. 2, lane 3). The fragments of 13 and 10 kb represent the border fragments of plasmid pA1 of Psg 7a/90. These fragments were found in all cor⁺ strains isolated in 1990, but not in strain Psg S8/83, which was isolated in 1983. *Bam*HI-digested plasmid DNA from PG4180 showed a hybridization pattern identical to Psg S8/83 when probed with pSAY1. Southern blot analysis

Fig. 2. Restriction fragment length polymorphism of *Bam*HI-digested plasmid DNA of two coronatine producing *P. syringae* pv. *glycinea* strains from field samples. The probe was digoxigenin-labelled DNA of plasmid pSAY1. Lanes: 1, *Psg* 7a/90; 2, *Psg* S8/83; 3, pSAY1. The sizes of internal *Bam*HI fragments are indicated; V, position of vector DNA.

indicated that *Bam*HI fragments adjacent to the poly-linker in the pSAY1 insert were 7.5 and 12 kb in the coronatine plasmids indigenous to PG4180 and *Psg*

S8/83. Therefore, restriction fragment polymorphisms were found which distinguished the pv. *glycinea* isolates of 1990 from *Psg* S8/83 and PG4180. Since none of the plasmids of the *cor*⁻ *Psg* strains showed hybridization signals to pSAY1, chromosomal DNA of those strains was probed. No clear signal was observed, which indicates that coronatine synthesis genes are not present in these strains.

Differentiation of *cor*⁺ and *cor*⁻ *P. syringae* pv. *glycinea* strains by PCR

The amplification reaction was carried out with chromosomal DNA of nine *cor*⁺ and nine *cor*⁻ pv. *glycinea* field isolates. For these strains the Tn5-derived primer give rise to the amplification of four distinct bands migrating at 3.4 kb, 2.7 kb, 1.6 kb and 1 kb in both types of strain (Fig. 3). An additional band of 4 kb was observed in the *cor*⁻ isolates *Psg* 36a/90, *Psg* 51a/90 and *Psg* 55a/90. The *cor*⁺ pv. *glycinea* strains produced a characteristic signal at 5 kb, which was not observed for the *cor*⁻ isolates. This implies that a 5 kb band is specific for pv. *glycinea* derivatives which are copper resistant and produce the phytotoxin coronatine. Furthermore, the PCR-amplified signals from *P. syringae* pv. *glycinea* were different from those of *P. syringae* pvs *tomato*, *syringae*, *phaseolicola* or *morsprunorum*, which did not produce the 5 kb band (S. Bereswill & K. Geider, unpublished data).

Fig. 3. PCR analysis of *P. syringae* pv. *glycinea* DNA with a single primer. Lanes 1–9 show coronatine-positive strains and lanes 10–18 coronatine-negative strains. Lanes: 1, *Psg* 7a/90; 2, *Psg* S161/83; 3, *Psg* S8/83; 4, *Psg* S30/85; 5, *Psg* 15a/90; 6, *Psg* 43a/90; 7, *Psg* 46a/90; 8, *Psg* 28a/90; 9, *Psg* 58a/90; 10, *Psg* 34a/90; 11, *Psg* 16a/90; 12, *Psg* 18a/90; 13, *Psg* 24a/90; 14, *Psg* 27a/90; 15, *Psg* 44a/90; 16, *Psg* 36a/90; 17, *Psg* 51a/90; 18, *Psg* 55a/90; M, 1 kb ladder marker DNA (the size of some fragments is indicated), *, A 5 kb band, characteristic of coronatine-producing strains; Δ, a 4 kb band, produced by some coronatine-negative strains.

Fig. 4. RFLP pattern of *Bam*HI-digested plasmid DNA from *P. syringae* pv. *glycinea*. Strains without coronatine synthesis are shown in lanes 1–4, positive strains in lanes 5–11. The sizes (kb) of *Bam*HI fragments of plasmid pSAY1 are indicated on the right and by arrowheads. Lanes: 1, GSPB 1561; 2, GSPB 2003; 3, *Psg* 51a/90; 4, *Psg* 44a/90; 5, GSPB 1974 (race 6); 6, GSPB 2033; 7, GSPB 1545; 8, GSPB 1971; 9, *Psg* 7a/90; 10, GSPB 2002; 11, GSPB 1988; P, pSAY1. V, Vector DNA.

Molecular characterization of *P. syringae* pv. *glycinea* strains from a culture collection

Eight strains which were used as reference for pv. *glycinea* races and 19 additional pv. *glycinea* isolates from different geographic origins and isolation dates, were assayed for coronatine production, copper resistance, and homology to pSAY1 (Table 1). The race reference strains and strain PG4180, which is well characterized for its molecular biology, were considered to be 'pathotypes'. The correlation between coronatine synthesis and copper resistance which was shown for field isolates was also confirmed for strains from the culture collection. Out of 27 GSPB strains, 13 produced hypertrophic outgrowth on potato tissue and grew on agar with 2 mM-CuSO₄. All 13 isolates were previously classified into race 4 (11 strains) and race 6 (2 strains) (Abo-Moch *et al.*, 1991). When plasmid DNA contained in these strains was digested with *Sst*I and probed with pSAY1 the bands hybridized to the coronatine-specific probe producing the six fragments of the plasmid insertion. However, three race 4 strains which were isolated in France (GSPB 1561), Italy (GSPB 1995) and Serbia (GSPB 1996) were sensitive to copper ions and did not produce coronatine in detectable amounts. The remaining 11 cor⁻ strains, which belonged to races 1, 2, 5, 9, 0 and X, were copper-sensitive and did not show homology to pSAY1. pSAY1 hybridized to bands of similar size in the *Bam*HI-digested cor⁺ GSPB strains (Fig. 4). Polymorphisms observed between strains probably indicated variability in the size of fragments which

Fig. 5. Plasmid profiles of various *P. syringae* pv. *glycinea* strains derived from the GSPB culture collection. Plasmids were isolated and separated on an agarose gel. Lanes 1–5, cor⁻ strains; lanes 6–12, cor⁺ strains. Lanes: 1, GSPB 1997 (race X); 2, GSPB 2034 (race 4); 3, GSPB 2003 (race 9); 4, *Psg* 51a/90; 5, *Psg* 44a/90 (race 9); 6, GSPB 1545 (race 4); 7, GSPB 1988 (race 4); 8, GSPB 1974 (race 6); 9, GSPB 1971 (race 4); 10, *Psg* 49a/90; 11, *Psg* 58a/90; 12, *Psg* 7a/90. Letters A to D indicate plasmid size classes according to Table 2. The arrow indicates fragments of chromosomal and plasmid DNA.

flank the coronatine region. These polymorphisms were not due to race specificity since GSPB 1974, which is a race 6 isolate, showed a hybridization pattern similar to a race 4 strain, pv. *glycinea* 7a/90 (Fig. 4, lanes 5 and 9).

The plasmid patterns in the pv. *glycinea* strains from the culture collection differed from those found in the field isolates (Fig. 5). Plasmids of subclass A1 were found in all pv. *glycinea* strains tested. Plasmids of size class B (30–60 kb) which were typical for cor⁺ and copper-resistant pv. *glycinea* strains, also occurred in cor⁻ and copper-sensitive GSPB isolates. Interestingly, the 8 kb plasmid (class D) occurred in cor⁺ and cor⁻ strains. It was claimed previously to be specific for coronatine producing pv. *glycinea* strains (Leary *et al.*, 1987). No correlation between race and plasmid profiles could be found for the GSPB isolates (Fig. 5, lanes 3 and 5).

Discussion

Strains of *P. syringae* pv. *glycinea* were characterized for coronatine production, copper resistance, and race affiliation. Coronatine synthesis as well as copper resistance have been genetically investigated in *P. syringae* pv. *tomato* strain PT23.2 (Bender & Cooksey, 1986, 1987; Bender *et al.*, 1989; Mellano & Cooksey,

1988). The DNA regions responsible for these traits are located on plasmids designated as pPT23A (coronatine synthesis), pPT23C (copper resistance) and pPT23D (copper resistance, *cop* operon). The localization of genes encoding these properties on different plasmids suggests an independent evolution of copper resistance in *P. syringae* pathovars synthesizing coronatine. Results obtained in the present study with p4180A support this hypothesis. Upon acquisition of p4180A, *P. syringae* pv. *syringae* PS61 acquired the ability to synthesize coronatine (Young, 1991), but did not become copper-resistant. DNA hybridization experiments were conducted in the present study using pSAY1, which contains a 30 kb insert from the 101 kb plasmid pPT23A of *P. syringae* pv. *tomato* strain PT23.2. The insert DNA in pSAY1 was shown to be required for coronatine production in pv. *tomato* and restored phytotoxin synthesis to coronatine-negative Tn5 mutants of PT23.2. Furthermore, the insert DNA of pSAY1 hybridized to large plasmids in other coronatine-producing pathovars of *P. syringae*, indicating a conservation of this DNA region in many producers (Bender *et al.*, 1991). The homology also found for pv. *glycinea* indicated a strong conservation of coronatine synthesis genes in plasmid DNA of coronatine-producing isolates. For digests with two restriction enzymes (*Sst*I and *Bam*HI) we observed hybridizing fragments identical to those contained in pSAY1. Hybridization of pSAY1 to *Bam*HI-digested plasmid revealed distinct polymorphisms for pv. *glycinea* isolates from different regions or years of isolation. In a previous report, homology of genes required for coronatine synthesis was demonstrated for four coronatine-producing pathovars of *P. syringae* (Bender *et al.*, 1991). Coronatine production and copper resistance were correlated for all pv. *glycinea* strains investigated. However, results obtained with p4180A indicate that the genes responsible for these properties are unlikely to be linked on a single plasmid. A mutual benefit to the plasmids deriving from co-residence in the same cell could still be a reason for coevolution of copper resistance and coronatine production in *P. syringae* pv. *glycinea*. Approximately one-third of the field isolates did not produce coronatine. These were all copper-sensitive and their plasmid and chromosomal DNA did not hybridize to plasmid pSAY1. A possible explanation for their copper-sensitivity is the presence of a plasmid incompatible with plasmids encoding copper resistance in *cor*⁺ strains. Incompatibility in *cor*⁻ strains may also occur for the plasmid with genes for coronatine synthesis.

In the present study, the classification of *cor*⁺ and *cor*⁻ strains in *P. syringae* pv. *glycinea* was achieved both by pathogenicity on soybean and by PCR analysis with a random primer. Common bands were generated by PCR in all pv. *glycinea* strains, and an additional band was

generated in all coronatine-producing strains. Random-primed PCR analysis has also been used to distinguish aggressive and non-aggressive strains of *Leptosphaeria maculans* (Schäfer & Wöstemeyer, 1992).

The plasmid pattern of the field isolates was determined and used for classification of the strains. They were ordered into six groups according to their plasmid profile. Denny (1988) used a similar approach to classify *P. syringae* pv. *tomato*. He found diversity for the plasmid profiles and similarity in other properties like phage sensitivity, fatty acid composition and sugar catabolism. A more homogeneous profile was found by Bender & Cooksey (1986) for pv. *tomato* strains isolated from Southern California, USA. DNA fingerprint analysis of plasmid DNA of plant pathogenic bacteria has revealed heterogeneity not only for *P. syringae* pv. *pisi* but also for pv. *glycinea* strains (King, 1988).

A classical criterion for the grouping of pv. *glycinea* strains is the determination of races. All of the coronatine-producing field isolates investigated in the current study belonged to race 4. The non-producing strains were assigned to race 9 or an undefined race reaction pattern. Since race 4 strains are the most common in Europe (Abo-Moch *et al.*, 1991), their ability to produce coronatine might favour distribution under natural conditions.

Strains from an established culture collection also showed a correlation between coronatine synthesis and copper resistance. These strains belonged to races 4 and 6 (Abo-Moch *et al.*, 1991). The heterogeneous history of their isolation is reflected by a remarkable number of RFLPs observed for the region bordering the biosynthesis cluster for coronatine synthesis. The plasmid and chromosomal DNA of coronatine-negative strains did not hybridize to probe pSAY1, indicating the specificity of this probe for detection of coronatine-producing pv. *glycinea* isolates. The coronatine non-producing strains were heterogeneous in their race affiliation. The plasmid profiles of the GSPB strains were complex and could not be used for classification. On the other hand, random-primed PCR analysis resembles the fingerprinting of genomic DNA and can be used for differentiation of bacterial species and pathovars (Welsh & McClelland, 1990; S. Bereswill & K. Geider, unpublished data). The PCR bands confirmed the data about strains with respect to coronatine synthesis and the classification of the isolates described to be pv. *glycinea*. It seems reasonable to assume that *cor*⁺ and *cor*⁻ strains may occur in a single lesion of a diseased soybean leaf. In this study, we avoided isolating more than one strain from each lesion. These independently derived strains were unambiguously identified as *P. syringae* pv. *glycinea* and differ in production of the phytotoxin coronatine. Further investigations are required to determine the

properties of cor^+ and cor^- strains isolated from soybean lesions or of cor^- mutants in order to address the question as to the advantage of either phenotype for certain stages in the development of bacterial blight on soybean under natural conditions.

We thank Dr C. L. Bender (Department of Plant Pathology, Oklahoma State University, Stillwater, USA) for generously providing cosmid pSAY1, for unpublished data, for fruitful discussions and very helpful comments on the manuscript. We are also grateful to Dr K. Rudolph, University of Göttingen, for the gift of bacterial cultures.

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