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Cloning and Expression of Genes Required for Coronamic Acid (2-Ethyl-1-Aminocyclopropane 1-Carboxylic Acid), an Intermediate in the Biosynthesis of the Phytotoxin Coronatine

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Coronamic acid (CMA; 2-ethyl-1-aminocyclopropane 1-carboxylic acid) is an intermediate in the biosynthesis of coronatine (COR), a chlorosis-inducing phytotoxin produced by *Pseudomonas syringae* pv. glycinea PG4180. Tn*5* mutagenesis and substrate feeding studies were previously used to characterize regions of the COR biosynthetic gene cluster required for synthesis of coronafacic acid and CMA, which are the only two characterized intermediates in the COR biosynthetic pathway. In the present study, additional Tn*5* insertions were generated to more precisely define the region required for CMA biosynthesis. A new analytical method for CMA detection which involves derivatization with phenylisothiocyanate and detection by high-performance liquid chromatography (HPLC) was developed. This method was used to analyze and quantify the production of CMA by selected derivatives of *P. syringae* pv. glycinea which contained mutagenized or cloned regions from the CMA biosynthetic region. pMU2, a clone containing a 6.45-kb insert from the CMA region, genetically complemented mutants which required CMA for COR production. When pMU2 was introduced into *P. syringae* pv. glycinea 18a/90 (a strain which does not synthesize COR or its intermediates), CMA was not produced, indicating that pMU2 does not contain the complete CMA biosynthetic gene cluster. However, when two plasmid constructs designated pMU234 (12.5 kb) and pKTX30 (3.0 kb) were cointroduced into 18a/90, CMA was detected in culture supernatants by thin-layer chromatography and HPLC. The biological activity of the CMA produced by *P. syringae* pv. glycinea 18a/90 derivatives was demonstrated by the production of COR in cosynthesis experiments in which 18a/90 transconjugants were cocultivated with CMA-requiring mutants of *P. syringae* pv. glycinea PG4180. CMA production was also obtained when pMU234 and pKTX30 were cointroduced into *P. syringae* pv. syringae B1; however, these two constructs did not enable *Escherichia coli* K-12 to synthesize CMA. The production of CMA in *P. syringae* strains which lack the COR biosynthetic gene cluster indicates that CMA production can occur independently of coronafacic acid biosynthesis and raises interesting questions regarding the evolutionary origin of the COR biosynthetic pathway.

Coronatine (COR) is a chlorosis-inducing non-host-specific phytotoxin produced by several *Pseudomonas syringae* pathovars including glycinea, atropurpurea, morsprunorum, tomato, and maculicola (17, 19, 30). COR induces a number of responses in host plants which can be reproduced by ethylene or indoleacetic acid, suggesting that the toxin alters host metabolism in a manner analogous to that of plant growth hormones (9, 13, 25).

COR consists of two distinct moieties, coronafacic acid (CFA) and coronamic acid (CMA), which are derived from separate biosynthetic pathways. CFA, which is derived from the polyketide pathway (23), is coupled via amide bond formation to CMA, a cyclized derivative of isoleucine (18) (Fig. 1). Unequivocal evidence for the role of CMA (2-ethyl-1-aminocyclopropane 1-carboxylic acid) as a distinct intermediate in the COR biosynthetic pathway was recently obtained (20). The biosynthetic block to COR in several mutants was eliminated when CMA was exogenously supplied, whereas other mutants were shown to secrete CMA when the coupling step or CFA biosynthesis was blocked (3, 20). Additional studies with strain G70, a coupling-defective (CPL^-) mutant which secretes CMA, showed that [^{14}C]isoleucine was incorporated into CMA; furthermore, an excess of unlabelled CMA reduced the incorporation of [^{14}C]isoleucine into COR (20). The latter result would be predicted only if CMA was a true pathway intermediate and the preferred substrate for coupling to CFA.

The genes for COR biosynthesis in strain PG4180 are encoded within a 30-kb region of a 90-kb plasmid designated p4180A (6, 31). Extensive mutagenesis with Tn*5* and phenotypic characterization of each mutation resulted in the recovery of mutants blocked in distinct biosynthetic steps (3). The present study has focused on the localization of the CMA biosynthetic gene cluster. To facilitate our genetic studies, a simple and sensitive method for the detection and quantification of CMA by using reverse-phase high-performance liquid chromatography (HPLC) was developed. Additional Tn*5* mutants of PG4180 were generated to more precisely define the DNA region required for CMA biosynthesis, and selected mutants were analyzed for CMA biosynthesis. These results were used to successfully clone the CMA biosynthetic region and produce CMA in two *P. syringae* strains which lack all parts of the COR biosynthetic gene cluster.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are listed in

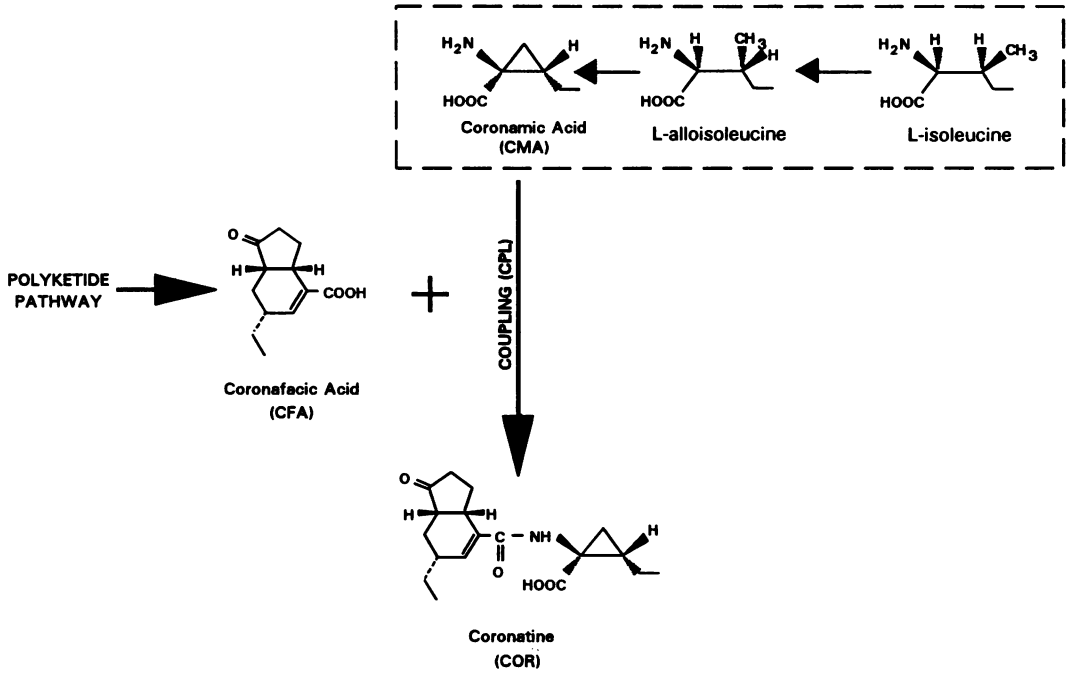

FIG. 1. Biochemical pathways involved in the synthesis of COR in *P. syringae* pv. glycinea PG4180. The structure of COR consists of a polyketide component, CFA, coupled via amide bond formation to an amino acid component, CMA. CFA is synthesized as a branched polyketide from five acetate units and one pyruvate unit via an unknown sequence of events (23). CMA is derived from isoleucine via alloisoleucine and cyclized by an unknown mechanism (18, 20, 22). Available evidence indicates that the intermediates CFA and CMA are coupled in the final step of the biosynthetic pathway.

Table 1. *Pseudomonas* strains were routinely cultured on King's medium B (14) or mannitol-glutamate medium (11) at 24 to 26°C. *Escherichia coli* HB101 and DH5α (16) were used as hosts in cloning experiments and were cultured in Luria-Bertani medium at 37°C. *E. coli* K-12 (16) was maintained on Luria-Bertani medium prior to studies involving CMA biosynthesis. Strains assayed for CMA production were cultivated in Hoitink-Sinden medium optimized for COR production (HSC) (21). Antibiotics were added to media in the following concentrations: tetracycline, 12.5 μg/ml; kanamycin, 10 μg/ml; ampicillin, 40 μg/ml; and streptomycin, 25 μg/ml.

Molecular genetic techniques. Agarose gel electrophoresis, DNA restriction digests, ligation reactions, transformations, and Southern hybridizations were performed by using standard procedures (16). When specific DNA fragments were excised from agarose gels, residual agarose was removed with the Qiaex DNA Extraction Kit manufactured by Qiagen (Chatsworth, Calif.).

Plasmid DNA was isolated from *E. coli* by standard procedures (16) and from *P. syringae* as described previously (4, 8). Selected clones were mobilized from *E. coli* into *P. syringae* by using the helper plasmid pRK2013 (6), and transconjugants were verified by agarose gel electrophoresis. Tn*5* insertions in p4180A were generated by marker exchange as described elsewhere (31).

The construction of pMU2, pMU23, and pMU234 involved the partial digestion of pSAY10 with *Sst*I, religation, and recovery of Tc^r *E. coli* transformants containing *Sst*I fragments as shown in Fig. 2. Subclones designated pMUB4, pMUE29, and pMUP31 were constructed by digesting pMU2 with either *Bam*HI, *Eco*RI, or *Pst*I to obtain subclones with inserts of 4.0, 2.9, and 3.1 kb, respectively. pMUH72, which contains a 7.2-kb insert, was constructed by partially digesting pSAY12 with *Hin*dIII. pKTX30 contains a 3.0-kb *Xho*I fragment from pMUH72 inserted into pKT230.

Analysis of coronafacoyl compounds. *P. syringae* strains were analyzed for production of coronafacoyl compounds (COR, CFA, and CFA-amino acid conjugates) in 7-day, 10-ml cultures grown in HSC at 18°C with 280 rpm on a rotary shaker. Coronafacoyl compounds were recovered as organic acids from culture supernatants and analyzed by HPLC as described previously (21). When necessary, organic acids were extracted from 600 ml (60 × 10 ml) of culture supernatant by methods described elsewhere (5).

Isolation and detection of CMA. Bacteria were cultured in 600 ml (60 × 10 ml) of HSC at 18°C for 7 days (*P. syringae*) or 24°C for 5 days (*E. coli* K-12). Cells were pelleted by centrifugation at 6,800 × *g* for 10 min, and supernatants were stored at 4°C. Free amino acids were isolated from bacterial supernatants by ion exchange on Amberlite resin CG-120 (30 g; Sigma Chemical Co., St. Louis, Mo.) which was freshly converted to the H^+ form as described previously (20). Residues were then dissolved in methanol (1 ml) and stored at 4°C for further analysis.

For thin-layer chromatography (TLC), 10-μl samples were applied to Sigmacell Type 100 plates (20 by 20 cm; Sigma Chemical Co.) in 10-mm bands, air dried, and then developed in a solution containing *n*-butanol, acetic acid, water, and pyridine in a 5:1:4:4 ratio (20). Plates were visualized (20) by being sprayed with a ninhydrin-cadmium acetate solution (1 g of ninhydrin, 0.1 g of cadmium acetate, 0.5 ml of acetic acid, 100 ml of methanol).

HPLC analysis and quantitation of CMA. Reverse-phase HPLC analyses were conducted by the following procedure. Samples (1 to 50 μl, depending on the estimated concentration of amino-containing molecules) were evaporated in siliconized

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic or properties	Reference or source
Bacterial strains		
E. coli		
HB101		16
DH5α		16
K-12	Prototroph	16
P. syringae pv. glycinea		
18a/90	COR^- CMA^- CFA^- CPL^-	29
PG4180	COR^+ CMA^+ CFA^+ CPL^+	3, 31
PG4180.C14	COR^- CMA^- CFA^+ CPL^+ Km^r	3
PG4180.C0	COR^- CMA^- CFA^+ CPL^+ Km^r	31
PG4180.C1	COR^- CMA^- CFA^+ CPL^+ Km^r	3
PG4180.C9	COR^- CMA^- CFA^+ CPL^+ Km^r	3
PG4180.F7	COR^- CMA^- CFA^- CPL^+ Km^r	3
PG4180.D4	COR^- CMA^- CFA^- CPL^+ Km^r	3
PG4180.D5	COR^- CMA^+ CFA^- CPL^+ Km^r	3
PG4180.B7	COR^- CMA^+ CFA^- CPL^+ Km^r	3
PG4180.B1	COR^- CMA^+ CFA^- CPL^+ Km^r	3
PG4180.B2	COR^- CMA^+ CFA^- CPL^+ Km^r	3
PG4180.N42	COR^+ CMA^+ CFA^+ CPL^+ Km^r	This study
PG4180.N9	COR^+ CMA^+ CFA^+ CPL^+ Km^r	This study
PG4180.N53	COR^+ CMA^+ CFA^+ CPL^+ Km^r	This study
PG4180.N10	COR^- CMA^- CFA^+ CPL^+ Km^r	This study
PG4180.N7	COR^- CMA^- CFA^+ CPL^+ Km^r	This study
PG4180.N13	COR^- CMA^- CFA^+ CPL^+ Km^r	This study
P. syringae pv. syringae		
B1	COR^- CMA^- CFA^- CPL^-	K. Geider
Plasmids		
p4180A	Contains COR genes	3
pKT230	Km^r Sm^r IncQ	1
pLAFR3	Tc^r IncP; RK2-derived cosmid	27
pRK2013	Km^r Mob^+ Tra^+	10
pRK415	Tc^r IncP; RK2-derived vector	12
pSAY10	Tc^r; contains a 29-kb insert from p4180A which is required for COR biosynthesis	31
pSAY10.11	Tc^r; contains a 4.8-kb *Sst*I fragment cloned in pRK415	31
pMU2	Tc^r; contains a 6.45-kb insert derived from pSAY10 in pLAFR3	This study
pMU23	Tc^r; contains an 8.7-kb insert derived from pSAY10 in pLAFR3	This study
pMU234	Tc^r; contains a 12.5-kb insert derived from pSAY10 in pLAFR3	This study
pMUB4	Tc^r; contains a 4.0-kb *Bam*HI insert derived from pMU2 in pRK415	This study
pMUE29	Tc^r; contains a 2.9-kb *Eco*RI insert derived from pMU2 in pRK415	This study
pMUP31	Tc^r; contains a 3.1-kb *Pst*I insert derived from pMU2 in pRK415	This study
pSAY12	Tc^r; contains a 28-kb insert from p4180A which partially overlaps with pSAY10	31
pMUH72	Tc^r; contains a 7.2-kb *Hin*dIII insert derived from pSAY12 in pRK415	This study
pKTX30	Sm^r; contains a 3.0-kb *Xho*I insert derived from pMUH72 in pKT230	This study

microcentrifuge tubes. Ten microliters of freshly prepared redrying solution containing ethanol, water, and triethylamine in a 2:2:1 ratio was added to each sample. Samples were mixed and reevaporated, and 20 μl of freshly prepared derivatization solution containing ethanol, triethylamine, water, and phenylisothiocyanate (7:1:1:1) was added. Samples were incubated in derivatization solution for 20 min at room temperature, evaporated, and stored at −20°C until further analyzed.

HPLC analyses were conducted with a Waters system (Millipore, Milford, Mass.) consisting of two model 6000 solvent delivery pumps, a WISP autosampler-injector (model 712), a model 680 gradient controller, a Baseline 810 chromatography workstation, a Pico-Tag C-18 reverse-phase column (3.9 by 100 mm), and detector model 490. Phenylthiocarbamyl (PTC) derivatives were dissolved in sample diluent (5 mM sodium phosphate, 5% acetonitrile [pH 7.4]), filtered through 0.45-μm-pore-size, 3-mm-diameter nylon filters (Micron Separations, Inc., Westboro, Mass.), and then injected in 10-μl volumes. PTC derivatives were selectively eluted from the column with a binary gradient consisting of an aqueous phase of 140 mM sodium acetate-trihydrate (with 6% acetonitrile–0.05% triethylamine [pH 6.4]) and an organic phase of 60% acetonitrile (7). PTC derivatives were detected by measuring the A_{254}. The total time for separation of the derivatives and reequilibration of the column was 22 min. Data were collected and processed with the Baseline 810 software package.

Authentic CMA was produced by acid hydrolysis of COR according to the method of Ferguson and Mitchell (9). The product after TLC produced the typical yellow spot after ninhydrin treatment. This product retained biological activity, since it restored COR production to mutants which required CMA for COR biosynthesis (3, 31). A calibration curve for CMA was constructed by injecting a dilution series of CMA at concentrations ranging from 50 to 750 pmol, measuring the A_{254}, and calibrating the HPLC by using Waters Baseline 810 chromatography software. Quantitative information concerning CMA yields was then obtained from the calibration curve.

Cosynthesis experiments. Conditions for the inoculation and incubation of strains utilized in cocultivation studies have been described previously (3, 31). In preliminary experiments, strains derived from *P. syringae* pv. glycinea 18a/90 showed a higher growth rate than mutants derived from PG4180. There-

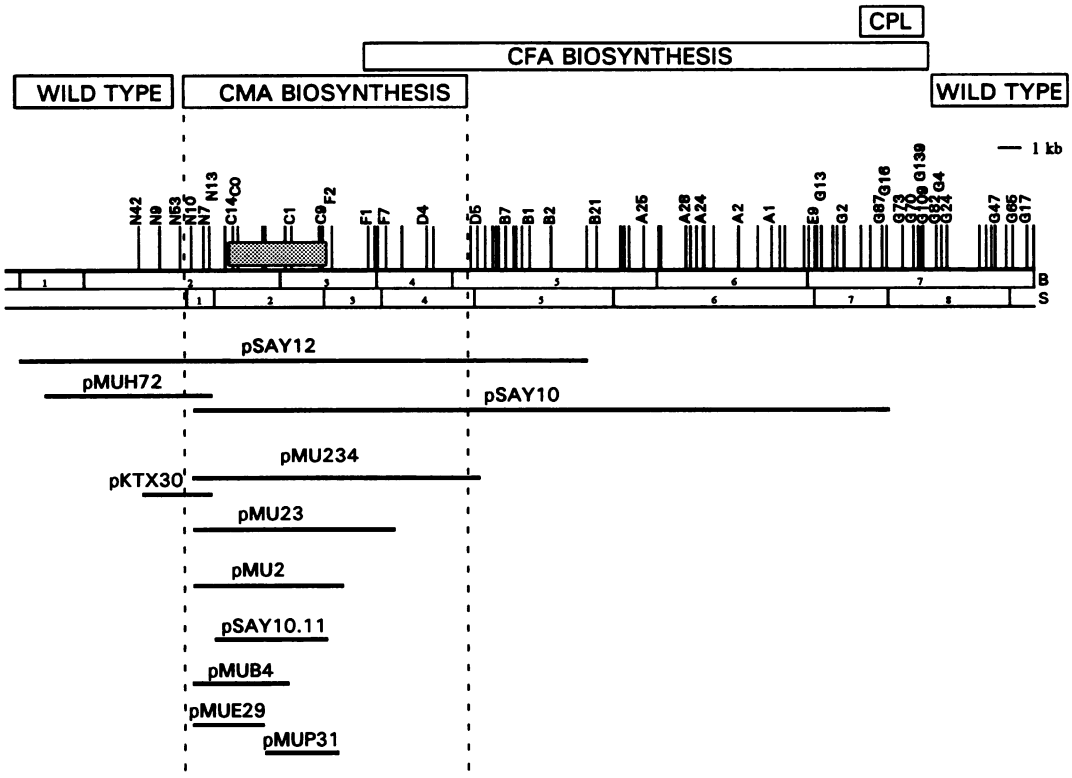

FIG. 2. Partial restriction map of plasmid p4180A from *P. syringae* pv. glycinea PG4180, illustrating the locations of Tn*5* insertions which span a 43-kb region. Enzymes used for restriction mapping were *Bam*HI (B) and *Sst*I (S). Functional regions of the COR biosynthetic cluster are shown in the boxed areas above the map. Tn*5* insertions in the regions designated wild type had no affect on COR production. Mutants containing mutations with the prefix C (see the shaded box) were COR^- CMA^- CFA^+ CPL^+; these were utilized in the genetic complementation studies described in the text. Mutants containing mutations with the prefix N were generated in this study and were used to define the left border of the COR biosynthetic gene cluster. All other mutations (labelled and unlabelled) have been described previously (3, 31). CPL, coupling activity.

fore, HSC broth was first inoculated with a PG4180 mutant, incubated for 24 h, and then supplemented with a second strain derived from 18a/90. This procedure compensated for the higher growth rate of 18a/90 and resulted in optimal growth of both strains utilized in the cocultivation experiments. Organic acids were extracted after a 7-day incubation and analyzed for production of coronafacoyl compounds as outlined above.

RESULTS

Detection of CMA by HPLC. Previous HPLC separation and detection conditions for CFA and COR were not suitable for CMA, since CMA does not absorb strongly at 208 nm, the maximum UV for COR. However, PTC derivatization of the amino group of CMA allowed efficient detection at 254 nm. PTC derivatives of CMA eluted at approximately 8.53 min (Fig. 3C) and were clearly distinguishable when cochromatographed with amino acid standards (Fig. 3A and B). These results indicated that CMA does not coelute with any of the 17 amino acids tested and can be differentiated in a mixture of amino acids. The elution time of CMA (8.53 min) was highly reproducible (± 0.05 min) in five different experiments consisting of four to eight replications (data not shown). This reproducibility was established regardless of whether CMA was measured separately or in combination with other amino acids.

The calibration curve for CMA (Fig. 4) was linear in the range of 50 to 750 pmol, permitting a precise quantification of CMA. Quantities of CMA as low as 10 pmol per injection were detected by this method. Differences in the quantities of CMA detected in purified samples subjected to ultrafiltration and/or ion-exchange chromatography were also investigated. The loss of CMA due to ultrafiltration was negligible, and only 10% of the CMA was unaccounted for after ion-exchange chromatography and ultrafiltration (data not shown). These results verify the accuracy of this detection method for quantifying levels of CMA.

Genetic complementation of CMA-requiring mutants. Mutants PG4180.C14, PG4180.C0, PG4180.C1, and PG4180.C9 were previously shown to be CFA^+ CPL^+ CMA^-, and they required the addition of exogenous CMA for COR production (3, 31). Initial attempts at defining the region(s) required for CMA biosynthesis were focused on genetically complementing these mutants with DNA from the COR biosynthetic cluster. Since the Tn*5* insertions designated C14, C0, C1, and C9 mapped within *Sst*I fragment 2 (Fig. 2), pSAY10.11, which contains *Sst*I fragment 2 subcloned in pRK415, was introduced into PG4180.C0 and PG4180.C9, and the transconjugants were analyzed for COR production. PG4180.C0 and PG4180.C9 transconjugants containing pSAY10.11 did not produce COR, indicating that *Sst*I fragment 2 (4.8 kb) was not sufficient to restore CMA biosynthesis to these mutants. pMUB4, pMUE29, and pMUP31, which contained inserts of 4.0, 2.9, and 3.1 kb, respectively (Fig. 2), also failed to complement the four CMA-requiring mutants. However, pSAY10, which contains a 29-kb insert from the COR biosynthetic cluster, enabled

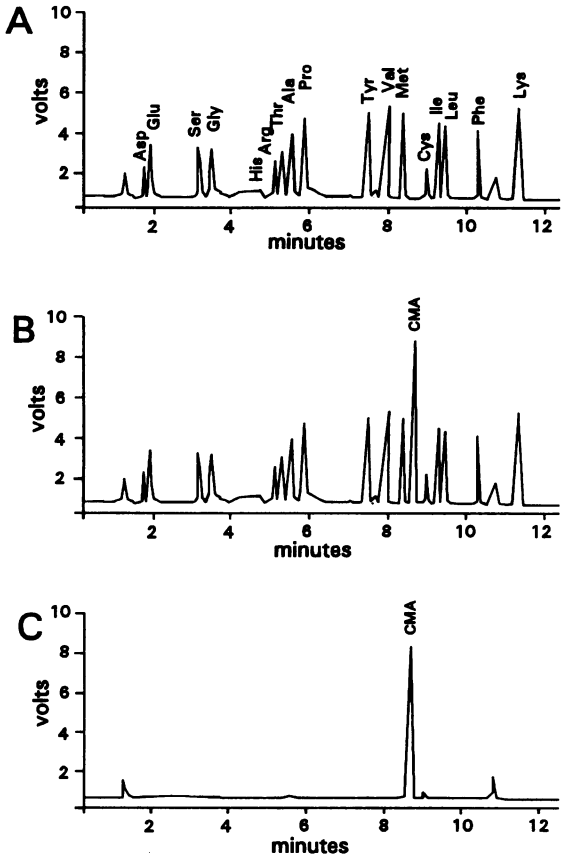

FIG. 3. HPLC fractionation of amino acids after derivatization with PTC. Separations of mixtures of 17 common amino acids (A) and amino acids plus CMA (B) and of authentic CMA alone (C) are shown. When CMA was chromatographed with a mixture containing 17 other amino acids, it eluted at a unique retention time (8.53 min). The concentration of CMA was approximately twice those of other amino acids in the mixture.

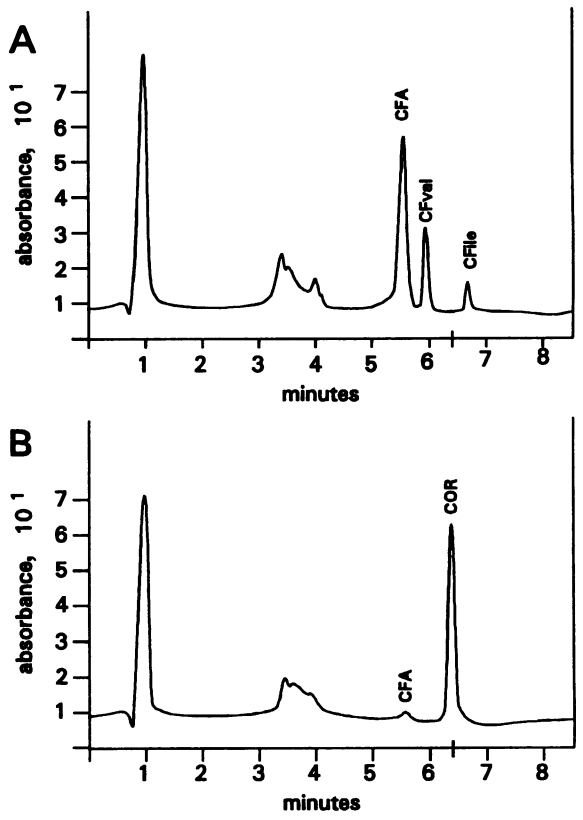

FIG. 5. HPLC analysis of organic acids extracted from PG4180.C0 and PG4180.C0(pMU2). (A) PG4180.C0. CMA biosynthesis is blocked, leading to the increased secretion of CFA, coronafacoylvaline (CFval), and coronafacoylisoleucine (CFile). The latter two compounds are coronafacoyl analogs produced from the coupling of CFA to valine and isoleucine, respectively. (B) The organic acid profile obtained from PG4180.C0(pMU2). In this transconjugant, CMA was produced and coupled to CFA to produce COR.

all four CMA-requiring mutants to synthesize COR. These results indicated that additional DNA adjacent to *Sst*I fragment 2 was required to complement the CMA-defective mutants. This hypothesis was investigated by introducing pMU2, which contains *Sst*I fragment 2 and most of *Sst*I fragment 1, into the four CMA-requiring mutants. The introduction of pMU2 into all four mutants resulted in the synthesis of COR, indicating that the biosynthetic block to CMA in these mutants was relieved by the introduction of pMU2. HPLC tracings supporting these results for PG4180.C0 and the transconjugant PG4180.C0(pMU2) are shown in Fig. 5.

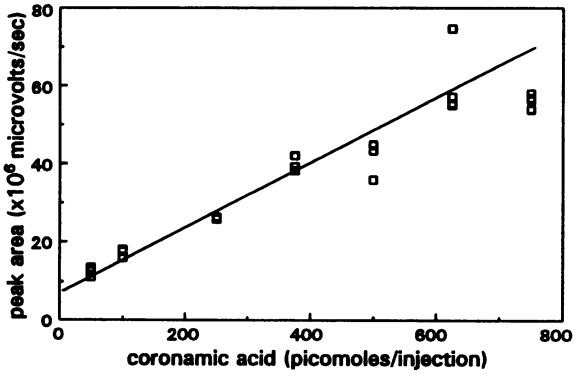

FIG. 4. Calibration curve for CMA quantification.

CMA biosynthesis by *P. syringae* pv. glycinea 18a/90. *P. syringae* pv. glycinea 18a/90 was previously reported to be a nonproducer of COR, and it was found to lack DNA homologous to the COR gene cluster by means of Southern hybridization experiments (29). In the present study, supernatants of 18a/90 were shown by HPLC analyses to be devoid of both CFA and coronafacoyl compounds and were shown by TLC and HPLC not to contain CMA. Furthermore, substrate feeding assays using exogenous CFA and CMA indicated that 18a/90 could not ligate CFA and CMA to form COR and therefore that it lacked coupling activity (i.e., it was CPL$^-$). Therefore, since 18a/90 had no biosynthetic activity in the COR pathway, several constructs from the COR biosynthetic gene cluster were introduced into it to determine the DNA region required for CMA biosynthesis. The amino acids isolated from supernatants of 18a/90, 18a/90(pLAFR3, pKT230), 18a/90(pSAY12), 18a/90(pMU234), 18a/90(pMU23), 18a/90 (pMU2), 18a/90(pMU234, pKTX30), and 18a/90(pMU23, pKTX30) were examined by TLC. Out of these, only 18a/

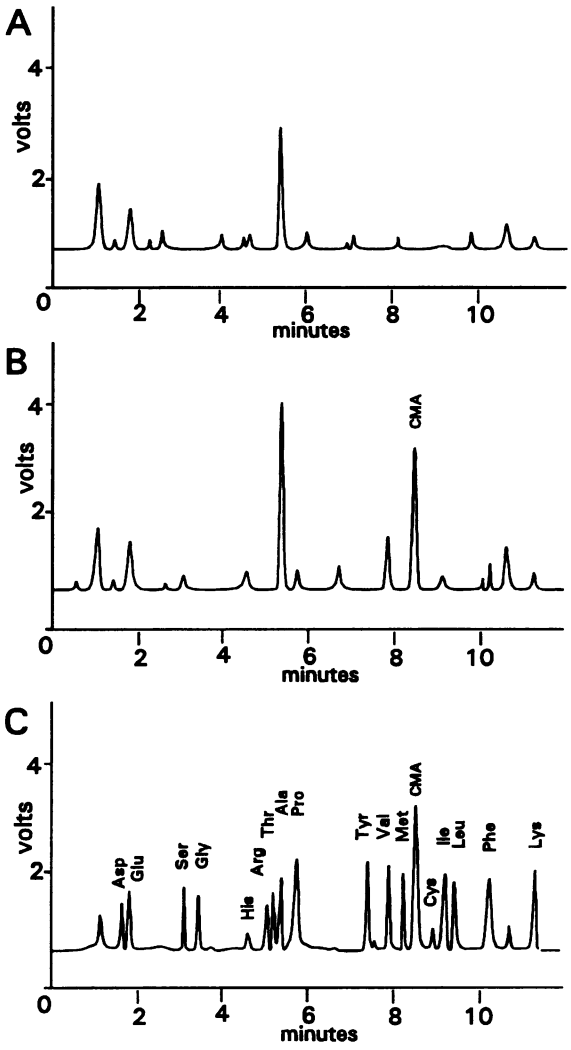

FIG. 6. HPLC analysis of PTC derivatives of amino acids secreted by *P. syringae* pv. glycinea 18a/90 and 18a/90(pMU234, pKTX30). (A) Amino acids isolated from the wild-type *P. syringae* pv. glycinea 18a/90. (B) Amino acid profile of the transconjugant 18a/90(pMU234, pKTX30). (C) Detection of CMA in a mixture of 17 standard amino acids.

90(pSAY12) and 18a/90(pMU234, pKTX30) contained an amino acid which comigrated with authentic CMA on TLC plates (data not shown), and the presence of CMA in these extracts was confirmed by HPLC analysis (Fig. 6B). HPLC analysis of supernatants from the 18a/90 wild type (Fig. 6A) and all other 18a/90 derivatives demonstrated an absence of CMA. These results provided unequivocal evidence that plasmids pMU234 and pKTX30 contain all of the DNA necessary for expression of CMA biosynthesis in *P. syringae* pv. glycinea 18a/90.

CMA biosynthesis in other bacterial strains. Cosmid pSAY12 as well as plasmids pMU234 and pKTX30 was introduced into *P. syringae* pv. syringae B1, a CFA^- CMA^- CPL^- nonproducer of COR, and into *E. coli* K-12, which lacks the COR biosynthetic gene cluster. Amino acids were isolated from 500-ml cultures of *P. syringae* pv. syringae B1, *E. coli* K-12, and selected transconjugants and analyzed by TLC and HPLC. CMA production was detected in *P. syringae* pv. syringae B1(pSAY12) and B1(pMU234, pKTX30) but not in *E. coli* K-12 derivatives containing these same constructs. CMA was not detected in either wild-type strain (*P. syringae* pv. syringae B1 or *E. coli* K-12).

TABLE 2. Evaluation for COR production by cocultivation of *P. syringae* pv. glycinea 18a/90 transconjugants with CFA^+ CPL^+ CMA^- mutants of PG4180

18a/90 transconjugant	Production of COR by cocultivation with PG4180 mutant[a,b,c]:			
	C14	C0	C1	C9
18a/90(pLAFR3)	−	−	−	−
18a/90(pSAY12)	+	+	+	+
18a/90(pSAY10)	−	−	−	−
18a/90(pMU2)	−	−	−	−

[a] The maintenance of the constructs in 18a/90 required the addition of tetracycline to the fermentation. Therefore, PG4180 mutants contained pRK415 (Tc^r) as a source of tetracycline resistance.
[b] COR was detected by HPLC analysis of organic acids extracted from supernatants of the cocultivation. +, COR detected; −, no COR detected.
[c] C14, PG4180.C14; C0, PG4180.C0; C1, PG4180.C1; C9, PG4180.C9.

Cosynthesis experiments. Cosynthesis studies generally involve the growth of two defined strains which individually lack the ability to make the end product of interest. The production of the biosynthetic end product in the cocultivation indicates that an intermediate has been produced by one strain, secreted, and utilized by the second strain to produce the end product. Feeding experiments had previously shown that exogenous CMA could be taken up by CFA^+ CPL^+ CMA^- mutants of PG4180 and used to synthesize COR (3). Consequently, the cocultivation experiments conducted in the present study were designed to screen for the biological activity of the CMA produced by derivatives of 18a/90. The secretion of CMA by 18a/90 derivatives would be indicated by the production of COR in the cocultivation, indicating that the CFA^+ CPL^+ CMA^- mutants of PG4180 had assimilated CMA and utilized it to synthesize COR.

The results for the cocultivation experiment are summarized in Table 2. COR was synthesized in significant quantities when the four CMA^- CFA^+ CPL^+ mutants, PG4180.C14, PG4180.C0, PG4180.C1, and PG4180.C9, were cocultivated with *P. syringae* pv. glycinea 18a/90(pSAY12). However, other combinations did not result in COR synthesis. *P. syringae* pv. glycinea 18a/90(pMU234, pKTX30) was not tested in these experiments.

Identification of the left border of the COR biosynthetic gene cluster in PG4180. Genetic complementation studies with CMA-requiring mutants and the production of CMA by *P. syringae* pv. glycinea 18a/90(pKTX30, pMU234) indicated that the region required for CMA biosynthesis extended further leftward of *Sst*I fragment 2. To more precisely define the left border of the CMA biosynthetic gene cluster, pMUH72, which contains a 7.2-kb *Hin*dIII fragment (Fig. 2), was saturated with Tn*5* insertions. Tn*5* insertions in p4180A were generated by homologous recombination using various pMUH72::Tn*5* constructs as described previously (31). Southern blot analysis of intact and digested plasmid DNA was used to confirm the locations of the Tn*5* insertions in p4180A (mutations with the prefix N in Fig. 2). *P. syringae* pv. glycinea PG4180 mutants containing Tn*5* insertions in p4180A were then analyzed for production of coronafacoyl compounds and CMA. The compounds produced by the new mutants generated in the present study were then compared with those produced by previously reported mutants with prefixes B, C, D, and F by isolation of

TABLE 3. Production of CFA, CMA, and COR by *P. syringae* pv. glycinea PG4180 and selected mutants containing Tn*5* insertions in the COR biosynthetic cluster

Strain or mutant	Amt of compound produced (mg/liter)		
	CFA[a]	CMA[b]	COR[a]
PG4180	2.2	1.5	9.8
PG4180.N42	0.57	1.07	8.54
PG4180.N9	0.83	0.97	8.52
PG4180.N53	0.59	0.98	6.60
PG4180.N10	4.46	ND[c]	ND
PG4180.N7	11.70	ND	ND
PG4180.N13	10.46	ND	ND
PG4180.C14	11.20	ND	ND
PG4180.C0	11.80	ND	ND
PG4180.C1	10.90	ND	ND
PG4180.C9	10.14	ND	ND
PG4180.F7	ND	ND	ND
PG4180.D4	ND	ND	ND
PG4180.D5	ND	1.07	ND
PG4180.B7	ND	1.02	ND
PG4180.B1	ND	1.08	ND
PG4180.B2	ND	1.04	ND

[a] Production of CFA and COR was determined by isolation of organic acids from culture supernatants and fractionation by HPLC (21). Quantities represent mean amounts recovered from three replications.

[b] Production of CMA was determined by isolation of free amino acids from supernatants by ion-exchange chromatography, PTC derivatization, ultrafiltration, and fractionation by HPLC. Quantities represent mean amounts recovered from six replications.

[c] ND, compound not detected.

CFA, CMA, and COR from the supernatants (Table 3). Mutants PG4180.N13, PG4180.N7, and PG4180.N10 did not produce CMA or COR but produced CFA and coronafacoylvaline. PG4180.N13 and PG4180.N7 also produced coronafacoylisoleucine and therefore resembled mutants with the prefix C. Tn*5* insertions in PG4180.N53, PG4180.N9, and PG4180.N42 did not interfere with the synthesis of either COR or CMA. These strains produced wild-type levels of COR, CMA, and CFA. Therefore, the left border of the CMA biosynthetic gene cluster was defined by mutations N10 (CMA^-) and N53 (CMA^+). Data obtained for CMA production in selected mutants with the prefixes B, C, D, and F established a right border for the CMA biosynthetic gene cluster. Although CMA production by PG4180.D4 was not detected, PG4180.D5, PG4180.B7, PG4180.B1, and PG4180.B2 produced substantial quantities of CMA (Table 3). Therefore, the results shown in Table 3 confirmed previously obtained indirect data from feeding and cocultivation studies (3) and demonstrated that the DNA located between Tn*5* insertions N10 and D4 (approximately 10.2 kb) is required for CMA biosynthesis.

DISCUSSION

The secretion of CMA by blocked mutants and wild-type PG4180 provided the first evidence for the occurrence of CMA as a natural product of the COR biosynthetic pathway (20). In contrast, CFA, the end product of the polyketide pathway to COR, is generally secreted with COR and can be detected in organic acid extracts (3, 31). In the present study, reverse-phase HPLC was used to accurately determine the concentrations of CMA in bacterial supernatants. The ease of sample preparation and the sensitivity and reproducibility of the method make it suitable for elucidating factors which regulate CMA production.

The results obtained in the present study suggest that at least 10.2 kb of the COR biosynthetic gene cluster is required for CMA biosynthesis in *P. syringae*. PG4180 mutants containing Tn*5* insertions in *Sst*I fragments 1, 2, 3, and 4 did not produce CMA in the present study. The complementation data suggest that three or more transcriptional units are contained in this region and are required for the biosynthesis of CMA in *P. syringae*. The four CMA-requiring mutants with mutations located at insertion positions C14, C0, C1, and C9 were complemented by the 6.45-kb insert in pMU2 but not by smaller subclones. This result suggests that one transcriptional unit which is required for CMA biosynthesis maps to the insert contained in pMU2. Results obtained from nucleotide sequencing (28) indicate that the transcript contained in pMU2 is oriented in the opposite direction relative to the *lacZ* promoter in pLAFR3. Thus, transcription from the vector promoter can be excluded. Although pMU2, pMU23, and pMU234 did not contain sufficient DNA to confer CMA biosynthesis to *P. syringae* glycinea 18a/90, transconjugants containing both pMU234 and pKTX30 produced CMA. This suggests that a second transcriptional unit could be situated on pKTX30, since this clone contained DNA which functioned in *trans* with pMU234 to enable *P. syringae* pv. glycinea 18a/90 and *P. syringae* pv. syringae B1 to produce CMA. Mutants containing Tn*5* insertions in a 2.65-kb region spanning *Sst*I fragments 3 and 4, including those located at positions F1, F7, and D4, required the addition of both exogenous CFA and CMA to restore COR biosynthesis (3). Complementation data for this region are presently lacking, so the possibility exists for the presence of one or more transcriptional units in this area. In summary, our current results indicate that at least three transcriptional units are required for CMA biosynthesis in *P. syringae*.

The lack of CMA production in *E. coli* K-12 was not surprising, since previous efforts aimed at expressing the coupling function in K-12 also failed (15). Although the reason for the lack of expression is not clear, failure to transcribe the CMA biosynthetic cluster remains a possibility, since *Pseudomonas* promoters are often not efficiently recognized in *E. coli*.

In the current study, CMA was produced and expressed independently of the CFA biosynthetic cluster. Previous studies with blocked mutants indicated that CFA biosynthesis could occur without CMA production. This raises interesting questions regarding the evolutionary origin of the COR biosynthetic pathway and whether production of CMA and production of CFA might occur independently in nature. Although neither CMA nor CFA causes chlorosis in plant hosts, these compounds may individually possess properties which benefit bacteria that produce them in undetermined ways.

The specific steps involved in CMA biosynthesis remain the subject of ongoing investigations. Parry et al. (22) demonstrated that alloisoleucine is a more immediate precursor to CMA than isoleucine is, which suggests that alloisoleucine functions as an intermediate in the CMA pathway. Experimental evidence is consistent with the oxidative cyclization of L-alloisoleucine to form CMA (24). For this cyclization to produce CMA, the CMA synthetase enzyme or enzyme complex would have to perform at least four different reactions: (i) recognition and binding of isoleucine; (ii) isomerization to alloisoleucine; (iii) oxidative cyclization; and (iv) release of CMA from the enzyme complex. In this respect, a large transcript which encodes several enzymes or a multifunctional protein fits the hypothetical scheme. Comparable processes in the biosynthesis of clavaminic acid (26), isopenicillin N (2), and deacetoxycephalosporin C (2) have been uncovered. Although the substrates for the enzymes which synthesize these compounds are different, they resemble each other and the puta-

tive CMA synthetase in several respects: isomerization of a precursor, oxidative cyclization, formation of a cyclic molecule of high intramolecular energy, and antimicrobial or biological activity of the end product. Certainly, nucleotide sequencing of the cloned DNA region derived from this study will reveal new insights regarding related enzymes or enzyme families and thus will provide the keys to elucidating the actual mechanisms leading to the synthesis of CMA.

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