JOURNAL OF BACTERIOLOGY, Mar. 1992, p. 1837–1843
0021-9193/92/061837-07$02.00/0

Vol. 174, No. 6

Physical and Functional Characterization of the Gene Cluster Encoding the Polyketide Phytotoxin Coronatine in *Pseudomonas syringae* pv. glycinea†

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Received 30 September 1991/Accepted 15 January 1992

***Pseudomonas syringae* pv. glycinea PG4180 produces the polyketide phytotoxin coronatine. The coronatine synthesis genes in PG4180 were previously shown to reside on a 90-kb plasmid designated p4180A. In the present study, clones containing a 34-kb region of p4180A were saturated with Tn*5*, and 71 unique mutations were recombined into p4180A by marker exchange. The effect of each mutation on coronatine synthesis was determined by analyzing the organic acids produced by the mutants by reverse-phase high-performance liquid chromatography. The organic acids of selected mutants were derivatized to their methyl esters and analyzed by gas chromatography and gas chromatography-mass spectrometry. Mutations in a 20.5-kb region of p4180A completely blocked the synthesis of coronafacic acid and coronatine. Mutations within a 4.4-kb region of p4180A prevented the formation of coronatine but allowed for production of coronafacic acid, coronafacoylvaline, coronafacoylisoleucine, and coronafacoylalloisoleucine. The phenotypes of selected mutants were further confirmed in feeding experiments in which coronafacic acid or coronamic acid was added to the culture media. The results of this study allow us to speculate on the likely sequence of steps in the later stages of coronatine biosynthesis.**

Coronatine is a non-host-specific phytotoxin which is produced by several pathovars of *Pseudomonas syringae*, including atropurpurea (30), glycinea (27), morsprunorum (20), and tomato (4, 26), 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 (4, 12, 34).

The structure of coronatine (Fig. 1a) is unusual and consists of a bicyclic component, coronafacic acid (Fig. 1f), linked by an amide bond to an ethylcyclopropyl amino acid, coronamic acid (14). Parry and Mafoti (32) demonstrated that coronafacic acid is derived via the polyketide pathway from 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 (32). The coronamic acid moiety of coronatine is derived from the isoleucine biosynthetic pathway (22, 32). Both [1-^{13}C]-L-isoleucine and [1-^{13}C]-L-alloisoleucine are incorporated into coronamic acid, but L-alloisoleucine is a much more efficient precursor (31). Although the cyclopropane moiety of coronatine is known to be derived from isoleucine (22), the mechanism of cyclization to form the cyclopropane ring and the point in the pathway at which this occurs have not been determined.

The coronatine (*cor*) synthesis genes in various *P. syringae* pathovars may reside on plasmids (3, 5, 33) or in the chromosome (29). Although progress in the physical characterization of DNA required for coronatine synthesis has been made (18), 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 (5). In the present study, a 34-kb region of p4180A was saturated with Tn*5*, and the effect of each mutation on coronatine synthesis was determined by examination of the organic acid products by reverse-phase high-performance liquid chromatography (RP-HPLC). The organic acids of selected mutants were derivatized and further analyzed by gas chromatography (GC) and GC-mass spectrometry (MS). The phenotypes of these mutants were further characterized in feeding experiments which involved the addition of either coronafacic acid or coronamic acid to the culture media. The results allow speculation on some aspects of the coronatine biosynthetic pathway.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *P. syringae* pv. glycinea PG4180 (20) was used for genetic analyses in the present study. PG4180 mutants containing Tn*5* insertions in the coronatine plasmid p4180A were designated by letter-number combinations. *Escherichia coli* HB101 (19) was used as a host in cloning experiments. The plasmids utilized in this study are described in Table 1. Derivatives of *P. syringae* pv. glycinea PG4180 were maintained on mannitol-glutamate (MG) (15) medium or King's medium B (17). *E. coli* HB101 was grown at 37°C on Luria-Bertani medium (19). PG4180 broth cultures were grown in either MG supplemented with yeast extract at 0.25 g/liter or King's medium B on a rotary shaker (250 rpm) at 20 to 24°C. The following

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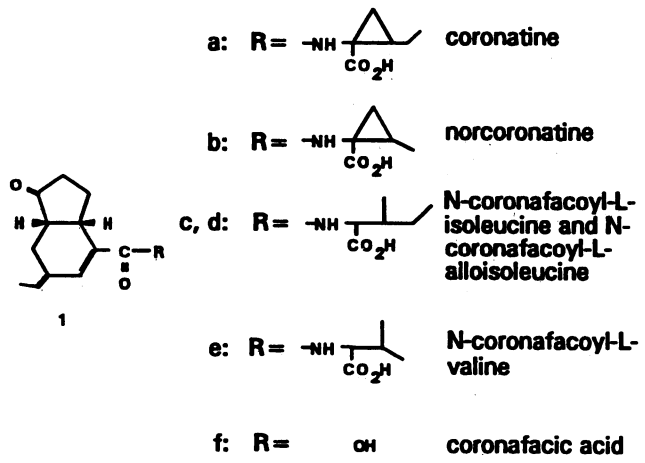

FIG. 1. Structures of coronatine and naturally occurring coronafacoyl compounds.

antibiotics were added to media in the following concentrations (μg/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 (19). Southern transfers were performed using Hybond N nylon membranes purchased from Amersham Corp., Arlington Heights, Ill. Conditions for prehybridizations, hybridizations, and posthybridization washes have been described previously (5). 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 Bio 101, La Jolla, Calif. DNA was labeled with ^{32}P by the random-primer labeling technique (9).

DNA isolations and cloning methods. Plasmid DNA was isolated and purified from *E. coli* by standard procedures (19). Plasmid DNA was isolated from *P. syringae* pv. glycinea as described previously (1, 6). Rapid, small-scale plasmid isolations from *E. coli* were performed by using boiling method 2 of Crouse et al. (7).

A cosmid library of PG4180 plasmid DNA was constructed in pLAFR3 as described previously (2). Restriction maps of selected cosmid clones were constructed with *Bam*HI, *Eco*RI, *Hin*dIII, and *Sst*I 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 (16).

Transposon mutagenesis. Cloned DNA was mutagenized with lambda::Tn*5* in *E. coli* HB101 as described previously (2, 8). Cloned DNA containing Tn*5* insertions was selected by transforming *E. coli* HB101 and plating transformants onto Luria-Bertani agar containing tetracycline and kanamycin.

Tn*5* insertions in p4180A were generated by first creating merodiploids. Clones containing Tn*5* insertions were mobilized from *E. coli* into PG4180 by using the helper plasmid pRK2013 as described previously (2). PG4180 transconjugants containing cloned DNA were verified by agarose gel electrophoresis and then subcultured in MG broth containing kanamycin to maintain selection pressure for Tn*5* (5). Putative PG4180 mutants containing Tn*5* insertions in p4180A were identified by replica plating and selecting Km^r Tc^s colonies. The insertion of Tn*5* into p4180A was verified by plasmid isolation and agarose gel electrophoresis. The location of Tn*5* insertions in cloned DNA and p4180A was established by using *Bam*HI, *Eco*RI, and *Sst*I in single and double digests and by Southern blot analysis.

Analyses for coronafacoyl compounds. PG4180 marker-exchanged mutants were analyzed for production of coronafacoyl compounds in 7-day, 10-ml cultures in the medium of Hoitink and Sinden (13). Supernatants were extracted directly (without concentration) by the method described by Bender et al. (3). The residues from the ethyl acetate extracts were dissolved in 0.5 ml of 1:1 acetonitrile-water, and a 0.1-ml aliquot was analyzed by using a Beckman RP-HPLC system. The HPLC 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 by 0.4 cm) at 208 nm. The initial conditions for gradient elution of organic acids were 10% acetonitrile in 0.05% 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.

Larger isolations (3 liters) of PG4180, PG4180.C0, and PG4180.E9 were undertaken as described by Bender et al. (3). The organic acids were analyzed directly by HPLC and also derivatized to methyl esters and analyzed by GC and GC-MS. GC analyses were undertaken as described previously (3), with an isothermal sector of the temperature program set at 120°C for 5 min. GC-MS analyses were as described by Bender et al. (3) on a DB1 capillary column programmed at 8°/min from 220 to 250°C.

Coronafacoyl standards for chromatographic analyses included coronafacic acid, coronatine, coronafacoylvaline (Fig. 1e), and coronafacoylisoleucine and coronafacoylalloisoleucine (Fig. 1c and d). Coronatine and coronafacic acid were purified from PG4180 as described previously (21). Coronafacoylisoleucine and coronafacoylalloisoleucine were synthesized by a slight modification of the method of Shiraishi and coworkers (35) with the corresponding L-amino

TABLE 1. Plasmids

Plasmid	Characteristic(s)	Source or reference
pLAFR3	Tc^r; RK2-derived cosmid	36
pRK2013	Km^r Mob^+ Tra^+	11
pRK415	Tc^r; RK2-derived vector	16
pSAY10	pLAFR3 containing a 29-kb insert required for coronatine synthesis	This study
pSAY10.11	Tc^r; a 4.8-kb *Sst*I insert cloned in pRK415	This study
pSAY10.12	Tc^r; a 6.0-kb *Sst*I insert cloned in pRK415	This study
pSAY10.13	Tc^r; an 8.7-kb *Sst*I insert cloned in pRK415	This study
pSAY12	pLAFR3 containing a 28-kb insert from p4180A	This study

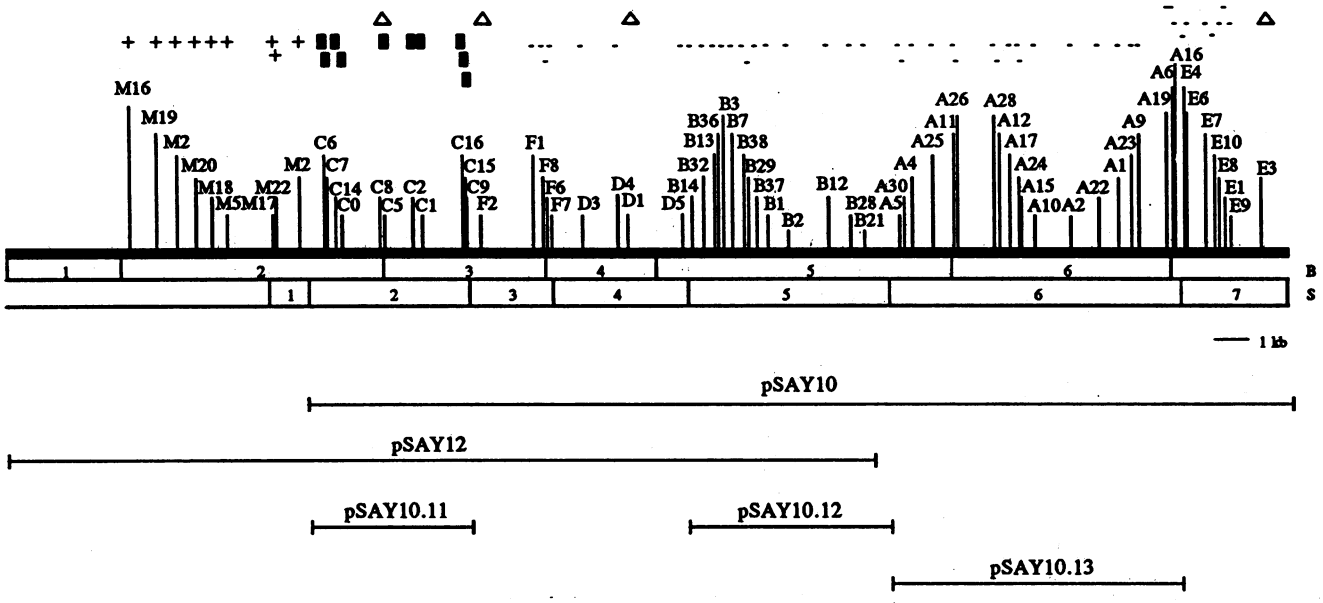

FIG. 2. Partial restriction map of plasmid p4180A from *P. syringae* pv. glycinea PG4180, illustrating the location of Tn*5* insertions and clones which span a 38-kb region. Phenotypes: +, coronatine (cor), coronafacic acid (cfa), coronafacoylvaline (cfval), coronafacoylisoleucine (cfile), and coronafacoylalloisoleucine (cfalloile) synthesized at levels equivalent to the wild-type PG4180; −, synthesis of all coronafacoyl compounds is completely blocked (cfa$^-$, cfval$^-$, cfile$^-$, cfalloile$^-$, cor$^-$); △, all coronafacoyl compounds are synthesized at levels lower than in the wild-type PG4180; ■, cfa$^+$, cfval$^+$, cfile$^+$, cfalloile$^+$, and cor$^-$. Enzymes used for restriction mapping were *Bam*HI (B) and *Sst*I (S).

acids, and the products were purified by column chromatography on silica gel. Coronafacoylvaline was isolated from *P. syringae* pv. atropurpurea as described previously (21) and purified by column chromatography on silica gel with freshly distilled chloroform.

Feeding experiments. Coronamic acid was obtained from the acid hydrolysis of coronatine (10), and coronafacic acid was purified from PG4180 (21). PG4180.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 600 nm, and 100 μl was inoculated into 10 ml of Hoitink-Sinden broth. The cultures were incubated at 18°C for 2 days at 250 rpm, and then PG4180.C0 was supplemented with coronamic acid and PG4180.E9 was supplemented with coronafacic acid (each compound was added to the media to give a final concentration of 7 μM). Cultures were then incubated an additional 5 days and analyzed by RP-HPLC, GC, and GC-MS.

RESULTS

Characterization of mutations in p4180A. pSAY10 and pSAY12 are two overlapping cosmid clones from the PG4180 plasmid DNA library which together contain the 38-kb contiguous region of p4180A shown in Fig. 2. Both pSAY10 and pSAY12 contain a 2.5-kb *Sst*I fragment (Fig. 2, *Sst*I fragment 3) which hybridizes to p4180A but not to other plasmid DNAs in PG4180. A restriction map of the 38-kb region of p4180A contained in these two cosmids was constructed with *Bam*HI and *Sst*I (Fig. 2). The restriction sites shown in the map were verified by digesting p4180A with *Bam*HI and *Sst*I. In another study, cosmid pSAY10 restored coronatine production to cor$^-$ mutants of PG4180 which contained Tn*5* insertions in *Sst*I fragments 3 and 7, indicating that pSAY10 contains genes required for coronatine synthesis (5, 38). However, pSAY10 did not confer coronatine production to a strain of *P. syringae* pv. phaseolicola, indicating that this clone does not contain all of the genes required for coronatine synthesis.

The 4.8-, 6.0-, and 8.7-kb *Sst*I fragments of pSAY10 (*Sst*I fragments 2, 5, and 6; Fig. 2), were subcloned into the *Sst*I site of pRK415 to yield subclones pSAY10.11, pSAY10.12, and pSAY10.13, respectively (Fig. 2). Tn*5* insertions in the two cosmid clones (pSAY10 and pSAY12) and three pRK415-derived subclones were selected to give an even distribution of mutations across the region contained in Fig. 2. Single and double digestions with *Bam*HI, *Eco*RI, *Hin*dIII, and *Sst*I were used to precisely map 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 of intact and digested plasmid DNA was used to confirm the location of Tn*5* insertions in p4180A (see the vertical lines in Fig. 2). Organic acids were extracted from each marker-exchanged PG4180 mutant and analyzed for coronatine by RP-HPLC.

Nine mutations in *Bam*HI fragment 2 (M2, M5, M16, M17, M18, M19, M20, M22, and M23) exhibited the same phenotype as PG4180 and produced wild-type levels of coronatine. Mutations C8, F2, D1, and E3, which were located in *Sst*I fragments 2, 3, 4, and 7, respectively (Fig. 2), produced coronatine at reduced levels (17 to 38% of that produced by PG4180). Tn*5* insertions in a 20.5-kb region of p4180A which spanned *Sst*I fragments 3, 4, 5, 6, and 7 completely blocked the synthesis of coronafacic acid and coronatine (Fig. 2). Mutant PG4180.E9, which contained a Tn*5* insertion in *Sst*I fragment 7 (Fig. 2), belonged to this phenotypic class (cfa$^-$ cor$^-$) and was chosen for further analysis by GC and GC-MS. Nine insertions in a 4.8-kb *Sst*I fragment (Fig. 2, *Sst*I fragment 2) prevented coronatine synthesis but allowed for the production of coronafacic acid. These mutations shared a common phenotype and produced two prominent

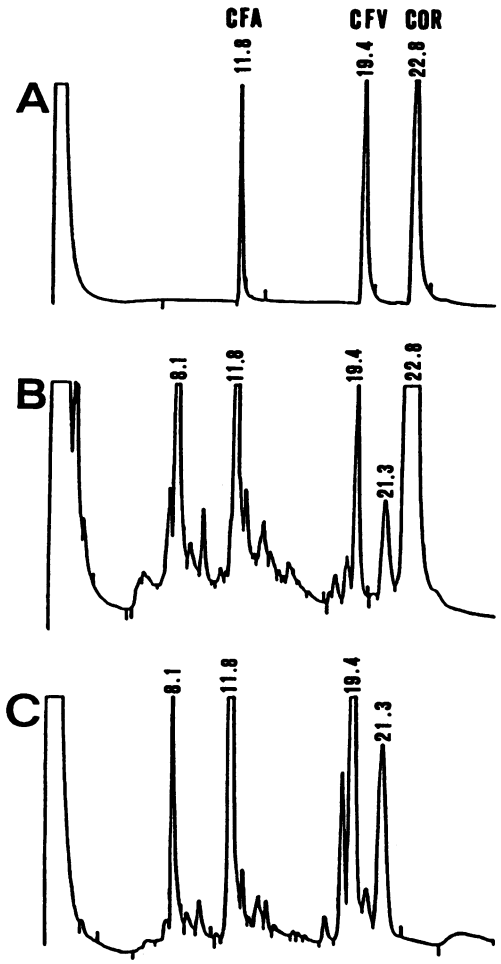

FIG. 3. GC analysis showing retention times of coronafacoyl compounds and methyl esters of organic acids extracted from *P. syringae* pv. glycinea. (A) Methyl esters of authentic coronafacic acid (CFA), coronafacoylvaline (CFV), and coronatine (COR); (B and C) methyl esters of organic acids extracted from PG4180 (B) and PG4180.C0 (C).

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 mutant (PG4180.C0) was selected for further analysis by GC and GC-MS.

GC and GC-MS analyses of PG4180, PG4180.C0, and PG4180.E9. The GC tracings of methyl-esterified organic acids produced by PG4180.E9 showed no trace of coronafacic acid, coronatine, or other coronafacoyl compounds, while those from PG4180 and PG4180.C0 indicated that both strains were producing coronafacic acid and coronafacoylvaline with retention times of 11.8 and 19.4 min, respectively (Fig. 3). Only PG4180 produced coronatine (retention time, 22.8 min; Fig. 3B). PG4180.C0 produced a 21.3-min peak (Fig. 3C) which was also present at a reduced level in PG4180 (Fig. 3B); the relative position of this peak corresponded to a mixture of compounds previously identified as norcoronatine (23), coronafacoylalloisoleucine, and coronafacoylisoleucine (28).

TABLE 2. GC-MS data for selected components of the methyl ester derivatives of organic acids isolated from *P. syringae* pv. glycinea PG4180 and PG4180.C0

Compound	Corresponding GC peak[a] (min)	Molecular ion (*m/z*)	Detection in[b]	
			PG4180	PG4180.C0
Coronafacic acid	11.8	222	+	++
Coronafacoylvaline	19.4	321	+	++
Coronafacoylisoleucine[c]	21.3	335	+	++
Coronafacoylalloisoleucine[c]	21.3	335	+	++
Norcoronatine[c]	21.3	319	+	−
Coronatine	22.8	333	+	−

[a] Retention time, as in Fig. 3.
[b] +, coronafacoyl compound detected at levels routinely synthesized by PG4180; ++, coronafacoyl compound synthesized at levels exceeding those produced by PG4180; −, coronafacoyl compound not detected.
[c] In GC-MS analysis, GC was on a capillary column which completely separated coronafacoylalloisoleucine from coronafacoylisoleucine; the coelution of norcoronatine with coronafacoylalloisoleucine was demonstrated by selective ion monitoring.

The identities of the 11.8-, 19.4-, 21.3-, and 22.8-min GC peaks in PG4180 and PG4180.C0 were confirmed by GC-MS analyses (Table 2). In these analyses, which used capillary GC and selective ion monitoring in the MS analysis, the 21.3-min GC peak of PG4180 was found to consist of a mixture of coronafacoylisoleucine, coronafacoylalloisoleucine, and norcoronatine (Table 2). However, the 21.3-min GC peak of PG4180.C0 was found to contain the first two coronafacoyl compounds but lacked norcoronatine (Table 2). Under the GC-MS conditions, the coronafacoylalloisoleucine and coronafacoylisoleucine in PG4180.C0 organic acids eluted separately at 22.3 and 22.8 min (Fig. 4). In PG4180, norcoronatine and coronafacoylalloisoleucine coeluted at 22.3 min, and coronafacoylisoleucine eluted separately at 22.8 min (data not shown). The elution profile of norcoronatine and coronafacoylalloisoleucine in the 22.3-

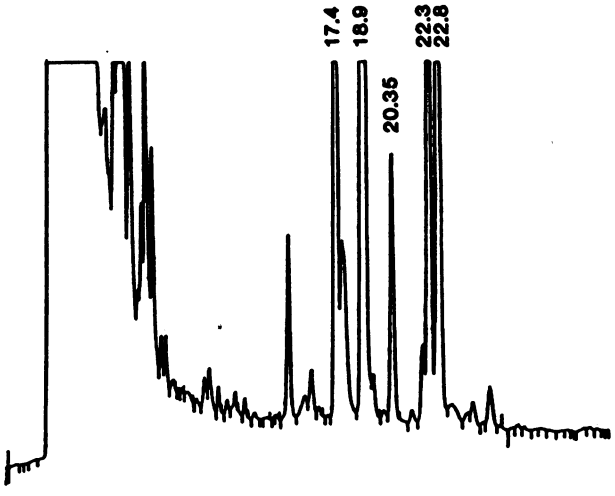

FIG. 4. Separation of components of PG4180.C0 by capillary GC prior to analysis by MS. Sample was run as a methyl ester derivative of the organic acid extract, and detection was by flame ionization. Peaks eluting at 18.9, 22.3, and 22.8 min were identified as coronafacoylvaline, coronafacoylalloisoleucine, and coronafacoylisoleucine, respectively, in the corresponding GC-MS analysis. Coronafacic acid was present in the solvent tail, while minor peaks at 17.4 and 20.35 min were unidentified coronafacoyl compounds.

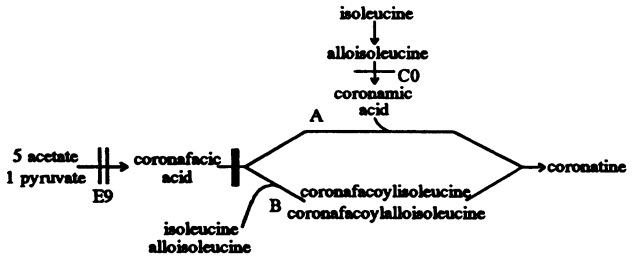

FIG. 5. Alternative routes to the synthesis of coronatine. Coronafacic acid is synthesized from two polyketide chains, with pyruvate serving as a starter unit for one chain. Several possible routes for the synthesis of coronatine from coronafacic acid exist: (A) L-isoleucine or L-alloisoleucine is cyclized to form coronamic acid, and the latter product is coupled with coronafacic acid to form coronatine; (B) L-isoleucine or L-alloisoleucine is coupled with coronafacic acid, and then the amino acid moiety undergoes an oxidative cyclization to form coronatine. The locations of the biosynthetic blocks in PG4180.C0 (——) (defective in the cyclization of alloisoleucine to form coronamate) and PG4180.E9 (‖) (defective in coronafacate synthesis) are indicated. The location of the coupling step which conjugates coronafacate to different amino acids via amide bond formation is also shown (▮).

min peak was visualized by monitoring the ions at *m/z* 319 and 335, respectively.

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 2.5, 3.6, and 3.0 times, respectively, relative to those 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 and the increased accumulation of coronafacoyl compounds lacking the cyclopropane ring in the products from PG4180.C0 strongly suggested that the Tn*5* insertion in this mutant had interrupted steps involved in the formation of the cyclopropane ring of coronatine. This hypothesis was tested by supplying PG4180.C0 with the cyclopropane-containing amino acid coronamic acid. Analysis of the organic acid products by HPLC, GC, and GC-MS unequivocally identified the presence of coronatine, supporting the conclusion that genes involved in cyclopropane ring formation of coronatine have been interrupted in PG4180.C0. The experiment also demonstrated that PG4180.C0, like the wild-type PG4180, retains the ability to couple a variety of amino acids with coronafacic acid. Since the synthesis of coronatine requires production of coronafacic acid (cfa$^+$), cyclopropane ring formation (cyclization$^+$), and amide bond formation (coupling$^+$), these results establish the phenotype of PG4180.C0 to be cfa$^+$, cyclization$^-$, and coupling$^+$ (Fig. 5).

Administration of coronafacoyl compounds to PG4180.E9. The absence of coronafacic acid, coronatine, and all other coronafacoyl compounds in PG4180.E9 organic acids strongly suggested that steps involved in the synthesis of coronafacic acid had been interrupted by the Tn*5* insertion. When PG4180.E9 was supplemented with coronafacic acid, HPLC and GC analyses of the products showed that the major component was coronatine. This confirmed that the Tn*5* insertion in PG4180.E9 interrupted coronafacic acid synthesis and also demonstrated that genes involved in amide bond or cyclopropane ring formation had not been interrupted. These results therefore establish the phenotype of PG4180.E9 to be cfa$^-$, cyclization$^+$, and coupling$^+$ (Fig. 5).

DISCUSSION

In the present study, a 34-kb region of p4180A was mutagenized with Tn*5* and 27 kb was shown to be required for coronatine synthesis. Additional mutations were recently obtained in a region immediately adjacent to *Sst*I fragment 7 (Fig. 2); the phenotype of several mutants containing Tn*5* insertions in this region was cfa$^-$ cor$^-$ (38). The involvement of this additional DNA in the synthesis of coronafacic acid may explain why cosmid pSAY10 does not contain all of the DNA required for coronatine synthesis.

In previous studies, coronafacoyl compounds were detected by extracting organic acids from large volumes (100 to 1,000 ml) of culture supernatant (3–5). This method was fairly slow because the supernatants required concentration by evaporation prior to extraction. In the present study, mutants were screened for the production of coronafacoyl compounds by directly extracting the supernatant of 10-ml culture volumes. This scaled-down, abbreviated procedure was possible because PG4180 produces high yields of coronatine in vitro (5 to 7 μg/ml) and the detection limit for coronatine by RP-HPLC was approximately 10 ng.

HPLC fractionation of coronafacoyl compounds produced by PG4180 revealed the presence of small peaks eluting approximately 1 and 3 min after coronafacic acid. The 1-min peak cochromatographed with coronafacoylvaline and corresponds to the 19.4-min peak shown in Fig. 3B. The peak eluting 3 min after coronafacic acid in the HPLC fractionation cochromatographed with authentic coronafacoylisoleucine and coronafacoyalloisoleucine and corresponds to the 21.3-min peak shown in Fig. 3B. The use of capillary GC and selective ion monitoring in the MS analysis showed that the 21.3-min peak in PG4180 consisted of coronafacoylisoleucine, coronafacoylalloisoleucine, and norcoronatine. Therefore, the HPLC method developed in the present study reliably separated coronafacic acid, coronafacoylvaline, and coronatine, but coronafacoylisoleucine, coronafacoylalloisoleucine, and norcoronatine coeluted as a single peak.

In the wild-type PG4180, coronatine is the most abundant coronafacoyl compound, while mutants containing insertions in *Sst*I fragment 2 (Fig. 2) synthesized elevated levels of products identified as coronafacic acid, coronafacoylvaline, coronafacoylisoleucine, and coronafacoylalloisoleucine, but neither coronatine nor norcoronatine. Two interpretations from this result are that coronamic acid is the preferred substrate for the coupling reaction and that these mutants accumulated increased levels of the observed products because synthesis of coronamic acid was blocked. On this basis, we propose that the most likely biosynthetic route to coronatine from coronafacic acid is route A (Fig. 5), during which alloisoleucine is cyclized to coronamic acid, which is then coupled to form coronatine, rather than route B (Fig. 5), which involves cyclization of coronafacoylalloisoleucine. Support for this proposal, but certainly not proof for it, was found when coronamic acid was fed to PG4180.C0, which resulted in production of coronatine. In the case of norcoronatine, which is a very minor product of wild-type PG4180, the inferences are that both valine and alloisoleucine are substrates in identical enzymatic steps and

that alloisoleucine retains a much higher affinity for the cyclization reaction than valine.

Since coronatine is the most toxic of all presently characterized coronafacoyl compounds, the cyclization reaction significantly increases the toxicity of coronafacoyl-derived compounds. Recently, coronafacoyl-derived compounds were shown to be produced by a xanthomonad, *Xanthomonas campestris* pv. phormiicola (24, 37). Mitchell (24) showed that three strains of *X. campestris* pv. phormiicola produced coronafacoylvaline and coronafacoylisoleucine but no coronafacoylalloisoleucine, coronatine, or norcoronatine. The administration of alloisoleucine to *X. campestris* pv. phormiicola did not result in coronatine production, but administration of coronamic acid did allow coronatine production (25). 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 coronamic acid and norcoronamic acid, respectively. This organism and the mutants developed in the present study will be used to elucidate the steps involved in the coupling and cyclization reactions.

ACKNOWLEDGMENTS

This work was supported by the Oklahoma Agricultural Experiment Station and by NSF grant DMB-8902561. S.K.P. acknowledges the support of a postdoctoral fellowship from the Korea Science and Engineering Foundation.

We thank Vivienne Paterson for compiling the GC-MS data.

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