ISOLATION OF GENES INVOLVED IN CORONATINE BIOSYNTHESIS FROM *P. SYRINGAE* PV. TOMATO PT23.2 AND *P. SYRINGAE* PV. GLYCINEA PG4180

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CHAPTER I

INTRODUCTION

Several phytopathogenic Pseudomonas spp. excrete toxic compounds which alter the metabolism of the host cells and elicit a symptom associated with the disease, such as chlorosis or necrosis. The pseudomonad phytotoxins that have been characterized are generally low molecular weight substances which often contain an amino acid or peptide moiety (Mitchell, 1984b). Seven distinct chemical toxin structures have been characterized from various Pseudomonas spp. including the tabtoxins, the phaseolotoxins, coronatine, syringomycin (syringotoxin), tagetitoxin, and recently rhizobitoxine and tropolone (Mitchell, 1984b; Mitchell, 1989).

Although there have been several studies and reviews (Gross and Cody 1985; Mitchell, 1982; Mitchell, 1984b; Durbin and Graniti, 1989; and Mitchell, 1991a), the mechanisms and complete effects of phytotoxins on host physiology are not clearly understood for any one phytotoxin. However, the phytotoxins appear to share one common biological function, that is, they disturb the metabolism of the host to enhance access to essential

nutrients for the proliferation of the pathogen, thereby creating their own ecological niche. By understanding the biosynthetic pathways of toxins our understanding of several plant-pathogen interactions will be enhanced.

Coronatine is an example of a phytotoxin which is produced by various Pseudomonas syringae pathovars, including P. s. pv. atropurpurea (Sato, 1983), P. s. pv. glycinea (Mitchell and Young, 1978), P. s. pv. morsprunorum (Mitchell, 1982), and P. s. pv. tomato (Bender et al., 1987 and Mitchell et al., 1983). The isolation of genes involved in coronatine biosynthesis will advance our knowledge of the genetics and regulation of phytotoxins. Also, since little is known regarding the synthesis of polyketide compounds such as coronatine, valuable information may be gained regarding the genetics of polyketide synthesis.

The main purpose of these studies was to isolate the coronatine biosynthesis genes from plasmid pPT23A, an indigenous plasmid in Pseudomonas syringae pv. tomato PT23.2. The first goal was to map the locations of Tn5 in four coronatine-defective mutants which have Tn5 insertions in pPT23A. The second objective was to clone coronatine biosynthesis genes by functional complementation using a cosmid library of plasmid pPT23A constructed in pLAFR3. Complementation of coronatine-defective mutants was accomplished by introducing cosmid clones into the mutants by conjugation or electroporation. Because gene transfer

into P. s. pv. tomato was difficult, and plasmid pPT23A was not stably maintained in the absence of the host, a revised strategy for cloning coronatine synthesis genes was adapted for P. s. pv. glycinea (PG4180). A cosmid library of PG4180 plasmid DNA was first constructed in pLAFR3. Selected clones from the library were examined for their ability to restore toxin production to two coronatine-defective mutants of PG4180. The third objective was to develop a fine structure map of the coronatine synthesis genes in P. s. pv. glycinea 4180. A number of mutations were generated in the PG4180 coronatine synthesis genes using site-directed mutagenesis. Complementation tests were performed to estimate the number and location of transcriptional units involved in coronatine production. Also, studies were conducted to determine if the polyketide synthetase genes actI, actIII, and gra3 had any functional significance in the pathway of coronafacic acid synthesis by probing these genes to plasmid DNA of P. s. pv. glycinea and P. s. pv. tomato. A similar experiment was conducted to determine if the indoleacetic acid-lysine synthetase gene from P. s. pv. savastanoi has a role in the conjugation of coronafacic acid to isoleucine or coronamic acid, a step which requires amide bond formation between the carboxyl of coronafacic acid and the amino group of isoleucine or coronamic acid.

CHAPTER II

LITERATURE REVIEW

Phytotoxin-producing pseudomonads

Bacteria in the genus Pseudomonas are rod-shaped, gram-negative aerobes that belong to the family Pseudomonadaceae in the kingdom Prokaryotae. These bacteria are motile by polar flagella and are chemoorganotrophs which inhabit soil, fresh water, and marine environments. The number of phytopathogenic species of Pseudomonas has been reduced from 100 to 23 by the International Committee of Systematic Bacteriology (ICSB) (Fahy and Lloyd, 1983). Nine of the phytopathogenic species have been identified as fluorescent pseudomonads. Most fluorescent phytopathogenic pseudomonads are presently classified as one species, Pseudomonas syringae. This reduction has caused problems in distinguishing strains that are distinct because of their differences in host range and the disease symptoms they cause. For this reason the term "pathovar" is now used to differentiate strains of P. syringae on the basis of pathogenic characteristics (Fahy and Lloyd, 1983). There are presently over 40 different pathovars (pv.) of this species.

Several Pseudomonas spp. produce phytotoxins which

cause chlorotic or necrotic areas in localized regions of the plant. The pseudomonad phytotoxins that have been characterized are low molecular weight substances which often contain a peptide or amino acid component (Mitchell, 1982). Seven distinct chemical structures have been identified from the various Pseudomonas spp. which produce toxins: the tabtoxins, the phaseolotoxins, coronatine, syringomycin (syringotoxin), tagetitoxin, and recently rhizobitoxine and tropolone (Mitchell, 1984b and Mitchell, 1991a).

Coronatine is a nonhost-specific phytotoxin which is produced by several pathovars, including Pseudomonas syringae pv. atropurpurea (Sato et al., 1983), P. syringae pv. glycinea (Mitchell and Young, 1978), P. syringae pv. morsprunorum (Mitchell, 1982), and P. syringae pv. tomato (Bender et al., 1987 and Mitchell et al., 1983) which are pathogens of ryegrass, soybean, Prunus spp., and tomato, respectively. Recently, Xanthomonas campestris pv. phormiicola, a pathogen of New Zealand flax (Mitchell, 1991b), was found to produce coronafacoyl compounds. Coronatine has also been noted for other biological effects in addition to the induction of leaf chlorosis. Using purified coronatine, Nishiyama and co-workers have induced hypertrophic growth in potato tubers (Sato et al., 1983), and Sakai et al. inhibited root-growth in wheat seedlings (1976). Recent reports suggest that coronatine is involved

in ethylene production. Ferguson and Mitchell (Ferguson and Mitchell, 1985) demonstrated that the intact molecule of coronatine is necessary for ethylene production in bean leaf discs and for the chlorotic response in the intact leaf. Therefore, ethylene may have a specific role in the biological effects of coronatine.

Importance of phytotoxins

The symptoms of plant diseases caused by pathogens may require joint or sequential interactions of various pathogenic factors, including phytotoxins. Phytopathogenic bacteria generally do not require toxic metabolites to cause disease. Yoder (Yoder, 1980) has developed various criteria that are commonly used to evaluate the role of toxins in pathogenesis. These include: a) host specificity of the toxin, b) presence of the toxin in infected hosts, c) production of the toxin during development of the disease, d) induction of typical disease symptoms by treatment with toxin alone, and e) correlation of virulence with the quantity of toxin produced in vitro (Yoder, 1980). A toxin may be classified as host-specific when it has high biological activity only for host plants of the producing pathogen; alternatively, a toxin is termed nonhost-specific when it affects a wider range of plant species than does the producing pathogen (Mitchell, 1984b).

Furthermore, toxins are classified as either

pathogenicity factors, which are essential for a pathogen to cause disease, or as virulence factors, which increase the extent of the disease. Generally, toxins that are virulence factors are also nonhost-specific toxins, whereas host-specific toxins are pathogenicity factors. Bender et al. demonstrated that coronatine acts as a virulence factor in the bacterial speck disease caused by Pseudomonas syringae pv. tomato (Bender et al., 1987). They found that coronatine-defective (Cor-) mutants produced lesions significantly smaller than those of the coronatine-producing strain and were unable to achieve the population levels attained by the toxigenic wild-type. They concluded that coronatine synthesis was important in the virulence of P. syringae pv. tomato and contributed significantly to both lesion expansion and multiplication of the bacterium in tomato leaves.

An understanding of the biosynthetic pathways that lead to phytotoxin production is important in comprehending how toxins alter the plant host metabolism and thereby enhance the virulence of the producing pathogen. Studying these biosynthetic pathways should enhance our understanding of the regulation of toxin production, of biosynthetic intermediates, and of the mode of action of the toxin in planta. Durbin has justified the work needed to determine the biosynthetic pathways of phytotoxins by naming several potential benefits: 1) taxonomic application in identifying

pathogens by chromatographic behavior, symptom expression, host specificity, and hybridization of probes, 2) biocontrol agents for controlling weeds, and 3) rational design of bioactive compounds for antibiotic, herbicidal, or other applicable activities (Durbin and Graniti, 1989).

Structure and biosynthesis of coronatine

The structure and absolute stereochemistry of coronatine is illustrated in Figure 1a (Ichihara et al., 1977). Coronatine consists of a polyketide component (coronafacic acid, Fig. 1f) joined to a cyclized isoleucine derivative (ethylcyclopropyl amino acid or coronamic acid, Fig. 1a). These two components of coronatine are synthesized by two different pathways. Parry and Mafoti have shown that the coronafacic acid moiety is synthesized as a branched polyketide consisting of one unit of pyruvate and five molecules of acetate (Figure 2) (Parry and Mafoti, 1986). The mechanism by which the polyketide chains are coupled together to give an intermediate bicyclic structure is not known.

The cyclopropyl moiety of coronatine was shown by Mitchell to be derived from the isoleucine biosynthetic pathway (Mitchell, 1984a). Both L-[U-^{14}C]threonine and L-[U-^{14}C]isoleucine were incorporated into the ethylcyclopropyl part of coronatine with a higher incorporation rate recorded

for isoleucine, suggesting that threonine is probably metabolized to isoleucine. Recently, Parry et al. demonstrated that coronamic acid is specifically derived from L-alloisoleucine by a novel cyclization reaction that involves the removal of only one hydrogen atom from the C-methyl group of the amino acid (Figure 2) (Parry, 1991).

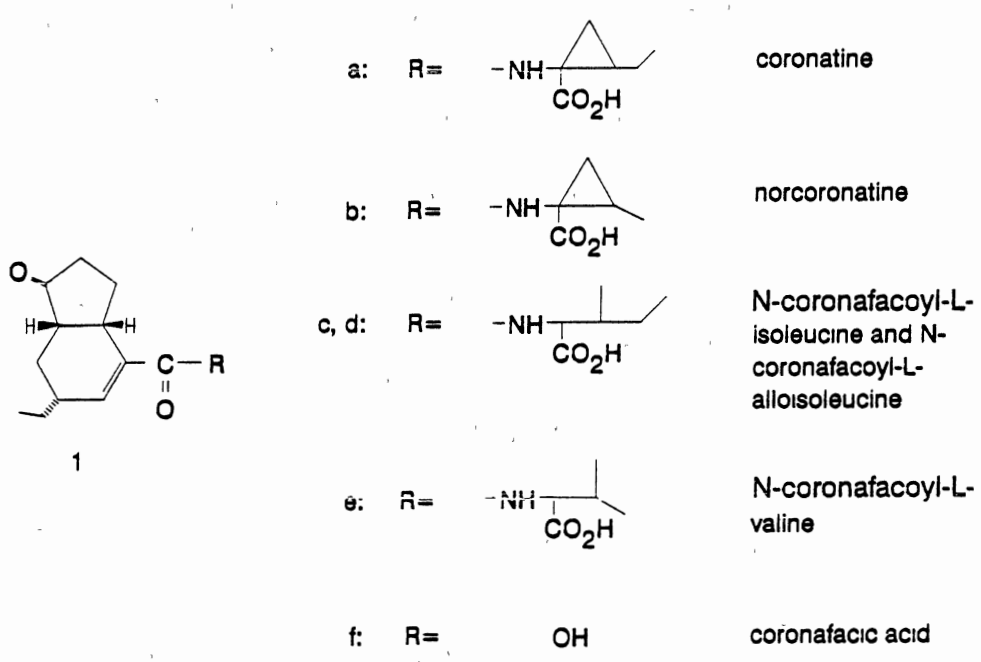

Figure 1. Different coupling combinations of R-amino groups with coronafacic acid.

Coronafacic acid is coupled to an isoleucine derivative by an amide bond to form the final product coronatine. The mechanism of cyclization to form the cyclopropane ring and the point at which this occurs are not known. Both N-

coronafacoyl-L-isoleucine and N-coronafacoyl-L-alloisoleucine (Figure 1c and 1d) are potential biosynthetic intermediates that may undergo an oxidative cyclization to form the cyclopropane ring present in the end-product, coronatine (Mitchell and Young, 1985). Because of stereochemical requirements, an alternative route may be the cyclization of the amino acid component prior to the coupling with coronafacic acid (Figure 3) (Mitchell, 1991a).

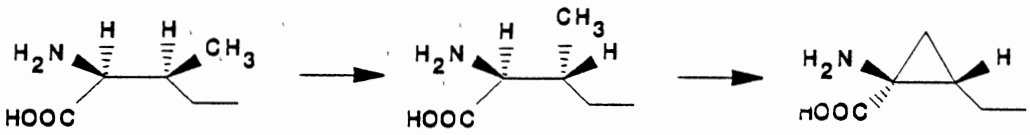

Figure 2. Synthesis of coronamic acid.

In addition to coronatine, Mitchell has identified other minor chlorosis-inducing compounds present in liquid cultures from strains of P. s. pv. atropurpurea (Mitchell, 1987 and Mitchell, 1984a) and P. s. pv. glycinea (Mitchell and Young, 1985 and Mitchell, 1985b). These naturally occurring compounds, termed "coronatines", include: N-coronafacoyl-L-valine, norcoronatine, N-coronafacoyl-L-isoleucine, and N-coronafacoyl-L-alloisoleucine (Figures 1 e, b, c, and d, respectively). Mitchell suggests that the coupling between coronafacic acid and the amino acids is

less specific compared to the synthesis of coronafacic acid which undergoes rigorous enzymatic control. However, coronatine, which contains the cyclopropyl moiety, is the most abundant component in liquid cultures of P. syringae (Mitchell and Frey, 1986).

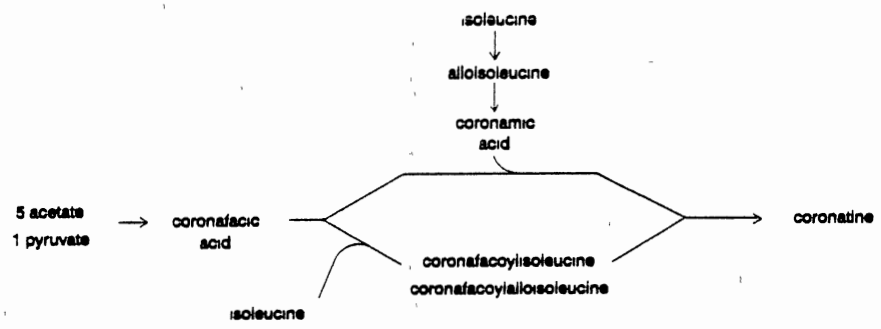

Figure 3. Proposed alternative biosynthetic pathways to coronatine.

Plasmids involved in phytotoxin production

Bacterial plasmids are generally not essential for the growth and metabolism of their hosts (Mills and Gonzalez, 1982). However, they may provide their hosts with added genetic capabilities, including antibiotic resistance, bacteriocin synthesis, metabolic pathways, phytohormone production, and toxin production (Lacy and Leary, 1979).

Gonzalez and Vidaver investigated P. syringae pv.

syringae for the possible involvement of plasmid DNA in the biosynthesis of syringomycin (Mills and Gonzalez, 1982). Some plasmid-free strains did not produce toxin and were nonpathogenic. Subsequent studies showed that plasmid-free strains produced the toxin syringomycin, indicating that plasmid DNA was not required for toxin synthesis (Gonzalez and Vidaver, 1980). Other researchers have investigated the possible involvement of plasmid DNA in phaseolotoxin production and showed no correlation between plasmid DNA and toxin production by P. syringae pv. phaseolicola (Jamieson et al., 1981). More recently, the locations of toxin synthesis genes in pathovars phaseolicola and syringae have been determined using transposon mutagenesis. In these studies, Tn5 insertions which inactivated toxin synthesis in P. syringae pvs. phaseolicola and syringae were chromosomally located (Morgan and Chatterjee, 1985 and Quigley et al., 1985). Similarly, Moore et al. identified a 30 kb chromosomal region of P. syringae pv. tomato DC3000 required for biosynthesis of coronatine (Moore et al., 1989). Also, Bender and coworkers have determined that genes for biosynthesis of the coronafacoyl compounds of Xanthomonas campestris pv. phormiicola are chromosomally encoded (unpublished data).

However, the involvement of plasmid DNA in the synthesis of coronatine by several Pseudomonas syringae pathovars has been firmly established (Bender et al., 1989;

Sato et al., 1983; Bender et al., 1991; and Sato, 1988). In P. syringae pv. tomato PT23.2, a 101 kb plasmid designated pPT23A was shown to have a role in coronatine production. This was demonstrated by introducing pPT23A into a non-producer of coronatine, P. syringae pv. syringae PS61; the acquisition of pPT23A by PS61 resulted in the biosynthesis of coronatine in the transconjugant. Similarly, pCOR1, a 58 Mdal plasmid in P. syringae pv. atropurpurea, was transferred into an avirulent strain of P. syringae pv. atropurpurea. Coronatine production and virulence were restored to the transconjugant containing pCOR1 (Sato, 1988). Leary et al. (Leary, 1987) detected an 8.3 kb plasmid (pPG1) in coronatine-producing strains of P. syringae pv. glycinea. Although pPG1 was absent in non-producing strains, further evidence for the role of pPG1 in coronatine production has not been obtained.

In a recent study, ^{32}P-labeled DNA fragments from a 30 kb region of pPT23A hybridized to plasmid DNAs from P. syringae pathovars atropurpurea, glycinea, and morsprunorum (Bender et al., 1991). These DNA fragments, which originated from pPT23A, are involved in coronatine synthesis and were strongly conserved in large plasmids (90-105kb) present in pvs. atropurpurea, glycinea, and morsprunorum. Marker-exchange mutagenesis experiments were used to introduce Tn5-inactivated regions of pPT23A into the three heterologous pathovars. These mutations resulted in an

interruption of coronatine synthesis in the heterologous pathovars. Therefore, these results indicate that coronatine biosynthesis genes are highly conserved in the plasmid DNAs of the four producing pathovars.

Genetics of coronatine production in *P. syringae* pv. tomato PT23.2

The toxigenic strain *P. syringae* pv. tomato PT23.2 typically induces necrotic lesions *in planta* which are surrounded by a yellow chlorotic halo. In a previous study, Bender *et al.* (1987) generated 1000 Tn5 mutants of PT23.2 and screened these for their ability to induce chlorosis *in planta*. Ten chlorosis-defective mutants were recovered; nine of these were further analyzed and found to be defective in production of coronatine. An additional 28 chlorosis-defective mutants of PT23.2 were provided by Cooperman and Cooksey (unpublished). Studies were then initiated to characterize physically the location of Tn5 insertions in these mutants. The results indicated that a 101 kb indigenous plasmid in PT23.2, designated pPT23A, was involved in coronatine production. Five mutants had Tn5 insertions in pPT23A, four mutants had deletions in the same plasmid, and the remaining 29 were lacking this plasmid entirely. A probe specific for pPT23A indicated that missing portions of the plasmid were not present in the

chromosome. Furthermore, a spontaneous nontoxigenic mutant of strain PT23, designated PT23.58, was also missing pPT23A.

The role of plasmid pPT23A in coronatine biosynthesis was further investigated by conjugating it into a coronatine non-producer, P. syringae pv. syringae PS61, in which the absence of coronafacoyl compounds were demonstrated by gas chromatography-mass spectrometry (Bender et al., 1989). The transconjugant PS61(pPT23A) was assayed for coronatine production using the potato slice bioassay developed by Sakai et al. (Sakai et al., 1979). These authors reported that potato tuber slices developed hypertrophic outgrowths when inoculated with coronatine-producing bacteria or purified coronatine. When PT23.2, PS61, and PS61(pPT23A) were inoculated to potato slices, outgrowths developed on tuber slices inoculated with PT23.2 and PS61(pPT23A); however, the surface of slices inoculated with the wild-type PS61 remained flat and smooth. This experiment suggested that the transfer of pPT23A to PS61 resulted in production of the toxin. Mitchell confirmed these results with chemical analyses. Organic acids were extracted from PT23.2, PS61, and the transconjugant PS61(pPT23A); these were then derivatized to their methylesters and analyzed by gas chromatography. The derivatized organic acids extracted from PT23.2 and PS61(pPT23A) contained peaks that corresponded to coronafacic acid, coronafacoylvaline, and coronatine, but these were absent in the extracts of the

wild type PS61. The identification of the components was confirmed by combined gas chromatography-mass spectrometry. These results demonstrated the involvement of pPT23A in coronatine production by P. syringae pv. tomato (Bender et al., 1989).

CHAPTER III

CLONING OF PLASMID DNA REQUIRED FOR CORONATINE SYNTHESIS IN P. SYRINGAE PV. TOMATO PT23.2 AND P. SYRINGAE PV. GLYCINEA PG4180

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ABSTRACT

Plasmid pPT23A is involved in coronatine biosynthesis in Pseudomonas syringae pv. tomato PT23.2. In the present study, a cosmid library of pPT23A was constructed in pLAFR3. A 52 kb cosmid clone, designated pSAY1, restored coronatine production to mutants containing Tn5 insertions or deletions in pPT23A but did not confer coronatine production to a mutant lacking pPT23A nor to nonproducers of coronatine. Because gene transfer into PT23.2 was difficult and plasmid pPT23A was not stably maintained in the absence of the host, a revised strategy for cloning coronatine synthesis genes was adapted for P. syringae pv. glycinea PG4180. Previously, DNA hybridizations and marker-exchange mutagenesis experiments suggested that coronatine synthesis genes resided on a 90 kb plasmid (p4180A) in PG4180. A

cosmid library of PG4180 plasmid DNA was constructed in pLAFR3. A 54 kb cosmid clone, designated pSAY10, restored coronatine production to two coronatine-defective mutants of PG4180, indicating that pSAY10 contains genes involved in coronatine synthesis. The polyketide synthase genes actI, actIII, and gra3, which are involved in antibiotic biosynthesis in Streptomyces, did not hybridize to the coronatine plasmid pPT23A nor to p4180A, indicating that they are not related to coronafacic acid synthesis genes at the nucleotide level. Although the indoleacetic acid-lysine synthetase gene iaaL hybridized to the coronatine plasmids, a functional role of this gene in the conjugation of coronafacic acid to amino acids could not be demonstrated.

Coronatine is a nonhost-specific phytotoxin which is produced by several pathovars of Pseudomonas syringae , including atropurpurea (Nishiyama et al., 1976), glycinea (Mitchell and Young, 1978), morsprunorum (Mitchell, 1982), and tomato (Mitchell, 1983 et al.; Bender et al., 1987). In addition to causing chlorosis on leaf tissue, coronatine induces hypertrophy in potato tubers (Sato et al., 1983), induces stunting (Sakai et al., 1979), interferes with ethylene production (Ferguson and Mitchell, 1985), and increases the virulence of producing organisms (Bender, et al., 1987). Coronatine is composed of a polyketide component (coronafacic acid) which is joined by an amide

bond to a cyclized isoleucine derivative (coronamic acid). These two moieties are synthesized by two separate pathways (polyketide and isoleucine) prior to coupling.

Polyketide-derived compounds include a large class of natural products which are constructed from acetate, propionate, or butyrate in a manner which resembles fatty acid synthesis (Hopwood and Sherman, 1990). Polyketide antibiotics may be synthesized by distinct polypeptides (Hopwood and Sherman, 1990) or by multifunctional enzymes (Donadio et al., 1991). Although the specific enzymatic steps required for synthesis of the polyketide coronafacic acid have not been elucidated, various polyketide synthase enzymes are functionally conserved among polyketide producers and may also have a role in coronafacic acid synthesis.

The synthesis of coronatine requires the coupling of coronafacic acid to either isoleucine or coronamic acid, a step which requires amide bond formation between the carboxyl of coronafacic acid and the amino group of isoleucine or coronamic acid. This step is functionally similar to the formation of IAA-lysine, a conjugate of IAA which is synthesized by P. syringae pv. savastanoi (Glass and Kosuge, 1986). The gene for IAA-lysine synthetase (iaaL) has been cloned in E. coli and has been detected in various P. syringae pathovars, including some which synthesize coronatine (Glass and Kosuge, 1986; White, 1989).

In previous studies (Bender et al., 1987; Bender et al., 1989), 2500 Tn5 mutants of the coronatine-producing strain P. syringae pv. tomato PT23.2 were generated and screened for their ability to induce chlorosis in planta. Studies were then initiated to physically characterize the location of mutations in the coronatine-defective mutants. The results indicated that pPT23A, a 101 kb plasmid, was involved in coronatine production (Bender et al., 1989). The role of pPT23A in coronatine production was demonstrated by transferring it into a nonproducer of coronatine, P. syringae pv. syringae PS61. Organic acids from PS61 containing pPT23A were analyzed by gas chromatography and found to contain coronatine, indicating that at least some of the genes required for coronatine synthesis reside on pPT23A (Bender et al., 1989).

The conservation of coronatine synthesis genes in P. syringae pv. tomato PT23.2 and pv. glycinea PG4180 was previously demonstrated (Bender et al., 1991). Regions of pPT23A which are required for coronatine synthesis were labeled with ^{32}P and hybridized to PG4180 plasmid DNA. The probes hybridized to p4180A, a 90 kb plasmid in PG4180. The functional significance of this homology was demonstrated by marker-exchange experiments in which regions of pPT23A which are required for coronatine synthesis were inactivated with Tn5 and recombined into PG4180. The coronatine-defective mutants generated using this approach contained Tn5

insertions in p4180A, further suggesting the role of this plasmid in coronatine synthesis. Whether p4180A contains all genes required for coronatine synthesis is not known.

In the present study, the physical location of Tn*5* insertions in pPT23A which inactivated coronatine production was identified. A cosmid library of PT23.2 plasmid DNA was constructed, and clones which restored toxin production to coronatine-defective mutants were recovered. Because gene transfer into PT23.2 was difficult and pPT23A was not stably maintained, a genetic analysis of coronatine production in *P. syringae* pv. glycinea PG4180 was undertaken. Several polyketide synthase genes from *Streptomyces* spp. and the *iaaL* gene from *P. syringae* pv. savastanoi were used as probes in this study to determine their possible role in coronatine production.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. Strains of *P. syringae* were maintained on mannitol-glutamate (MG) medium supplemented with yeast extract (MGY; Keane, 1970) or on King's medium B (KMB; King *et al.*, 1954). *Escherichia coli* HB101 was grown at 37 C on Luria-Bertani medium (LB; Maniatis *et al.*, 1982). *P. syringae* broth cultures were grown in either MGY or KMB on a rotary shaker (250 rpm) at 20 to 24 C. To analyze for coronatine production, *P. syringae* pv. tomato and glycinea

were grown for 6 days at 18 C in the media of Woolley, and of Hoitink and Sinden, respectively, as described previously (Bender et al., 1989). Antibiotics were added to media in the following concentrations (ug/ml): tetracycline, 12.5; ampicillin, 40; and kanamycin, 10 for P. syringae and 25 for E. coli.

DNA isolations. Plasmid DNA was isolated and purified from E. coli by standard methods (Maniatis et al., 1982). Plasmid DNA was extracted from P. syringae as described previously (Crosa and Falkow, 1981) with slight modifications (Bender and Cooksey, 1986). Preparative amounts of plasmid DNA were further purified on CsCl-EtBr gradients. Rapid, small-scale plasmid isolations from E. coli were performed using plasmid boiling method 2 of Crouse et al. (1983).

Molecular genetic techniques. Agarose gel electrophoresis, DNA restriction digests, and Southern transfers were done by standard procedures (Maniatis et al., 1982). Methods for lambda::Tn5 mutagenesis of cloned DNA and site-directed mutagenesis have been described elsewhere (Bender and Cooksey, 1987; Bender et al., 1991). Bacterial colonies were transferred to nylon membranes purchased from Amersham Corp., Arlington Heights, Ill.. When specific DNA fragments were to be labeled with ^{32}P, they were separated from vector fragments on agarose gels and excised. Residual agarose was removed with the Geneclean Kit manufactured by

BIO101, La Jolla, Calif. DNA was labeled with ^{32}P by the random primer labeling technique (Feinberg and Vogeistein, 1983). Prehybridizations (4 h at 68 C) and hybridizations (12 to 16 h at 68 C) were in aqueous solutions as described previously (Maniatis, 1982). Post-hybridization washes proceeded for 1 h at the following temperatures and salt conditions: (1) high stringency was 0.1X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.5% sodium dodecyl sulfate (SDS) at 68 C; (2) moderately high stringency was 2X SSC-0.5% SDS at 55 C; and (4) low stringency was 2X SSC-0.5% SDS at 42 C.

Cloning methods and characterization of Cor$^-$ mutants. Cosmid libraries of P. syringae pv. tomato PT23.2 and P. syringae pv. glycinea PG4180 plasmid DNA were constructed in pLAFR3 as described previously (Bender and Cooksey, 1987). Restriction maps of selected cosmids were constructed by digesting with BamHI, EcoRI, HindIII, and SstI in single and double digests. When necessary, restriction fragments from cosmid clones were subcloned into the broad-host-range vector, pRK415, to assist in mapping and in colony hybridizations.

PT23.20, PT23.22, PT23.23, PT23.26, and PT23.55 are five coronatine-defective (Cor$^-$) mutants of P. syringae pv. tomato that contain Tn5 insertions in pPT23A (Bender et al., 1989). In a previous study, Tn5 and flanking DNA were cloned from PT23.20, PT23.22, and PT23.26 in pCLB2, pCLB7,

and pCLB5, respectively (Bender et al., 1991). In the present study, Tn5 and flanking pPT23A DNA from PT23.23 and PT23.55 were cloned into pLAFR3 as described previously (Bender and Cooksey, 1987; Bender et al., 1991). Resulting plasmids were named pCLB8 and pCLB6, respectively. Clones containing Tn5 and flanking DNA from the mutated plasmids were transfected into E. coli HB101 and selected on LB medium containing tetracycline and kanamycin. HindIII, BamHI, and SstI were used to map the location of the Tn5 insertions contained in these clones.

In PT23.24 and PT23.25, the approximate extent of DNA deleted from pPT23A was estimated by sizing the pPT23A deletion derivatives on 0.4% agarose gels. Selected DNA fragments from the pPT23A cosmid library were hybridized to PT23.24 and PT23.25 plasmid digests to define the region deleted from pPT23A.

Gene transfer experiments. Conjugation or electroporation was used to transfer cosmids into two non-producers of coronatine, E. coli K12 and P. syringae pv. syringae PS51, and into the following classes of Cor$^-$ mutants of PT23.2: (1) mutants containing Tn5 insertions in pPT23A, (2) mutants missing pPT23A, and (3) mutants containing deletions in pPT23A. Cor$^-$ mutants of PT23.2 were prepared for electroporation using a procedure described by Cooksey (1990) except that KMB broth and 0.5 M sucrose were utilized for growing and washing the cells. Electroporation

was conducted at 12.5 kV/cm with a single pulse delivered from a 25-uF capacitor at 400 ohms. MGY broth (1 ml) was added to the cells immediately after electroporation, cells were incubated 1 h at 28 C, and then plated onto MGY agar with the appropriate antibiotics. Triparental matings using the helper plasmid pRK2013 were used to mobilize cosmids into marker-exchanged mutants of P. syringae pv. glycinea PG4180 as described previously (Bender and Cooksey, 1987). Transconjugants were selected on MG agar containing tetracycline and kanamycin, and plasmid transfer was verified by agarose gel electrophoresis.

Transfer of p4180A into PS7. Southern hybridization and marker-exchange mutagenesis experiments previously indicated that the coronatine synthesis genes in P. syringae pv. glycinea PG4180 were located on a 90 kb plasmid designated p4180A (Bender et al., 1991). In another study (discussed in Chapter 4), a Tn5 insertion was recovered in SstI fragment B of p4180A (Fig. 4C) which did not eliminate coronatine production and thus provided a cor^+ plasmid bearing a selectible marker. Plasmid DNA was isolated from PG4180.41, which contained this altered form of p4180A, and transferred into P. syringae pv. syringae PS7 by electroporation. PS7 was chosen as a recipient in this experiment because it does not contain plasmid DNA. PS7 transformants containing Tn5-labeled p4180A were selected on media containing kanamycin and confirmed by agarose gel

electrophoresis. BamHI and SstI were used to develop a restriction map of p4180A.

Assays for coronatine. Organic acids were extracted from transconjugants and transformants with ethyl acetate (Bender et al., 1989) and analyzed using a Beckman high-performance liquid chromatographic (HPLC) system which was equipped with two model 116 solvent delivery modules, a model 166 programmable detector, and System Gold chromatography software. Organic acids were analyzed on an Ultrasphere C-8 reverse-phase column (15 x 0.4 cm) at 208 nm. Initial conditions for gradient elution of organic acids were 10% acetonitrile in 0.05% aqueous trifluoroacetic acid (pH 3.0). Organic acids were fractionated with a linear gradient (slope = 1.0) from 10 to 50% acetonitrile for 15 min at 1 ml min^{-1}. Isocratic conditions (50% acetonitrile) were maintained for 3 min, and the system was then returned to initial conditions (10% acetonitrile) in 2 min.

Hybridization of PT23.2 and PG4180 plasmid DNA with selected DNA probes. Homology experiments were conducted to determine if the polyketide synthase genes actI, actIII (actinhorodin synthesized from Streptomyces coelicolor), and gra3 (granaticin synthesized from Streptomyces violaceoruber) were related to those which control coronafacic acid synthesis. Previous studies indicated that genes for coronafacic acid synthesis were contained on

pPT23A and p4180A (Bender et al., 1989; Bender et al., 1991). Consequently, plasmid DNA was isolated from PT23.2 and PG4180, separated by agarose gel electrophoresis, blotted to nylon membranes, and probed with selected polyketide synthase genes. DNA fragments used as probes for polyketide synthase genes included the following: (i) the 2.2 kb BamHI insert in pIJ2345 which contained actI (Malpartida and Hopwood, 1986); (ii) the 1.1 kb BamHI insert in pIJ2346 which contained actIII (Malpartida and Hopwood, 1986); and (iii) a 450 bp NcoI fragment containing gra3 (Sherman, 1989). The functional products of actI, actIII, and gra3 are beta-ketoacyl synthase (condensing enzyme), ketoreductase, and acyl carrier protein, respectively (Malpartida, 1987; Hallam, 1988; and Sherman, 1989).

A similar experiment was conducted to investigate if the indoleacetic acid-lysine synthetase (iaaL) gene from P. syringae pv. savastanoi has a potential role in the conjugation of coronafacic acid to amino acid moieties. In this experiment, a 2.0 kb EcoRV fragment containing the iaaL gene from pLG87 (Glass and Kosuge, 1986) was probed to PT23.2 and PG4180 plasmid DNA.

RESULTS

Characterization of mutations in pPT23A. PT23.20, PT23.22, PT23.26, PT23.23, and PT23.55 were previously shown to contain Tn5 insertions in pPT23A (Bender et al., 1989).

The Tn5 insertion sites in these mutants were further localized by probing SstI-digested plasmid DNA from each mutant with ^{32}P-labeled ColE1::Tn5. These results indicated that Tn5 had inserted into SstI fragments which were 2.5 kb in PT23.20 and PT23.23, 3.25 kb in PT23.22, and 8.7 kb in PT23.26 and PT23.55 (data not shown). Single and double digestions with BamHI, HindIII, and SstI were used to more precisely map the Tn5 insertion sites in pCLB2, pCLB5, pCLB6, pCLB7, and pCLB8 which contain Tn5 and pPT23A flanking DNA from PT23.20, PT23.26, PT23.55, PT23.22, and PT23.23, respectively. The Tn5 insertion site mapped in each clone was corroborated with that present in the five mutants by probing BamHI-, HindIII-, and SstI-digested plasmid DNA isolated from the latter with ^{32}P-labeled ColE1::Tn5. The location of the five Tn5 insertions with respect to SstI fragments B (2.5 kb), E (8.7 kb), and F (3.25 kb) is shown in Fig. 4A.

Electrophoresis of PT23.24 and PT23.25 plasmid DNA on 0.4% agarose gels indicated that the extent of pPT23A DNA deleted from the two mutants was about 30 and 8 kb, respectively. Hybridization of SstI fragments B, E, and F and EcoRI fragments A, C, and D (Fig. 4A) to SstI- and EcoRI-digested PT23.24 plasmid DNA indicated that these restriction fragments from pPT23A were missing. In PT23.25, Southern analysis of EcoRI-digested plasmid DNA indicated that EcoRI fragment A was absent but EcoRI fragments B and C

were present. The approximate regions of pPT23A DNA deleted in PT23.24 and PT23.25 are indicated in Fig. 4B.

One thousand clones from the PT23.2 plasmid DNA library were screened for homology to a 2.4 kb HindIII fragment. This probe was previously used as a pPT23A-specific probe because it showed no homology to other plasmids in PT23.2. The 2.4 kb HindIII fragment was previously shown to map adjacent to a 2.5 kb SstI fragment which was shown to have a role in coronatine synthesis (Bender et al., 1989). Sixty-four colonies from the PT23.2 plasmid library hybridized to the 2.4 kb HindIII fragment. From these hybridizing clones, cosmid pSAY1 (Fig. 4A) was chosen for complementation studies because it contained SstI fragments B, E, and F, the sites of Tn5 insertions in the five Cor$^-$ mutants. Plasmid pSAY1 (52 kb) completely restored the wild-type level of coronatine production to PT23.20, PT23.23, PT23.24, PT23.25, and PT23.26 when transformed into these mutants by electroporation (Table 2). A lower level of coronatine was produced in PT23.22 and PT23.55 containing pSAY1 (Table 2). However, cosmid pSAY2, which contains additional DNA to the right of pSAY1 (Fig. 4A), completely restored the wild-type level of coronatine production to PT23.22 and PT23.55 (Table 2). pSAY1 did not confer coronatine production to PT23.29, a mutant lacking pPT23A altogether, nor to two nonproducers of coronatine, E. coli K12 and P. syringae pv. syringae PS51 (Table 2). This demonstrated that pSAY1 contained some but

not all of the genes necessary for coronatine production.

Coronatine production in P. syringae pv. glycinea PG4180. Gene transfer by conjugation or electroporation into P. syringae pv. tomato PT23.2 was consistently unreliable. Mass matings resulted in Km^rTc^r transconjugants at a frequency of 1.0×10^{-11} per donor cell. Electroporation was only partially successful for the introduction of cosmid clones. Another problem encountered with PT23.2 was the small amount of coronatine synthesized in vitro (0.3 mg/L); this made it necessary to grow up large amounts of liquid culture (250-500 ml) for routine assessment of coronatine synthesis. Furthermore, the coronatine plasmid pPT23A was not stably maintained in the absence of the host. The sporadic loss of the plasmid made it difficult to analyze mutations by marker-exchange mutagenesis.

These difficulties led to the investigation of coronatine production in P. syringae pv. glycinea PG4180. In contrast to PT23.2, PG4180 synthesized relatively large amounts of coronatine in vitro (6.7 mg/L), which made it possible to analyze small amounts of culture supernatant (10-50 ml) for the presence of the toxin. In this study, cosmid clones were reliably transferred into PG4180 derivatives at a frequency of 2×10^{-4} per donor cell. Coronatine synthesis was found to be a stable characteristic of PG4180 and was not diminished through repeated

subculturing of the strain in the laboratory.

Previously, DNA fragments of pPT23A which were required for coronatine production were shown to be present in p4180A, a 90 kb plasmid in PG4180 (Bender et al., 1991). In the same study, Tn5-containing fragments of pPT23A were used to inactivate coronatine synthesis genes in p4180A by marker-exchange. PG4180.11 and PG4180.31, which contain Tn5 insertions in 2.5 and 3.2 kb SstI fragments (fragments B and F, Fig. 4C), were two Cor⁻ mutants developed previously (Bender et al., 1991). In the present study, cosmid pSAY1, which originated from pPT23A, restored coronatine production to both PG4180.11 and PG4180.31, further demonstrating the conservation of coronatine synthesis genes between PT23.2 and PG4180 (Table 2).

Cloning coronatine synthesis genes from PG4180. The cosmid library of PG4180 plasmid DNA, which consisted of 620 clones, contained 21 clones which hybridized with the 2.5 kb SstI fragment in pSAY1 (fragment B, Fig. 4A). This fragment was used as a probe for the coronatine region in p4180A because it was previously shown to have a role in coronatine synthesis in PG4180 (Bender et al., 1991), and it hybridized to a single 2.5 kb SstI fragment in p4180A and not to other plasmids present in this strain. A 54 kb cosmid clone, designated pSAY10, was chosen for complementation studies because many of the restriction sites in pSAY10 were identical to those present in pSAY1 (Fig. 4A and C). Cosmid

pSAY10 restored coronatine production to PG4180.11 and PG4180.31 (Table 2), indicating that it contains genes which are required for coronatine production in PG4180.

Hybridization of PT23.2 and PG4180 plasmid DNA with selected DNA probes. The three polyketide synthase probes from Streptomyces spp. used in this study (actI, actIII, and gra3) did not hybridize to plasmids in PT23.2 or PG4180 at any stringency level, suggesting that the polyketide synthase genes involved in coronafacic acid synthesis are not related at the nucleotide level to those in Streptomyces spp. However, a 2.0 kb EcoRV fragment containing the iaaL gene from P. syringae pv. savastanoi hybridized to both pPT23A and p4180A (data not shown). Hybridization of iaaL to BamHI-digested p4180A indicated that the homology was contained in 3.7 and 2.2 kb fragments (Fig. 5, BamHI fragments H and K).

The above results indicated that the iaaL gene, which couples IAA to lysine, was conserved in both pPT23A and p4180A, plasmids which contain coronatine synthesis genes. This suggested that the iaaL homologues in these plasmids might encode the amino acid synthetase(s) which couples coronafacic acid to amino acid derivatives. The functional significance of the observed homology was investigated in p4180A by subcloning BamHI fragment "K" into pRK415, mutating the clone with Tn5, and recombining the mutations into p4180A. The location of six mutations generated in

BamHI fragment K is indicated in Fig. 5. Organic acids were extracted from PG4180.51, PG4180.61, PG4180.71, PG4180.81, PG4180.91, and PG4180.101, the six mutants containing Tn5 insertions in BamHI fragment "K", and analyzed for the presence of coronatine by HPLC. All six mutants produced coronatine, which indicated that this region of p4180A does not have a role in the coupling of coronafacic acid to amino acids.

DISCUSSION

In this study, regions required for coronatine synthesis in P. syringae pv. tomato PT23.2 and P. syringae pv. glycinea PG4180 were cloned from pPT23A (101 kb) and p4180A (90 kb), the large plasmids present in these strains. Cosmid pSAY1 completely restored coronatine production to Cor⁻ mutants containing Tn5 insertions (PT23.20, PT23.23, and PT23.26) and deletions (PT23.24 and PT23.25) in pPT23A. Cosmid pSAY1 restored a lower level of coronatine production to PT23.22 and PT23.55, which is unclear presently. Because plasmid pSAY1 did not confer coronatine production to PT23.29, a Cor⁻ mutant lacking pPT23A or to P. syringae pv. syringae PS51 and E. coli K-12, other genes outside the region cloned in pSAY1 must be involved in the synthesis of coronatine.

Many restriction sites are conserved in both pPT23A and p4180A in the regions required for coronatine synthesis. In

another study, the coronatine synthesis genes in P. syringae pathovars atropurpurea and morsprunorum were shown to be closely related to those present in pPT23A and were also localized in large plasmid DNAs (Bender et al., 1991). In P. syringae pv. tomato DC3000, coronatine synthesis genes are chromosomally located and have been cloned in cosmid pEC18 (Moore et al., 1989). pEC18 contains three contiguous SstI fragments which are also present in pSAY1 and pSAY10 (see SstI fragments D, E, and F in Fig. 4A and 4C; Young and Bender, unpublished). This result further supports the conservation of coronatine synthesis genes among different producing organisms.

The polyketide synthase genes are responsible for condensing acyl-CoA esters of B-keto acids by a head-to-tail polymerization mechanism, which yields aliphatic chains of varying length. The polyketide synthase gene actI encodes the condensing enzyme required for actinorhodin synthesis (Malpartida et al., 1987), actIII codes for a ketoreductase (Hallam et al., 1988), and the protein product of gra3 is an acyl carrier protein (Sherman et al., 1989). The condensing enzyme may be common to all organisms which produce polyketide-derived secondary metabolites. Malpartida et al. (1987) observed hybridization to 14 of 18 polyketide producers using the actI and actIII genes as probes. Although the hybridizations with the actI, actIII, and gra3 genes were conducted at various stringency levels in the

present study, these three polyketide synthase genes did not hybridize to pPT23A or p4180A. However, it would be interesting to see if polyketide synthase genes from other bacteria were related to those which are involved in coronafacic acid synthesis, since the genes used in the present study orginated from a G+ bacteria Streptomyces coelicolor and violaceoruber.

The DNA hybridization studies conducted with the indoleacetic acid lysine synthetase (iaaL) gene showed homology to both pPT23A and p4180A. One of the regions homologous to iaaL in p4180A was inactivated with Tn5. Since PG4180 strains containing mutations in this region still produced coronatine, there is no evidence for the role of this region in the coupling of coronafacic acid to amino acids. Additional studies are currently in progress to isolate the genes involved in conjugating coronafacic acid to amino acid derivatives, a step which is essential for the biological activity of coronafacoyl-derived compounds.

Table 1. Bacterial strains and plasmids for Chapter 3.

Designation	Relevant characteristics	Source
Escherichia coli		
HB101		Maniatis, 1982
K12		Bachmann, 1983
Pseudomonas syringae pv. tomato		
PT23.2	Cor^+; contains pPT23A; isolated from diseased tomatoes, San Diego County, Calif., 1983	Bender, 1987
PT23.20	Cor^- Km^r; contains pPT23A::Tn*5*	Bender, 1987
PT23.22	Cor^- Km^r; contains pPT23A::Tn*5*	Bender, 1989
PT23.23	Cor^- Km^r; contains pPT23A::Tn*5*	Bender, 1989
PT23.24	Cor^- Km^r; deletion in pPT23A	Bender, 1989
PT23.25	Cor^- Km^r; deletion in pPT23A	Bender, 1989
PT23.26	Cor^- Km^r; contains pPT23A::Tn*5*	Bender, 1989
PT23.29	Cor^- Km^r; lacking pPT23A	Bender, 1989
PT23.55	Cor^- Km^r; contains pPT23A::Tn*5*	Bender, 1989
Pseudomonas syringae pv. glycinea		
PG4180	Cor^+; isolated from soybeans cultivated in New Zealand, 1975	Mitchell, 1982
PG4180.11	Cor^- Km^r; a marker-exchanged mutant contains p4180A::Tn*5*	Bender, 1991
PG4180.31	Cor^- Km^r; a marker-exchanged mutant contains p4180A::Tn*5*	Bender, 1991
PG4180.41	Cor^+ Km^r; a marker-exchanged mutant contains p4180A::Tn*5*	This study
PG4180.51	Cor^+ Km^r; a marker-exchanged mutant contains p4180A::Tn*5*	This study
PG4180.61	Cor^+ Km^r; a marker-exchanged mutant contains p4180A::Tn*5*	This study
PG4180.71	Cor^+ Km^r; a marker-exchanged mutant contains p4180A::Tn*5*	This study
PG4180.81	Cor^+ Km^r; a marker-exchanged mutant contains p4180A::Tn*5*	This study
PG4180.91	Cor^+ Km^r; a marker-exchanged mutant contains p4180A::Tn*5*	This study
PG4180.101	Cor^+ Km^r; a marker-exchanged mutant contains p4180A::Tn*5*	This study
Pseudomonas syringae pv. syringae		
PS51	Cor^-	Bender & Cooksey, 1986

Table 1. (continued)

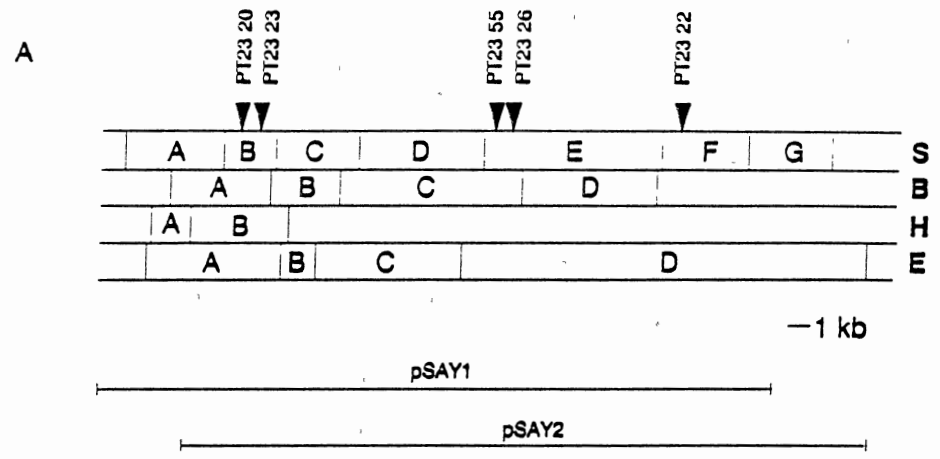
Designation	Relevant characteristics	Source
Plasmids		
pLAFR3	IncP Tc^r, cos^+	Staskawicz, 1987
pRK2013	Mob^+, Km^r, Tra RK2	Figurski, 1979
pRK415	Tc^r, RK2-derived cloning vector	Keen, 1988
pSAY1	pLAFR3 containing a 30-kb insert required for coronatine synthesis in PT23.2	This study
pSAY2	pLAFR3 containing a 31-kb insert required for coronatine synthesis in PT23.2	This study
pSAY10	pLAFR3 containing a 32-kb insert required for coronatine synthesis in PG4180	This study
pSAY12	pLAFR3 contianing a 30-kb insert required for coronatine synthesis in PG4180	This study
pSAY12.1	Tc^r; a 3.4 kb BamHI insert from pSAY12 cloned in pRK415	This study
pCLB2	Tc^r Km^r; contains Tn5-inactivated coronatine genes from PT23.20 cloned in pRK415	Bender, 1991
pCLB5	Tc^r Km^r; contains Tn5-inactivated coronatine genes from PT23.26 cloned in pLAFR3	Bender, 1991
pCLB7	Tc^r Km^r; contains Tn5-inactivated coronatine genes from PT23.22 cloned in pLAFR3	Bender, 1991
pCLB6	Tc^r Km^r; contains Tn5-inactivated coronatine genes from PT23.55 cloned in pLAFR3	This study
pCLB8	Tc^r Km^r; contains Tn5-inactivated coronatine genes from PT23.23 cloned in pLAFR3	This study
pIJ2345	Amp^r; contains a 2.2 kb BamHI fragment representing actI cloned in pBR329	Malpartida & Hopwood, 1986
pIJ2346	Amp^r; contains a 1.1 kb BamHI fragment representing actIII cloned in pBR329	Malpartida & Hopwood, 1986
pIJ5200	contains a 450 bp NcoI fragment representing gra3 cloned in M13	Sherman, 1989
pLG87	Amp^r; contains a 2.0 kb EcoRV fragment required for iaaL synthetase activity in P. syringae pv. savastanoi	Glass & Kosuge, 1986

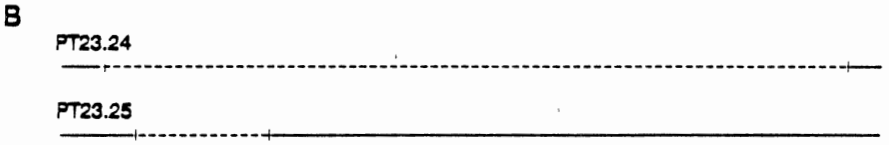
Table 2. Phenotypes of *P. syringae* PS51 and *E. coli* K12 and coronatine-defective mutants of *P. syringae* pv. tomato and pv. glycinea following transformation with cosmids pSAY1, pSAY2, and pSAY10.

	Cosmid clones		
Strain	pSAY1	pSAY2	pSAY10
PT23.20	++[a]	nt	nt
PT23.22	+	++	nt
PT23.23	++	nt	nt
PT23.24	++	nt	nt
PT23.25	++	nt	nt
PT23.26	++	nt	nt
PT23.29	-	-	nt
PT23.55	+	++	nt
PS51	-	nt	nt
K12	-	nt	nt
PG4180.11	++	nt	++
PG4180.31	+	++	+

[a]++, coronatine production restored to level characteristic of wild-type strain; +, coronatine production restored to a level lower than that of the wild-type strain (partial complementation); -, no coronatine production; and nt, not tested.

Fig. 4. (A) Partial restriction map of plasmid pPT23A from P. syringae pv. tomato PT23.2. Arrows indicate the Tn5 insertion sites in five coronatine-defective mutants of PT23.2. pSAY1 and pSAY2 are two overlapping cosmid clones which contain coronatine synthesis genes and were used to develop the restriction map shown in 1A. (B) The dashed line (---) indicates the approximate extent of pPT23A DNA deleted from PT23.24 and PT23.25. (C) Partial restriction map of p4180A DNA, the coronatine plasmid in P. syringae pv. glycinea 4180, which was cloned in pSAY10. The location of Tn5 insertions in the Cor^- mutants PG4180.11 and PG4180.31 and in the Cor^+ strain PG4180.41 is indicated. Enzymes used for restriction mapping were BamHI (B), EcoRI (E), HindIII (H), and SstI (S).


A
PT23 20
PT23 23
PT23 55
PT23 26
PT23 22
A B C D E F G S
A B C D B
A B H
A B C D E
—1 kb
pSAY1
pSAY2


B
PT23.24
PT23.25

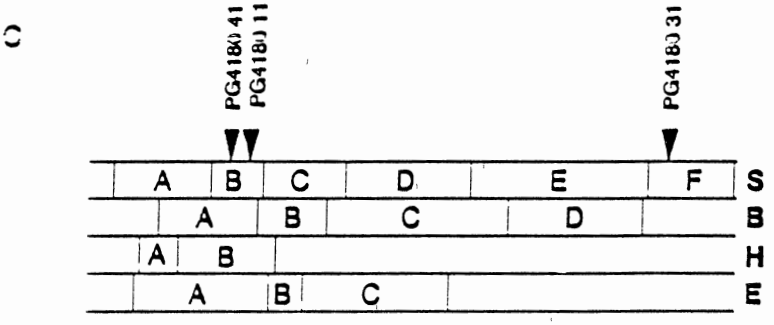
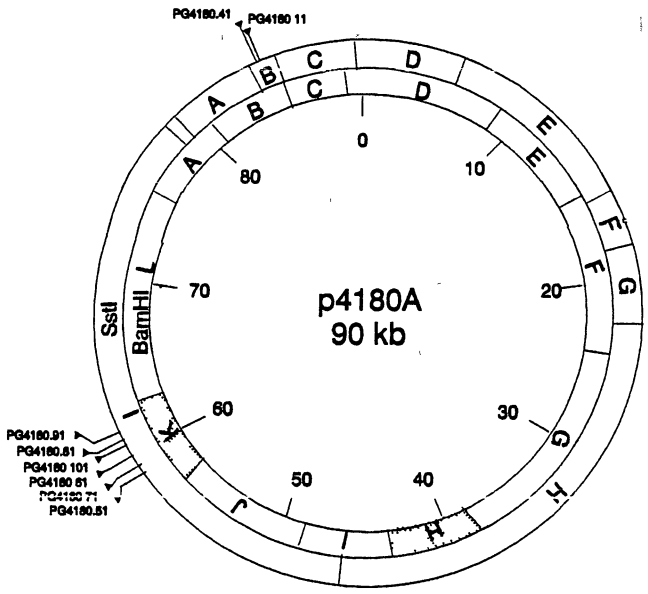

C
PG4180 41
PG4180 11
PG4180 31
A B C D E F S
A B C D B
A B H
A B C E

Fig. 5. Restriction map of p4180A, the coronatine plasmid in P. syringae pv. glycinea PG4180. Shaded areas hybridized to the indoleacetic-acid lysine synthetase gene in P. syringae pv. savastanoi. Solid triangles indicate the location of Tn5 insertions in p4180A. Restriction enzymes used to develop the map were BamHI (B) and SstI (S).


PG4180.41
PG4180 11
A
B
C
D
E
F
G
H
J
K
L
0
10
20
30
40
50
60
70
80
p4180A
90 kb
SstI
BamHI
PG4180.91
PG4180.81
PG4180 101
PG4180 61
PG4180.51

CHAPTER IV

PHYSICAL AND FUNCTIONAL CHARACTERIZATION OF THE GENE CLUSTER FOR THE POLYKETIDE PHYTOTOXIN CORONATINE IN PSEUDOMONAS SYRINGAE PV. GLYCINEA PG4180

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ABSTRACT

Pseudomonas syringae pv. glycinea PG4180 produces the polyketide phytotoxin coronatine. The coronatine synthesis genes in PG4180 were previously shown to reside on a 90 kilobase (kb) plasmid designated p4180A. pSAY10, a 52 kb cosmid clone containing p4180A DNA, previously restored coronatine production to two coronatine-defective mutants of PG4180, indicating that pSAY10 contained some of the genes required for coronatine synthesis. In the present study, a 41-kb region of p4180A was saturated by Tn5 mutagenesis. Seventy-seven unique mutations were recombined into p4180A by marker-exchange. The effect of these mutations on coronatine synthesis was determined by analyzing the organic acid extracts produced by the mutants by reverse-phase high

performance liquid chromatography. Mutations in a 20.5-kb region of pSAY10 completely blocked the synthesis of coronafacic acid and coronatine. Mutations within a 4.4-kb region of pSAY10 prevented the formation of coronatine but allowed for the production of coronafacic acid, coronafacoylvaline, coronafacoylisoleucine, and coronafacoylalloisoleucine. Complementation analyses indicated that eight distinct complementation groups were clustered across a 27-kb region of p4180A. Five tightly-clustered complementation groups (corD, corE, corF, corG, and corH) were shown to be involved in the synthesis of the polyketide coronafacic acid, since mutations in these regions completely inactivated production of this compound. Three of the complementation groups (corA, corB, and corC) were shown to have a role in the formation of the cyclopropane ring of coronatine.

Coronatine is a nonhost-specific phytotoxin which is produced by several pathovars of Pseudomonas syringae, including atropurpurea (Nishiyama et al., 1976), glycinea (Mitchell and Young, 1978), morsprunorum (Mitchell, 1982), and tomato (Mitchell et al., 1983; Bender et al., 1987), which are pathogens of ryegrass, soybean, Prunus spp., and tomato, respectively. Symptoms associated with coronatine production include chlorosis, hypertrophy, and stunting of plant tissue. Several investigators have shown that the

synthesis of coronatine significantly enhances the virulence of producing organisms (Bender et al., 1987; Gnanamanickam et al., 1982; Sato et al., 1983).

The structure of coronatine is unusual and consists of a bicyclic component, coronafacic acid, which is joined to an ethylcyclopropyl amino acid, coronamic acid (Ichihara et al., 1977). Parry and Mafoti (1986) demonstrated that coronafacic acid is derived from the polyketide pathway and consists of five acetate units and one pyruvate unit. Although coronafacic acid appears to be formed from two distinct polyketide chains, the specific steps involved in the synthesis and coupling of the separate chains are presently obscure (Parry and Mafoti, 1986). The coronamic acid moiety of coronatine was previously shown to be derived from the isoleucine biosynthetic pathway (Mitchell, 1985a). Although both (1-^{13}C)-L-isoleucine and (1-^{13}C)-L-alloisoleucine are incorporated into coronamic acid, L-alloisoleucine was shown to be a much more efficient precursor (Parry et al., 1991). The administration of (6-^{13})-DL-alloisoleucine demonstrated that the cyclopropane bridge of coronamate is derived from the methyl group of L-alloisoleucine, and carbon-13 NMR spectra indicated that the conversion of L-alloisoleucine to coronamic acid proceeds with the removal of one hydrogen atom from the methyl group of the precursor (Parry et al., 1991). The enzymatic steps involved in the cyclization of L-alloisoleucine are unknown.

The coronatine (*cor*) synthesis genes in various *P. syringae* pathovars may reside on plasmids (Bender *et al.*, 1989; Bender *et al.*, 1991; Sato, 1988) or in the chromosome (Moore *et al.*, 1989). Although progress has been made in the physical characterization of DNA required for coronatine synthesis (Ma *et al.*, 1991), the functional analysis of regions which control coronatine synthesis has not been previously undertaken. In *P. syringae* pv. glycinea PG4180, the coronatine synthesis genes reside on a 90-kb plasmid designated p4180A (Bender *et al.*, 1991; Young, Chapter 3). In the present study, a 41-kb region of p4180A was saturated with Tn*5*, and the effect of each mutation on coronatine synthesis was determined by reverse-phase high performance liquid chromatography (RP-HPLC). Mobilizable cloning vectors containing selected *cor*::Tn*5* insertions were introduced into coronatine-defective (Cor$^-$) mutants of PG4180 to estimate the number and location of transcriptional units required for coronatine synthesis in this strain.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 3. Derivatives of *P. syringae* pv. glycinea PG4180 were maintained on mannitol-glutamate (MG; Keane *et al.*, 1970) medium or King's medium B (KMB; King *et al.*, 1954).

Escherichia coli HB101 was grown at 37 C on Luria-Bertani medium (LB; Maniatis et al., 1982). PG4180 broth cultures were grown in either MG supplemented with yeast extract at 0.25 g/liter (MGY) or KMB on a rotary shaker (250 rpm) at 20 to 24 C. Antibiotics were added to media in the following concentrations (ug/ml): tetracycline, 12.5; and kanamycin, 10 for PG4180 derivatives and 25 for E. coli.

Molecular genetic techniques. Agarose gel electrophoresis, DNA restriction digests, and Southern transfers were done by standard procedures (Maniatis et al., 1982). Southern transfers were performed using Hybond N nylon membranes purchased from Amersham Corp., Arlington Heights, Ill. Conditions for prehybridizations, hybridizations, and post-hybridization washes have been described previously (Bender et al., 1991). When specific DNA fragments were to be labeled with ^{32}P, they were separated from vector fragments on agarose gels and excised. Residual agarose was removed with the Geneclean kit manufactured by BIO101, La Jolla, Calif. DNA was labeled with ^{32}P by the random primer labeling technique (Feinberg and Vogelstein, 1983).

DNA isolations and cloning methods. Plasmid DNA was isolated and purified from E. coli by standard procedures (Maniatis et al., 1982). Plasmid DNA was isolated from P. syringae pv. glycinea as described previously (Crosa and Falkow, 1981; Bender and Cooksey, 1986). Rapid, small scale

plasmid isolations from E. coli were performed using plasmid boiling method 2 of Crouse et al. (1983).

A cosmid library of PG4180 plasmid DNA was constructed in pLAFR3 as described previously (Bender and Cooksey, 1987). Restriction maps of selected cosmid clones were constructed using BamHI, EcoRI, HindIII, and SstI in single and double digests. When necessary, selected restriction fragments were isolated from cosmid clones, gel-purified, and subcloned into the broad-host-range vector pRK415.

Transposon mutagenesis. Cloned DNA was mutagenized with lambda::Tn5 in E. coli HB101 as described previously (DeBruijn and Lupski, 1984; Bender and Cooksey, 1987). Cloned DNA containing Tn5 insertions was selected by transforming E. coli HB101 and plating transformants onto LB agar containing tetracycline and kanamycin.

Tn5 insertions in p4180A were generated by first creating merodiploids. Clones containing Tn5 insertions were mobilized from E. coli into PG4180 using the helper plasmid pRK2013 as described previously (Bender and Cooksey, 1987). PG4180 transconjugants containing cloned DNA were verified by agarose gel electrophoresis and then subcultured in MG broth containing kanamycin to maintain selection pressure for Tn5 (Bender et al., 1991). Putative PG4180 mutants containing Tn5 insertions in p4180A were identified by replica plating and selecting Km^r Tc^s colonies. The insertion of Tn5 into p4180A was verified by agarose gel

electrophoresis. The location of Tn*5* insertions in cloned DNA and p4180A was established using *Bam*HI, *Eco*RI, and *Sst*I in single and double digests.

Complementation analyses. Complementation was defined as the restoration of coronatine production in a PG4180 Cor⁻ mutant following the construction of a merodiploid strain containing a second *cor* mutation in cloned DNA. These merodiploids were constructed by mobilizing selected Tn*5*-containing clones into PG4180 mutants containing Tn*5* insertions in p4180A. Transconjugants were verified by agarose gel electrophoresis, and then assayed for coronatine production as described below.

Assays for coronatine. PG4180 marker-exchanged mutants and merodiploid strains were analyzed for coronatine production by growing cultures for 7 days in 10 ml aliquots of Hoitink and Sinden (HS; Hoitink and Sinden, 1970) medium on a rotary shaker (250 rpm) at 18 C. Cultures were centrifuged for 15 min at 11,000 x g to pellet cells. The supernatant was adjusted to pH 8.0, and hydrophobic compounds were extracted twice with an equal volume of ethyl acetate. The aqueous phase was then adjusted to pH 3.0 and organic acids were extracted three times with an equal volume of ethyl acetate. The ethyl acetate extractions were dried with anhydrous Na_2SO_4 (25 g) and rotary evaporated. The residues were dissolved in 0.25 ml sterile distilled water and 0.25 ml acetonitrile, and 0.1 ml was analyzed by

reverse-phase high performance liquid chromatography (RP-HPLC) as described previously (Young and Bender, Chapter 3).

Large-scale isolations (3 L) of PG4180 and PG4180.C0 were extracted as described previously (Bender et al., 1989) and analyzed by HPLC as described above. The extracts from PG4180 and PG4180.C0 were further analyzed by gas chromatography-mass spectrophotometry (GC-MS) (Bender et al., 1989).

Feeding experiments. Coronamic acid was obtained from the acid hydrolysis of coronatine as described by Ferguson and Mitchell (1985). Coronafacic acid was purified from PG4180 as described by Mitchell (1984a) and coronafacoylisoleucine and coronafacoylalloisoleucine were synthesized according to Shiraishi et al. (1979). Strains PG4810.C0 and PG4180.E9 were incubated overnight at 28 C on MG agar supplemented with kanamycin. Each culture was then suspended in sterile distilled water to an optical density of 0.6 at A_{600}, and 100 ul was inoculated to 10 ml of HS broth. The cultures were incubated at 18 C for 2 days at 250 rpm and then PG4180.C0 was supplemented with 7 uM of coronamic acid and PG4180.E9 was supplemented with 7 uM of coronafacic acid, coronafacoylisoleucine, or coronafacoylalloisoleucine. Cultures were then incubated an additional 5 days, and analyzed by RP-HPLC.

RESULTS

Characterization of mutations in p4180A. Cosmid pSAY10, which contains a 32-kb insert of p4180A DNA, was previously recovered from a PG4180 plasmid DNA library constructed in pLAFR3 (Chapter 3). pSAY10 restored coronatine production to PG4180.11 and PG4180.31, two mutants containing Tn*5* insertions in p4180A (Chapter 3), indicating that pSAY10 contains genes required for coronatine production in PG4180. To further characterize the *cor* region, pSAY10 and pSAY12, a clone which overlaps pSAY10, were subjected to Tn*5* mutagenesis. Southern blot analysis was used to confirm the overlaps between pSAY10 and pSAY12 (the latter cosmid also originated from the PG4180 plasmid DNA library constructed in Chapter III). Figure 6 shows a *Bam*HI-*Sst*I restriction map of the 41-kb region of p4180A contained among these two cosmid clones. The 4.8, 6.0, and 8.7 kb *Sst*I fragments of pSAY10 (*Sst*I fragments 2, 5, and 6, Fig.6), were subcloned into the *Sst*I site of pRK415 to yield subclones pSAY10.11, pSAY10.12, and pSAY10.13, respectively (Fig. 6). Tn*5* insertions in the two cosmid clones and three pRK415-derived subclones were selected to give an even distribution of mutations across the region contained in Fig. 6. Single and double digestions with *Bam*HI, *Eco*RI, *Hin*dIII, and *Sst*I were used to map precisely the mutations contained in Tn*5*-containing cosmid clones and subclones. Each clone containing a Tn*5*

insertion was conjugated into PG4180 and recombined into p4180A. Southern blot analysis was used to confirm the location of Tn*5* insertions in p4180A (see vertical lines, Fig. 6). Organic acids were extracted from each marker-exchanged PG4180 mutant and analyzed for coronatine by RP-HPLC.

Six mutations in *Bam*HI fragment 1 (Fig. 6; PG4180.51-PG4180.101) which were generated in a previous study (Chapter III), had no effect on coronatine production. In the present study, nine additional mutations were generated in *Bam*HI fragment 2; these also exhibited the same phenotype as PG4180 and produced coronatine. Mutations C8, F2, D1, and E3, which are located in *Sst*I fragments 2, 3, 4, and 7, respectively (Fig. 6), produced reduced levels of coronatine. Tn*5* insertions in a 20.5 kb region of p4180A which spanned *Sst*I fragments 3, 4, 5, 6, and 7 completely blocked synthesis of coronafacic acid and coronatine (Fig. 6). Nine insertions in a 4.8-kb *Sst*I fragment prevented coronatine synthesis, but allowed for the production of coronafacic acid (fragment 2, Fig. 6). These mutations shared a common phenotype and produced two prominent peaks which eluted approximately 1 and 3 min after coronafacic acid when analyzed by RP-HPLC (data not shown). These two peaks were either barely detectable or absent in organic acids extracted from the wild-type PG4180 and other mutants. Because these mutants produced coronafacic acid but failed

to synthesize coronatine, one of them (PG4180.C0) was selected for further analysis by GC-MS.

GC-MS analyses of PG4180 and PG4180.C0. The GC tracings of methyl-esterified organic acids produced by PG4180 and PG4180.C0 indicated that both strains were producing coronafacic acid and coronafacoylvaline with retention times of 11.8 and 19.4, respectively (Fig. 7). Only PG4180 produced coronatine (retention time 22.8 min; Fig. 7B). PG4180.C0 produced a 21.3 min peak (Fig. 7C) which was also present at a reduced level in PG4180 (Fig. 7B).

The identities of the 11.8, 19.4, 21.3, and 22.8 min peaks in PG4180 and PG4180.C0 were confirmed by GC-MS analyses. Coronafacoylisoleucine (Cfile), Coronafacoylalloisoleucine (Cfalloile), and norcoronatine all elute at 21.3 min. Norcoronatine was distinguished from the other two compounds both of which have molecular ion peaks at m/z 335, by its molecular ion peak at m/z 319. Separation of Cfile and Cfalloile was accomplished by capillary GC and flame ionization detection. Using this approach, the Cfile and Cfalloile eluted separately at 22.3 and 22.8 min, respectively (data not shown). These results revealed that PG4180 contained the three compounds; however PG4180.C0 contained Cfile and Cfalloile but lacked norcoronatine (Table 4).

The integrations of specific GC peaks provided an estimation of the quantities of coronafacoyl compounds synthesized by PG4180.C0 relative to the wild-type PG4180.

In PG4180.C0, the levels of coronafacic acid, coronafacoylvaline, and the coronafacoylisoleucine/coronafacoylalloisoleucine mixture were increased approximately 2.5x, 3.6x, and 3.0x, respectively, relative to the amounts of these compounds synthesized by PG4180. In both PG4180 and PG4180.C0, coronafacoylalloisoleucine and coronafacoylisoleucine were synthesized in a 1:2 relative ratio.

Administration of coronamic acid to PG4180.C0. The absence of coronatine and norcoronatine (a cyclized derivative of coronafacoylvaline) and increased accumulation of non-cyclized coronafacoyl compounds in PG4180.C0 suggested that the Tn*5* insertion in this mutant had interrupted steps involved in the formation of the cyclopropane ring of coronatine. However, an alternative reason for the absence of coronatine in PG4180.C0 was the possible interruption of a region involved in coupling coronafacic acid to coronamic acid. This hypothesis was tested by supplying PG4180.C0 with coronamic acid. RP-HPLC analysis of organic acids extracted from PG4180.C0 supplemented with coronamic acid indicated the presence of a peak which co-chromatographed with coronatine (data not shown). This experiment provides further evidence that coronatine is not synthesized by PG4180.C0 because gene(s) involved in the formation of the cyclopropane ring of coronatine have been interrupted.

Administration of coronafacic acid, coronafacoyl-isoleucine, and coronafacoylalloisoleucine to PG4180.E9. The Tn*5* insertion PG4180.E9, eliminated the production of coronafacic acid, coronatine, and all other coronafacoyl compounds. Since coronafacic acid is a precursor for all coronafacoyl compounds, this result suggested that steps involved in the synthesis of coronafacic acid had been interrupted. When PG4180.E9 was supplemented with coronafacic acid, RP-HPLC analysis of organic acid extracts indicated the presence of a peak which co-chromatographed with coronatine. This demonstrated that the Tn*5* insertion in PG4180.E9 did not interrupt genes involved in amide bond or cyclopropane ring formation. To investigate whether coronafacoylisoleucine (Cfile) or coronafacoylalloisoleucine (Cfalloile) were biosynthetic intermediates in the pathway to coronatine, PG4180.E9 was supplied with Cfile or Cfalloile. RP-HPLC analysis showed that coronatine was absent from the organic acid extracts of PG4180.E9 supplemented with Cfile or Cfalloile. Because neither Cfile or Cfalloile functioned as an intermediate to coronatine, this suggests that the formation of the cyclopropane ring occurs before coronamate and coronafacate are coupled to form coronatine.

Genetic analysis of the p4180A *cor* region. The mutational analyses described above identified areas of p4180A which were required for the synthesis of coronatine.

The complementation tests presented in the following sections enabled us to group the p4180A cor region into eight transcriptional units (corA - corH, Fig. 6, Table 5). The Tn5-containing clones which were originally recombined into p4180A were tested for their ability to restore coronatine production to Cor⁻ mutants of PG4180. The restoration of coronatine synthesis to a merodiploid strain (PG4180 Cor⁻ mutant containing one Tn5 insertion in p4180A and a second Tn5 mutation in a clone containing the cor region) indicated that the two mutations were in different complementation groups. Since Tn5 normally causes polar mutations, complementation analyses between different cor::Tn5 insertions was used to establish the boundaries of transcriptional units but not the boundaries of genes within cor operons.

The complementation groups corA, corB, and corC are clustered within a 4.8 kb region which is located on the left border of the cor region (SstI fragment 2, Fig. 6). PG4180 mutants containing Tn5 insertions in corA, corB, and corC (including PG4180.C0 described above) shared a common phenotype and produced peaks which co-chromatographed with coronafacic acid (Cfa), coronafacoylvaline (Cfval), coronafacoylisoleucine (Cfile), and coronafacoylalloisoleucine (Cfalloile). However, mutants containing insertions in corA, corB, and corC did not produce coronatine.

The mutations located at C6, C7, C14, and C0 mapped within corA. The production of coronatine in the PG4180.C14(pC1) and PG4180.C0(pC1) merodiploids indicated that the mutation located at C1 mapped in another complementation group (see corB below). The minimum size for the corA complementation group is 650 bp, the distance between mutations C6 and C0; the maxium size for corA is 2.5 kb, the interval between the M2 and C8 mutations which result in wild-type and reduced levels of coronatine production, respectively.

The mutations located at C5, C2, and C1 mapped within a second complementation group, corB (Fig. 6, Table 5). The production of coronatine in the PG4180.C9(pC1) merodiploid indicated that the mutation at C9 mapped within a different transcriptional unit (see corC below). The minimum size for the corB complementation group is 1.2 kb (the distance between the C5 and C1 mutations). corB is bordered on the left by the C8 mutation, which resulted in reduced levels of coronatine production and on the right by the mutation C16, which mapped within corC, indicating that the maximum size for corB is 2.4 kb (the distance from C8 to C16).

Complementation analyses performed with C15, C16 and C9 indicated that these three mutations mapped within a very small complementation group, corC. This complementation group was bordered on the left by C1, which mapped within corB, and on the right by the mutation at F2, which resulted

in reduced levels of coronatine production. The minimum size of corC is 220 bp (the distance from C16 to C9), and the maximum size is 1.7 kb (the distance between mutations C1 and F2).

The mutations located at F1, F8, F6, and F7 resulted in a Cfa$^-$ Cor$^-$ phenotype and mapped within a fourth transcriptional unit corD (Fig.6, Table 5). The PG4180.F7(pF1) merodiploid exhibited the same phenotype as the PG4180.F7 and PG4180.F1 mutants (Cfa$^-$ Cor$^-$), indicating that these two mutations both mapped within corD. The production of coronatine in the PG4180.C9(pF1) and PG4180.D3(pF1) merodiploids established that the F1 mutation mapped in a different complementation group (corD) than the mutations at C9 (corC) and D3 (corE, see below). The minimum size for corD is 540 bp (distance between the F1 and F7 mutations), and the maxium size is 3.0 kb (the distance between the F2 and D3 mutations.

The mutations at D3 and D4 exhibited a common phenotype (Cfa$^-$ Cor$^-$). The absence of coronatine synthesis in the PG4180.D3(pD4) merodiploid indicated that the D3 and D4 mutations are in the same complementation group (corE). The production of coronatine in the PG4180.D3(pD5) merodiploid established that the D5 mutation is located in a separate transcriptional unit, corF (see below). Since PG4180.D1 produces coronatine at reduced levels, this mutation established a right border for corE. The minimum size for

corE is 1.1 kb (distance from D3 to D4), and the maximum size is 2.4 kb (distance from F7 to D1).

The mutations located at D5, B14, B32, B13, B36, B3, B7, B38, B29, B37, B1, and B2 all resulted in a common phenotype (Cfa$^-$ Cor$^-$) and spanned an area approximately 3.3 kilobases. The absence of coronatine production in PG4180.B36(pD5), PG4180.B37(pD5), PG4180.B36(pB2) and PG4180.B37(pB2) merodiploids indicated that the corF transcriptional unit encompassed the region contained between the mutations at D5 and B2. The production of coronatine in the PG4180.B37(pB12) merodiploid established the existence of another transcriptional unit (corG, see below). The minimum size of corF is 3.3 kb (distance from D5 to B2), and the maximum size is 5.9 kb (distance from D1 to B12).

The absence of coronatine production in the PG4180.B21(pB12), PG4180.B21(pB28), PG4180.B21(pA5), PG4180.A28(pA5), and PG4180.A28(pA24) merodiploids indicated that the mutations at B12, B21, B28, A5, A28, and A24 all map within a common transcriptional unit, corG. The production of coronatine in the PG4180.B21(pB2) and PG4180.B21(pB37) merodiploids clearly distinguished mutations in corF (B2 and B37) from corG. The production of coronatine in the PG4180.B21(pA23), PG4180.B21(pE10), and PG4180.A28(pE10) merodiploids established that another transcriptional unit, corH, was located to the right of corG

and contained the mutations A23 and E10. The minimum size of corG is 5.4 kb (distance from B12 to A24), and the maximum size is 6.9 kb (distance from B2 to A10).

The absence of coronatine synthesis in the PG4180.A2(pA23), PG4180.E9(pA23), PG4180.A2(pE10), and PG4180.E9(pE10) merodiploids indicated that mutations at A2, A23, E10, and E9 mapped to a common transcriptional unit, corH. The production of coronatine in the PG4180.E9(pA24) and PG4180.E9(pA5) merodiploids confirmed that the mutations at A24 and A5 mapped to a separate transcriptional unit, corG (see above). The reduced levels of coronatine produced in the PG4180.A28(pA10), PG4180.A28(pA23), and PG4180.A2(pA24) merodiploids suggested that corG and corH overlap as indicated in Fig. 6. The minimum size for the corH complementation group is 6.8 kb (the distance from A28 to E9). corH is bordered on the left by the A26 mutation, which mapped within corG and on the right by the E3 mutation, which resulted in reduced levels of coronatine production, indicating that the maximum size for corH is 8.6 kb (the distance from A26 to E3).

DISCUSSION

In the present study, a 41-kb region of p4180A was mutagenized with Tn5, and 27 kb was shown to be required for coronatine synthesis. Eight distinct complementation groups were clustered within this region. Complementation groups corA, corB, corC, corD, and corE are fairly small (220 bp to

1.1 kb) and could be single genes under the control of separate promoters. However, corF, corG, and corH were significantly larger (3.3 to 6.9 kb) and could represent large open reading frames or operon-like structures containing multiple genes. Several large open reading frames have been shown to control synthesis of the polyketide erythromycin (Donadio et al., 1991). However, the polyketide actinorhodin is synthesized by multiple operons containing one or more genes (Hopwood and Sherman, 1990). At present, steps required for synthesis of the polyketide component of coronatine, coronafacic acid, remain obscure. However, the minimum amount of DNA devoted to synthesis of coronafacic acid (20.5 kb) and the number of transcriptional units required (corD, corE, corF, corG, and corH) indicate the complexity of its synthesis.

Southern blot analysis (Young and Bender, unpublished) indicated that complementation groups corF, corG, and corH correspond to corI, corII, and corIII, three transcriptional units involved in the synthesis of coronatine by P. syringae pv. tomato DC3000 (Ma et al., 1991). Although Ma et al. (1991) did not investigate the function of the cor transcriptional units in the biosynthesis of coronatine, our results (failure of corF, corG, and corH mutants to produce any coronafacoyl compounds) suggest that corI, corII, and corIII may also be involved in the synthesis of coronafacic acid. The conservation of coronatine synthesis genes among

different producing P. syringae pathovars has been previously established (Bender et al., 1991).

Mutants containing Tn5 insertions in corA, corB, and corC, such as PG4180.C0, failed to produce coronatine but instead produced elevated levels of coronafacic acid, coronafacoylvaline, coronafacoylisoleucine, and coronafacoylalloisoleucine. We believe this phenotype has obvious significance in terms of the biosynthetic route to coronatine from coronafacic acid. Two alternative routes to coronatine from the intermediate compound, coronafacic acid, are possible: (1) coronafacic acid may be coupled with isoleucine or alloisoleucine to form coronafacoylisoleucine or coronafacoylalloisoleucine, followed by formation of the cyclopropane ring on the coronafacoyl conjugate; or (2) alloisoleucine may be cyclized to form coronamic acid, and the latter is coupled to coronafacic acid to form coronatine (Mitchell, 1991a; Parry et al., 1991). The natural occurrence of coronafacoylisoleucine and coronafacoylalloisoleucine suggests that the former biosynthetic route is possible; however, when PG4180.E9 was supplied with Cfile or Cfalloile, no coronatine was synthesized, suggesting that these two coronafacoyl compounds are not true intermediates in the coronatine biosynthetic pathway. Also, in the present study, the administration of coronamic acid to PG4180.C0 resulted in coronatine production. Consequently, we believe these

experiments indicate that the route to coronatine probably involves the formation of coronamic acid from alloisoleucine, and the coupling of coronamate and coronafacate to form coronatine (Fig. 8).

In the wild-type PG4180, coronatine is the most abundant coronafacoyl compound; howver, mutants containing defects in corA, corB, and corC synthesized elevated levels of peaks which co-chromatographed with Cfa, Cfval, Cfile, and Cfalloile. We believe this indicates that coronamic acid is the preferred substrate for the coupling reaction, and that these mutants accumulated increased levels of Cfa, Cfval, Cfile, and Cfalloile because the cyclization of alloisoleucine to form coronamate was blocked (Fig. 7). Additional evidence for this phenotype is the absence of norcoronatine, a cyclized derivative of Cfval (Mitchell, 1985b) in PG4180.C0. Since norcoronatine was present in PG4180, this indicates that the cyclization of valine and alloisoleucine may involve identical enzymatic steps. Norcoronatine is produced at relatively low levels in PG4180 as compared to coronatine, which suggests the increased affinity of the cyclization reaction for alloisoleucine as compared to valine.

The three complementation groups involved in cyclization, corA, corB, and corC, may represent three independent genes involved in the structural synthesis and regulation of the cyclization reaction. Since coronatine is

the most toxic of all presently-characterized coronafacoyl compounds, corA, corB, and corC represent genes which significantly increase the toxicity of coronafacoyl-derived compounds. Recently, coronafacoyl-derived comopounds were shown to be produced by a xanthomonad, (Xanthomonas campestris pv. phormiicola (Mitchell, 1991b; Tamura et al., 1988). Mitchell (1991b) showed that three strains of X. campestris pv. phormiicola produced Cfval and Cfile, but no Cor, Cfalloile or norcoronatine. The administration of alloisoleucine to X. campestris pv. phormiicola did not result in coronatine production (Mitchell, unpublished) These results suggest that X. campestris pv. phormiicola may lack steps necessary for the isomerization of isoleucine and for the cyclization of alloisoleucine and valine to form coronatine and norcoronatine, respectively. This organism and mutants with defects in corA, corB, and corC will be used in future studies designed to elucidate the steps involved in the cyclization reaction.

Table 3. Bacterial strains and plasmids.

Designation	Characteristics[a]	Source
E. coli		
HB101		Maniatias, 1982
P. syringae pv. glycinea		
PG4180	Cor$^+$; contains p4180A	Mitchell, 1982; Bender, 1991
PG4180.A1	*cor*H; p4180A::Tn*5*	This study
PG4180.A2	*cor*H; p4180A::Tn*5*	This study
PG4180.A4	*cor*G; p4180A::Tn*5*	This study
PG4180.A5	*cor*G; p4180A::Tn*5*	This study
PG4180.A6	*cor*H; p4180A::Tn*5*	This study
PG4180.A9	*cor*H; p4180A::Tn*5*	This study
PG4180.A10	*cor*H; p4180A::Tn*5*	This study
PG4180.A11	*cor*G; p4180A::Tn*5*	This study
PG4180.A12	*cor*G *cor*H; p4180A::Tn*5*	This study
PG4180.A15	*cor*G *cor*H; p4180A::Tn*5*	This study
PG4180.A16	*cor*H; p4180A::Tn*5*	This study
PG4180.A17	*cor*G *cor*H; p4180A::Tn*5*	This study
PG4180.A19	*cor*H; p4180A::Tn*5*	This study
PG4180.A22	*cor*H; p4180A::Tn*5*	This study
PG4180.A23	*cor*H; p4180A::Tn*5*	This study
PG4180.A24	*cor*G *cor*H; p4180A::Tn*5*	This study
PG4180.A25	*cor*G; p4180A::Tn*5*	This study
PG4180.A26	*cor*G; p4180A::Tn*5*	This study
PG4180.A28	*cor*G *cor*H; p4180A::Tn*5*	This study
PG4180.A30	*cor*G; p4180A::Tn*5*	This study
PG4180.B1	*cor*F; p4180A::Tn*5*	This study
PG4180.B2	*cor*F; p4180A::Tn*5*	This study
PG4180.B3	*cor*F; p4180A::Tn*5*	This study
PG4180.B7	*cor*F; p4180A::Tn*5*	This study
PG4180.B12	*cor*G; p4180A::Tn*5*	This study
PG4180.B13	*cor*F; p4180A::Tn*5*	This study
PG4180.B14	*cor*F; p4180A::Tn*5*	This study
PG4180.B21	*cor*G; p4180A::Tn*5*	This study
PG4180.B28	*cor*G; p4180A::Tn*5*	This study
PG4180.B29	*cor*F; p4180A::Tn*5*	This study
PG4180.B32	*cor*F; p4180A::Tn*5*	This study
PG4180.B36	*cor*F; p4180A::Tn*5*	This study
PG4180.B37	*cor*F; p4180A::Tn*5*	This study
PG4180.B38	*cor*F; p4180A::Tn*5*	This study
PG4180.C0	*cor*A; p4180A::Tn*5*	This study
PG4180.C1	*cor*B; p4180A::Tn*5*	This study
PG4180.C2	*cor*B; p4180A::Tn*5*	This study

Table 3. (continued)

Designation	Characteristics	Source
PG4180.C5	*corB*; p4180A::Tn*5*	This study
PG4180.C6	*corA*; p4180A::Tn*5*	This study
PG4180.C7	*corA*; p4180A::Tn*5*	This study
PG4180.C8	Cor$^{+/-}$; p4180A::Tn*5*	This study
PG4180.C9	*corC*; p4180A::Tn*5*	This study
PG4180.C14	*corA*; p4180A::Tn*5*	This study
PG4180.C15	*corC*; p4180A::Tn*5*	This study
PG4180.C16	*corC*; p4180A::Tn*5*	This study
PG4180.D1	Cor$^{+/-}$; p4180A::Tn*5*	This study
PG4180.D3	*corE*; p4180A::Tn*5*	This study
PG4180.D5	*corF*; p4180A::Tn*5*	This study
PG4180.E1	*corH*; p4180A::Tn*5*	This study
PG4180.E3	Cor$^{+/-}$; p4180A::Tn*5*	This study
PG4180.E4	*corH*; p4180A::Tn*5*	This study
PG4180.E6	*corH*; p4180A::Tn*5*	This study
PG4180.E7	*corH*; p4180A::Tn*5*	This study
PG4180.E8	*corH*; p4180A::Tn*5*	This study
PG4180.E9	*corH*; p4180A::Tn*5*	This study
PG4180.E10	*corH*; p4180A::Tn*5*	This study
PG4180.F1	*corD*; p4180A::Tn*5*	This study
PG4180.F2	Cor$^{+/-}$; p4180A::Tn*5*	This study
PG4180.F6	*corD*; p4180A::Tn*5*	This study
PG4180.F7	*corD*; p4180A::Tn*5*	This study
PG4180.F8	*corD*; p4180A::Tn*5*	This study
PG4180.M2	Cor^{+}; p4180A::Tn*5*	This study
PG4180.M5	Cor^{+}; p4180A::Tn*5*	This study
PG4180.M16	Cor^{+}; p4180A::Tn*5*	This study
PG4180.M17	Cor^{+}; p4180A::Tn*5*	This study
PG4180.M18	Cor^{+}; p4180A::Tn*5*	This study
PG4180.M19	Cor^{+}; p4180A::Tn*5*	This study
PG4180.M20	Cor^{+}; p4180A::Tn*5*	This study
PG4180.M22	Cor^{+}; p4180A::Tn*5*	This study
PG4180.51	Cor^{+}; p4180A::Tn*5*	Chapter 3
PG4180.61	Cor^{+}; p4180A::Tn*5*	Chapter 3
PG4180.71	Cor^{+}; p4180A::Tn*5*	Chapter 3
PG4180.81	Cor^{+}; p4180A::Tn*5*	Chapter 3
PG4180.91	Cor^{+}; p4180A::Tn*5*	Chapter 3
PG4180.101	Cor^{+}; p4180A::Tn*5*	Chapter 3
Plasmids		
pLAFR3	Tc^{r}; RK2-derived cosmid vector	Staskawicz, 1987
pRK2013	Km^{r}; Mob^{+}; Tra^{+}	Figurski and Helinski, 1979
pRK415	Tc^{r}; RK-2 derived cloning vector	Keen, 1988

Table 3. (continued)

Designation	Characteristics	Source
pSAY10	pLAFR3 containing a 32 kb insert required for coronatine synthesis	Young, Chapter 3
pSAY10.11	Tc^r; a 4.8 kb SstI insert cloned in pRK415	This study
pSAY10.12	Tc^r; a 6.0 kb SstI insert cloned in pRK415	This study
pSAY10.13	Tc^r; a 8.7 kb SstI insert cloned in pRK415	This study
pSAY12	pLAFR3 cosmid containing cor genes from p4180A	This study
pC6	pSAY10 derivative with Tn5 insertion	This study
pC1	pSAY10 derivative with Tn5 insertion	This study
pF1	pSAY10 derivative with Tn5 insertion	This study
pD4	pSAY10 derivative with Tn5 insertion	This study
pB14	pSAY10.12 derivative with Tn5 insertion	This study
pB2	pSAY10 derivative with Tn5 insertion	This study
pA5	pSAY10 derivative with Tn5 insertion	This study
pA24	pSAY10 derivative with Tn5 insertion	This study
pA23	pSAY10 derivative with Tn5 insertion	This study
pE10	pSAY10 derivative with Tn5 insertion	This study
pC2	pSAY10 derivative with Tn5 insertion	This study
pB12	pSAY10.12 derivative with Tn5 insertion	This study
pB37	pSAY10.12 derivative with Tn5 insertion	This study
pC15	pSAY12 derivative with Tn5 insertion	This study
pD5	pSAY12 derivative with Tn5 insertion	This study

Table 3. (continued)

Designation	Characteristics	Source
pB28	pSAY10.12 derivative with Tn*5* insertion	This study
pA10	pSAY10 derivative with Tn*5* insertion	This study

[a]Cor^{+}, coronatine produced at levels equivalent to PG4180; $^{+/-}$, coronatine produced at levels lower than PG4180.

Table 4. GC-mass spectrometic data for selected methyl ester derivatives of organic acids isolated from P. syringae pv. glycinea PG4180 and PG4180.C0.

Corresponding GC peak[a] (min)	Molecular ion (m/z)	Detection in[b] PG4180	PG4180.C0
11.8; coronafacic acid	222	+	++
19.4; coronafacoylvaline	321	+	++
21.3; coronafacoylisoleucine	335	+	++
21.3; coronafacoylalloisoleucine	335	+	++
21.3; norcoronatine	319	+	-
22.8; coronatine	333	+	-

[a]Retention time, as in Fig. 7.
[b]+, coronafacoyl compound detected at levels routinely synthesized by PG4180;
++, coronafacoyl compound synthesized at levels exceeding PG4180;
-, coronafacoyl compound not detected at > 0.1%.

Table 5. Genetic complementation of Cor⁻ mutants of PG4180.

	PG4180 Cor⁻ mutant	cor::Tn5 clone	Phenotype[a]
corA	PG4180.C7	pC6	-
	PG4180.C14	pC6	-
	PG4180.C0	pC6	-
	PG4180.C14	pC1	+
	PG4180.C0	pC1	+
corB	PG4180.C5	pC2	-
	PG4180.C5	pC1	-
	PG4180.C1	pC2	-
	PG4180.C9	pC1	+
corC	PG4180.C16	pC15	-
	PG4180.C9	pC15	-
	PG4180.C9	pC6	+
	PG4180.C9	pC2	+
	PG4180.C9	pF1	+
corD	PG4180.F7	pF1	-
	PG4180.C9	pF1	+
	PG4180.D3	pF1	+
corE	PG4180.D3	pD4	-
	PG4180.D3	pD5	+
	PG4180.D3	pB14	+
corF	PG4180.B36	pD5	-
	PG4180.B37	pD5	-
	PG4180.B36	pB2	-
	PG4180.B37	pB2	-
	PG4180.B37	pB12	+
	PG4180.B37	pB28	+
corG	PG4180.B21	pB12	-
	PG4180.B21	pB28	-
	PG4180.B21	pA5	-
	PG4180.A28	pA5	-
	PG4180.A28	pA24	-
	PG4180.A28	pA10	+/-
	PG4180.A28	pA23	+/-
	PG4180.B21	pB2	+
	PG4180.B21	pB37	+
	PG4180.B21	pA23	+
	PG4180.B21	pE10	+
	PG4180.A28	pE10	+

Table 5. (continued)

	PG4180 Cor⁻ mutant	cor::Tn5 clone	Phenotype
corH	PG4180.A2	pA23	-
	PG4180.E9	pA23	-
	PG4180.A2	pE10	-
	PG4180.E9	pE10	-
	PG4180.E9	pA24	+
	PG4180.E9	pA5	+
	PG4180.A2	pA24	+/-

[a](-), no production of coronatine; (+), coronatine produced at levels equivalent to the wild-type strain PG4180; (+/-), coronatine produced at levels lower than the wild-type PG4180.

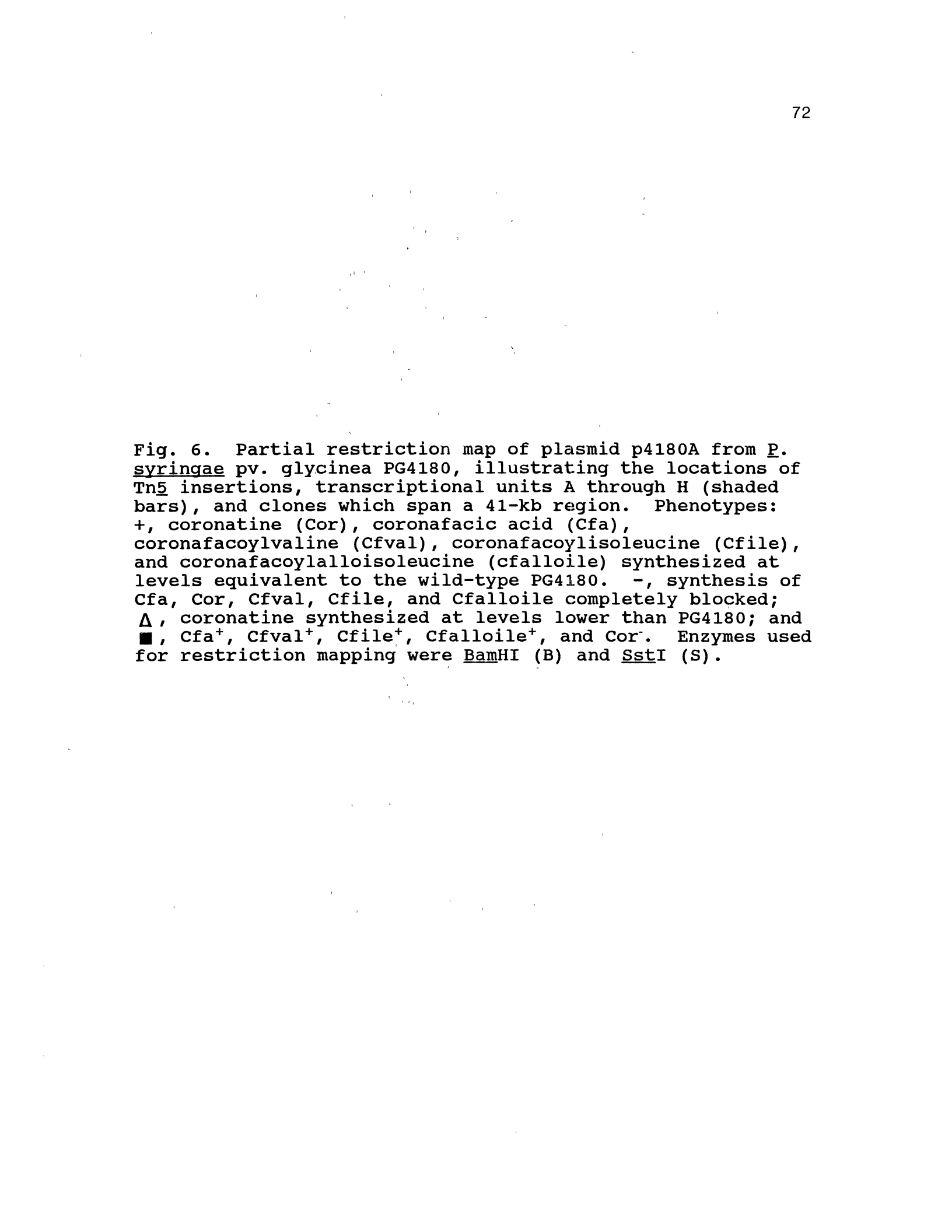


Fig. 6. Partial restriction map of plasmid p4180A from P. syringae pv. glycinea PG4180, illustrating the locations of Tn5 insertions, transcriptional units A through H (shaded bars), and clones which span a 41-kb region. Phenotypes: +, coronatine (Cor), coronafacic acid (Cfa), coronafacoylvaline (Cfval), coronafacoylisoleucine (Cfile), and coronafacoylalloisoleucine (cfalloile) synthesized at levels equivalent to the wild-type PG4180. -, synthesis of Cfa, Cor, Cfval, Cfile, and Cfalloile completely blocked; Δ, coronatine synthesized at levels lower than PG4180; and ■, Cfa^+, $Cfval^+$, $Cfile^+$, $Cfalloile^+$, and Cor^-. Enzymes used for restriction mapping were BamHI (B) and SstI (S).

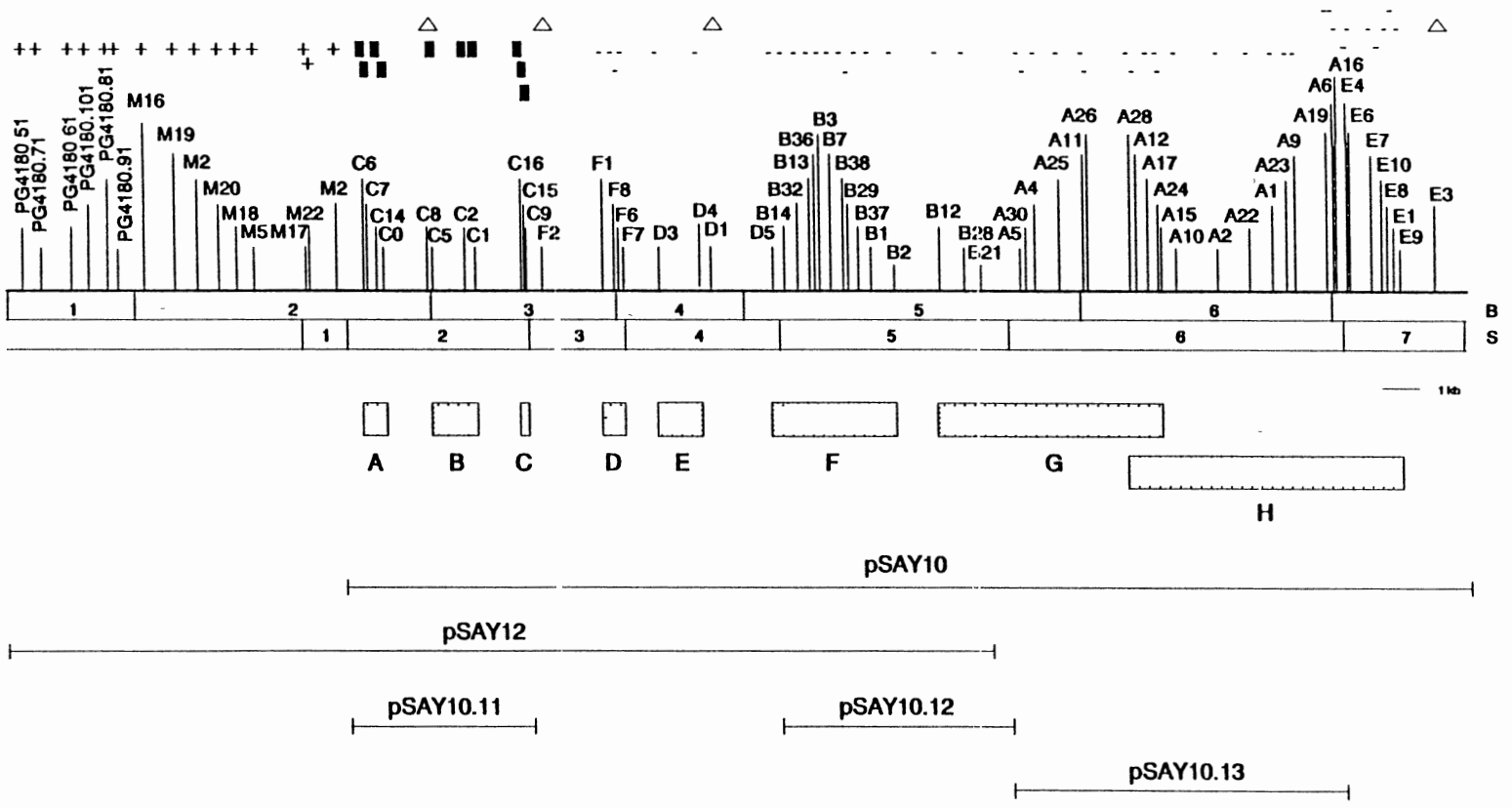

PG4180.51
PG4180.71
PG4180.61
PG4180.101
PG4180.81
PG4180.91
M16
M19
M2
M20
M18
M5
M17
M22
M2
C6
C7
C14
C0
C8
C5
C2
C1
C16
C15
C9
F2
F1
F8
F6
F7
D3
D4
D1
B14
D5
B32
B13
B36
B3
B7
B38
B29
B37
B1
B2
B12
B28
E21
A30
A5
A4
A25
A11
A26
A28
A12
A17
A24
A15
A10
A2
A22
A1
A23
A9
A19
A16
A6
E4
E6
E7
E10
E8
E1
E9
E3
1
2
3
4
5
6
B
1
2
3
4
5
6
7
S
1 kb
A
B
C
D
E
F
G
H
pSAY10
pSAY12
pSAY10.11
pSAY10.12
pSAY10.13

Fig. 7. GC analysis showing retention times of coronafacoyl compounds and methyl esters of organic acids extracted from P. syringae pv. glycinea. (A) Methyl esters of authentic coronafacic acid (CFA), coronafacoylvaline (CFV), and coronatine (COR). (B and C) Methyl esters of organic acids extracted from PG4180 (B) and PG4180.C0 (C).

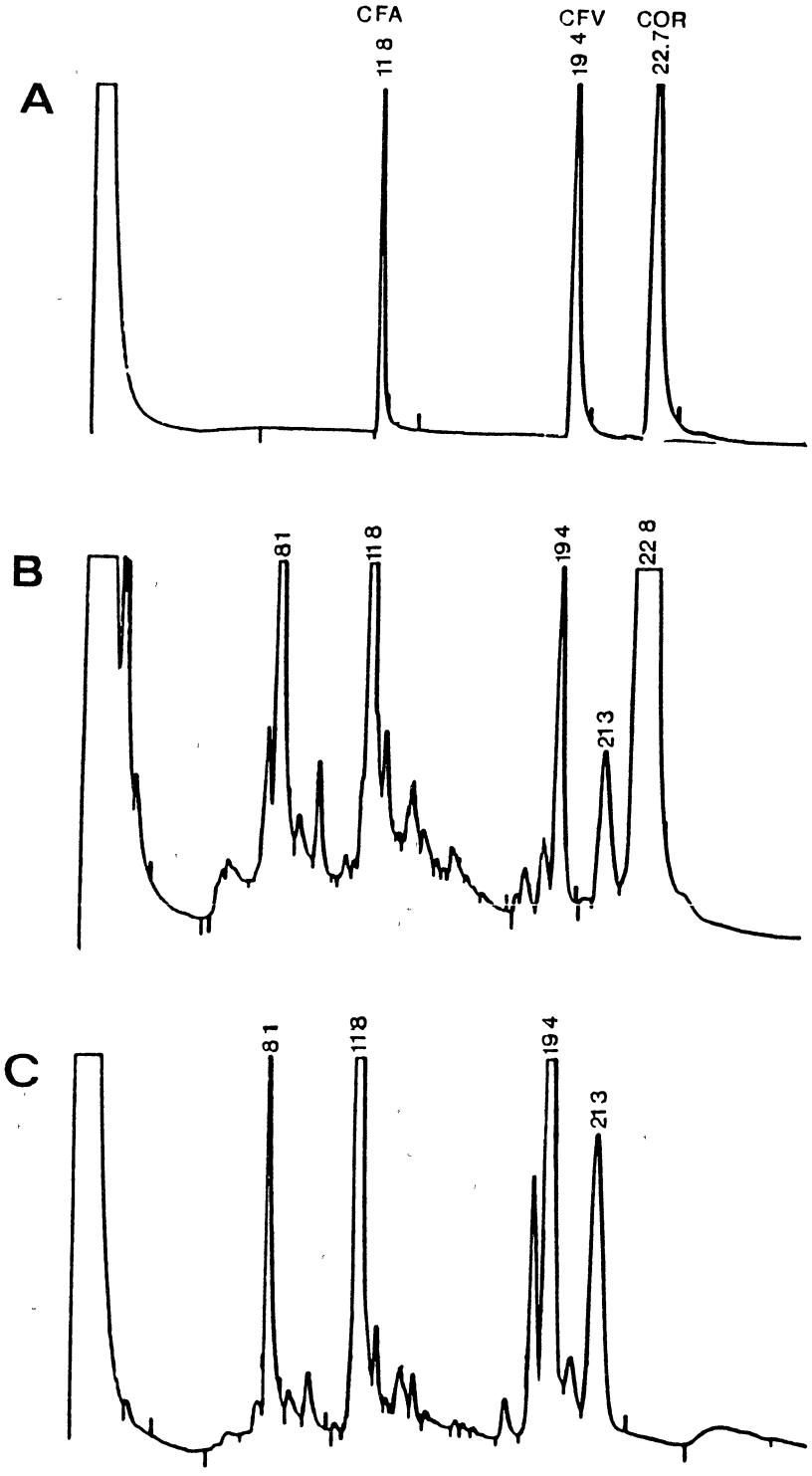
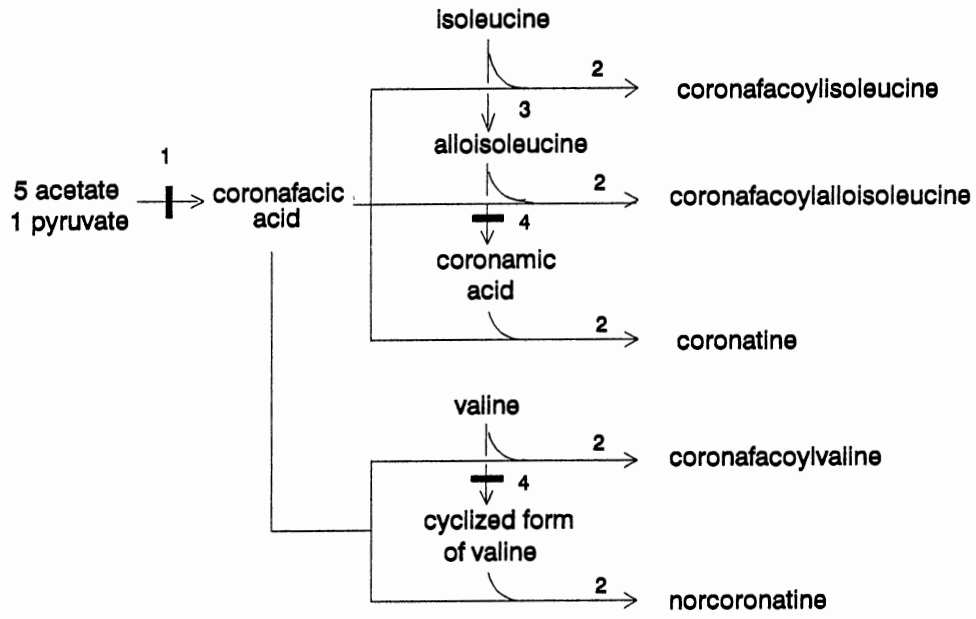

A
CFA
11 8
CFV
19 4
COR
22.7
B
8 1
11 8
19 4
21 3
22 8
C
8 1
11 8
19 4
21 3

Fig. 8. Hypothetical representation of coronatine biosynthesis. The shaded rectangles indicate where blocks occurred in the pathway with various mutations obtained in the present study. The steps in the pathway are labelled: 1, synthesis of coronafacic acid; 2, coupling reactions between coronafacic acid and amino acid derivatives; 3, isomerization of isoleucine to alloisoleucine; and 4, cyclization of alloisoleucine.


isoleucine
2
coronafacoylisoleucine
3
alloisoleucine
1
5 acetate
1 pyruvate
coronafacic
acid
2
coronafacoylalloisoleucine
4
coronamic
acid
2
coronatine
valine
2
coronafacoylvaline
4
cyclized form
of valine
2
norcoronatine

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