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

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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 ethylcyclpropyl 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

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Abbreviation: HR, hypersensitive reaction.

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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 PC18 (Ma et al., 1991). Interestingly, PC18 showed homology to three SsrI 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 plasmidic 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 (Schafer & Wöstemeyer, 1992). The latter approach was applied here to distinguish coronatine producing and non-producing strains of *P. glycinea*. A preliminary report on plasmid profiles and coronatine production in *P. syringae* pv. *glycinea* strains has been published elsewhere (Ulrich 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 Dormburg 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 day incubation at 28 °C, colonies typical for *P. syringae* were identified by microscopic, physiological and biochemical tests (Bradbury, 1988; B. Völkke et al., unpublished data), including pigment production (King et al., 1954), the LOPAT tests and nitrate reduction (Leblott 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
were prehybridized for 2 h and hybridized in 5x SSC (1 x 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 2x SSC plus 0.1% SDS at room temperature for 5 min, and twice in 0.1x 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 Volksch's (Volksch et al., 1979) 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 1 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(p418OA) on standard I agar containing 2.0 mM-CuSO_{4}.

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, Merritt, 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^{6} c.f.u. ml^{-1} (OD_{590} 0.1-0.15). Trifoliate 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 CAGGACGCTACTTGTGT 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-C_{18} 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-MgCl_{2}, 10 mM-2-mercaptoethanol, 160 μg bovine serum albumin ml^{-1}, 10% (v/v) dimethylsulphoxide, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 70 ng primer (12.5 pmol), 10 ng template DNA and 0.5 U of Tth DNA polymerase (from *Thermus thermophila*, 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* 58b/90; 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.](attachment:image)
Coronatine production by *P. syringae* pv. *glycinea*

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Toxin phenotype</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>B1</th>
<th>B2</th>
<th>C1</th>
<th>C2</th>
<th>D</th>
<th>Plasmid profile group</th>
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<td>++</td>
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<td>+</td>
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<td></td>
<td></td>
<td></td>
<td>+</td>
<td>VI</td>
</tr>
<tr>
<td><em>Psg</em> S8/83</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>VI</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>III</td>
</tr>
<tr>
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<td>cor+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td>I</td>
</tr>
<tr>
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<td>cor-</td>
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<td>+</td>
<td>+</td>
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<td></td>
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<td>+</td>
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<td>+</td>
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<td></td>
<td></td>
<td>+</td>
<td>II</td>
</tr>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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<td></td>
<td>+</td>
<td>V</td>
</tr>
</tbody>
</table>

The cor- *P. syringae* pv. *glycinea* isolates obtained from the field. Class C plasmids were present in both cor+ and cor- strains. The *P. syringae* 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+ *P. syringae* 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 *P. syringae* isolates were tested for growth on solid medium containing CuSO₄ (Table 1). Both cor+ and cor- isolates of *P. syringae* 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+ *P. syringae* 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 *P. 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.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Acme</th>
<th>Bicentennial</th>
<th>Chippewa</th>
<th>Flambeau</th>
<th>Harosoy</th>
<th>Merrit</th>
<th>Norchief</th>
<th>Peking</th>
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<td>16a/90, 18a/90, 24a/90, 27a/90, 44a/90</td>
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* 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 SstI-digested plasmid DNA of strains isolated in the present study, hybridization occurred to six SstI fragments in the cor+ isolates which were similar in size to six SstI fragments contained in pSAY1 (8.3, 5.4, 4.6, 3.7, 3.3 and 2.4 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 BamHI 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 BamHI 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. BamHI-digested plasmid DNA from PG4180 showed a hybridization pattern identical to Psg S8/83 when probed with pSAY1. Southern blot analysis
Coronatine production by \textit{P. syringae} pv. glycinea

58/33. Therefore, restriction fragment polymorphisms were found which distinguished the pv. glycinea isolates of 1990 from \textit{Psg} S8/83 and PG4180. Since none of the plasmids of the cor$^-$ \textit{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$^-$ \textit{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 \textit{Psg} 36a/90, \textit{Psg} 51a/90 and \textit{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 \textit{P. syringae} pv. glycinea were different from those of \textit{P. syringae} pvs tomato, syringae, phaseolicola or morsprunorum, which did not produce the 5 kb band (S. Bereswill & K. Geider, unpublished data).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{coronatine_production.png}
\end{figure}
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 SstI 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 produce homology to pSAY1. pSAY1 hybridized to bands of similar size in the BamHI-digested cor+ GSPB strains (Fig. 4). Polymorphisms observed between strains probably indicated variability in the size of fragments which 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,
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 phytoalexin 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 (SstI and BamHI) we observed hybridizing fragments identical to those contained in pSAY1. Hybridization of pSAY1 to BamHI-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 phytoalexin 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.

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References


