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The Biosynthetic Gene Cluster for Coronamic Acid, an Ethylcyclopropyl Amino Acid, Contains Genes Homologous to Amino Acid-Activating Enzymes and Thioesterases

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Coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine, functions as an intermediate in the biosynthesis of coronatine, a chlorosis-inducing phytotoxin produced by *Pseudomonas syringae* pv. glycinea PG4180. The DNA required for CMA biosynthesis (6.9 kb) was sequenced, revealing three distinct open reading frames (ORFs) which share a common orientation for transcription. The deduced amino acid sequence of a 2.7-kb ORF designated *cmaA* contained six core sequences and two conserved motifs which are present in a variety of amino acid-activating enzymes, including nonribosomal peptide synthetases. Furthermore, CmaA contained a spatial arrangement of histidine, aspartate, and arginine residues which are conserved in the ferrous active site of some nonheme iron(II) enzymes which catalyze oxidative cyclizations. The deduced amino acid sequence of a 1.2-kb ORF designated *cmaT* was related to thioesterases of both procaryotic and eucaryotic origins. These data suggest that CMA assembly is similar to the thiotemplate mechanism of nonribosomal peptide synthesis. No significant similarities between a 0.9-kb ORF designated *cmaU* and other database entries were found. The start sites of two transcripts required for CMA biosynthesis were identified in the present study. pRG960sd, a vector containing a promoterless glucuronidase gene, was used to localize and study the promoter regions upstream of the two transcripts. Data obtained in the present study indicate that CMA biosynthesis is regulated at the transcriptional level by temperature.

Coronatine (COR) is a chlorosis-inducing, non-host-specific phytotoxin produced by several *Pseudomonas syringae* pathovars and functions as an important virulence factor in the diseases incited by these bacteria (36, 37, 64). COR induces a number of responses in 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 (13, 24, 49). Recently, striking structural and functional homologies between COR, methyl jasmonate, and 12-oxo-phytodienoic acid were found, suggesting that COR mimics the octadecanoid signalling molecules of higher plants (15, 19, 63).

COR consists of two distinct moieties, a polyketide component, coronafacic acid (CFA), and a cyclized amino acid, coronamic acid (CMA) (2-ethyl-1-aminocyclopropane 1-carboxylic acid). These are derived from separate biosynthetic pathways and coupled via amide bond formation (Fig. 1) (42). Both moieties were previously shown to be required for the phytotoxic effects of COR (53, 63).

The genes for COR biosynthesis in *P. syringae* pv. glycinea PG4180 are encoded within a 30-kb region of a 90-kb plasmid designated p4180A (67). Tn*5* mutagenesis, phenotypic characterization, and feeding studies with exogenous CFA and CMA resulted in the recovery of mutants blocked in distinct biosynthetic steps (4). Genetic complementation studies with CMA-defective mutants and expression of CMA synthesis in another bacterium were used to localize a DNA region (6.9 kb) specifically required for CMA biosynthesis (59).

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The biosynthesis of CMA initially involves the isomerization of L-isoleucine to L-alloisoleucine, which is presumably followed by an oxidative cyclization to form the cyclopropane ring (41, 42) (Fig. 1). Recently, CMA was shown to be a distinctive intermediate in the COR biosynthetic pathway. The biosynthetic block to COR in several mutants of *P. syringae* pv. glycinea PG4180 was eliminated when CMA was exogenously supplied (4, 38). Furthermore, an excess of unlabelled CMA reduced the incorporation of [^{14}C]isoleucine into COR, indicating that CMA is a free intermediate of COR biosynthesis (38).

Obvious differences in the structures of the two moieties which form COR prompt questions regarding the mechanistic and evolutionary origins of CFA and CMA. Moreover, the disclosure of a unique reaction cascade which utilizes a branched-chain amino acid as a substrate and results in a cyclopropyl compound could be of immense biochemical interest. To further our understanding of CMA biosynthesis, the nucleotide sequence required for CMA production was determined and compared with other DNA and protein sequences.

CMA biosynthesis was previously shown to require two transcripts (59), and the start sites for these transcripts were identified in the present study. DNA fragments containing the promoter sequences for each transcript were localized in a vector containing a promoterless β-glucuronidase gene, and the constructs were used to investigate the effects of temperature on transcription.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *Pseudomonas* strains were routinely cultured on King's medium B (25) or mannitol-glutamate (MG) medium

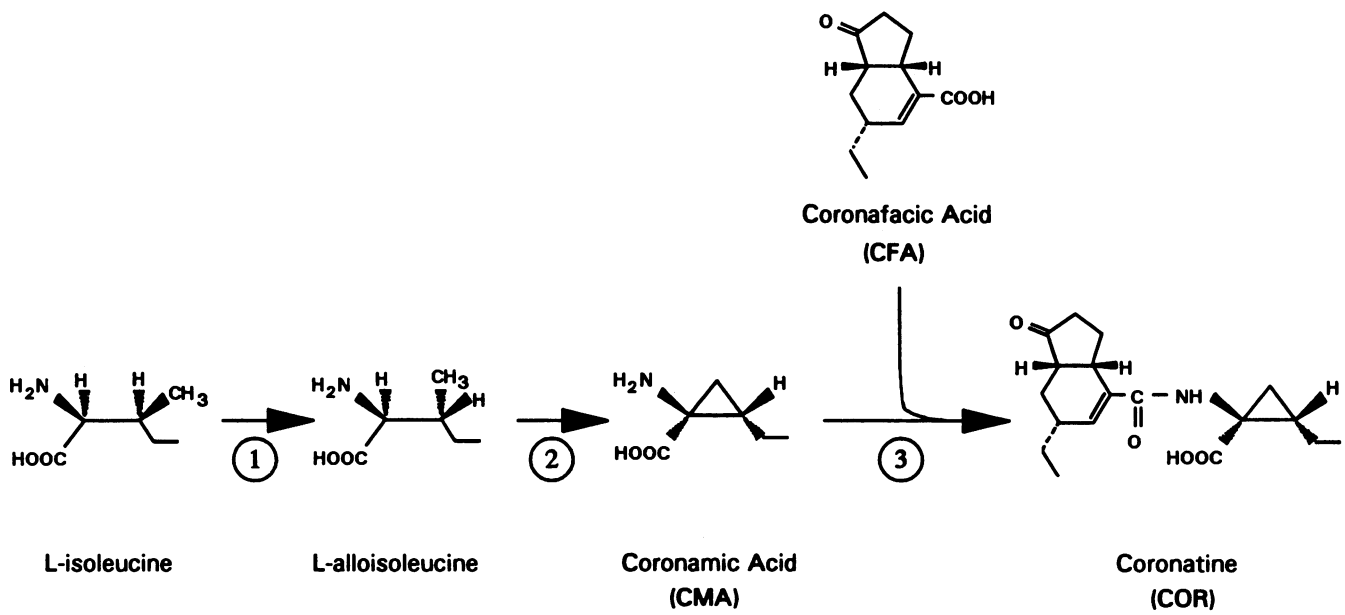

FIG. 1. Biochemical pathway involved in CMA synthesis and incorporation of CMA into COR by *P. syringae* pv. glycinea PG4180. COR consists of a polyketide component, CFA, coupled via amide bond formation (step 3) to CMA, a cyclopropyl amino acid. CFA is synthesized from acetate and pyruvate, but the precise steps of assembly remain obscure. CMA biosynthesis involves isomerization of L-isoleucine to L-alloisoleucine (step 1) and oxidative cyclization by an unknown mechanism (step 2) (42).

(23) at 24 to 26°C. *Escherichia coli* HB101 and DH5α (51) were used as hosts in cloning experiments and were cultured in Luria-Bertani medium at 37°C. For RNA isolation, *P. syringae* pv. glycinea PG4180 was cultivated in Hoitink-Sinden medium optimized for COR production (HSC) (40) at 18°C. The following antibiotics were added to media in the indicated concentrations (in micrograms per milliliter): tetracycline, 12.5; kanamycin, 10; ampicillin, 40; spectinomycin, 25; and streptomycin, 25.

DNA procedures. Agarose gel electrophoresis, polyacrylamide gel electrophoresis, DNA restriction digests, ligation reactions, small-scale plasmid DNA preparations, and transformations were performed by standard procedures (51). DNA fragments were excised from agarose gels and residual agarose was removed with the GeneClean DNA extraction kit (Bio 101, La Jolla, Calif.). Large-scale preparations of plasmid DNA from *E. coli* were isolated by alkaline lysis and purified with Qiagen columns (Qiagen, Chatsworth, Calif.). Plasmid DNA was isolated from *Pseudomonas* strains as described previously (3, 12). Selected clones were mobilized from *E. coli* into *P. syringae* by using the helper plasmid pRK2013 (5), and transconjugants were verified by agarose gel electrophoresis of plasmid DNA preparations.

DNA sequencing and analysis. Sequencing reactions were performed by the dideoxynucleotide method (51) with Sequenase version 2.0 (U.S. Biochemicals, Cleveland, Ohio) and α-^{35}S-dATP (DuPont-NEN, Boston, Mass.). For DNA sequencing, a series of subclones (0.5 to 1.5 kb) was generated in pBluescript SK$^+$ (Stratagene, La Jolla, Calif.) and sequenced by using T3 and T7 primers. Sequencing gaps were filled by synthesizing internal primers (18 bp) or by generating nested deletions with the Promega Erase-a-base kit (Promega, Madison, Wis.). The locations of Tn*5* insertions in CMA-defective mutants of PG4180 were determined by sequencing the DNA flanking the transposon by using the oligonucleotide 5′GGT TCCGTTCAGGACGCTAC, which is derived from the border region of IS*50* (47). The internal *Bam*HI site in Tn*5* was utilized for generating subclones containing the mutated DNA. All oligonucleotide primers used in this study were synthesized by the Recombinant DNA/Protein Resource Core Facility at Oklahoma State University. All materials for 8% polyacrylamide gels were purchased from National Diagnostics (Atlanta, Ga.) and used as directed by the manufacturer. Sequence data were aligned and processed with the software program MacVector 4.1 (International Biotechnologies Inc., New Haven, Conn.). DNA and protein sequence homology searches of the GenBank, EMBL, PIR, and SWISSPROT databases were performed by using the University of Wisconsin Genetics Computer Group (UWGCG) programs BLASTX, FAST EMBL, and BESTFIT.

RNA isolation and primer extension reactions. Total RNA was isolated from strain PG4180 by the method of Salmeron and Staskawicz (50). The 5′ ends of the two transcriptional units were determined by primer extension using the following two oligonucleotides: 5′ATTGGCTCTTGATCACCC, the reverse complement of nucleotides 890 to 907, and 5′CTCCA GACCGCAGCAGCA, the reverse complement of nucleotides 5904 to 5921 (primers Pr1 and Pr2, respectively; see Fig. 3). RNA (20 μg) and primer (20 ng) were mixed in 5 μl of water, boiled for 60 s, and cooled on ice. The mixture was incubated with 30 U of avian myeloblastosis virus reverse transcriptase (U.S. Biochemicals) in 10 μl of reaction buffer (100 mM Tris HCl, pH 8.3; 140 mM KCl; 50 mM MgCl; 10 mM dithioerythritol; 50 μM [each] dATP, dGTP, and dTTP; and 3,000 Ci of [^{32}P]dCTP per mmol) at 42°C for 30 min. After adding 3 μl of chase solution (deoxynucleoside triphosphates at 25 mM), the mixture was incubated at 30°C for 30 min. The reaction was stopped by phenol-chloroform extraction, and the labelled cDNA was precipitated with ethanol at −20°C for 1 h. The pellet was redissolved in 5 μl of Tris-EDTA and 5 μl of stop buffer (95% formamide, 20 mM EDTA, 0.1% bromophenol blue). Primer extension products (1 μl) were boiled for 2 min and electrophoresed in 8% polyacrylamide gels. The sequenc-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference(s) or source
E. coli		
HB101		51
DH5α		51
P. syringae pv. glycinea		
PG4180	COR$^+$ CMA$^+$ CFA$^+$ CPL$^+$[a]	4, 67
PG4180.N53	COR$^+$ CMA$^+$ CFA$^+$ CPL$^+$ Km^r	59
PG4180.N10	COR$^-$ CMA$^-$ CFA$^+$ CPL$^+$ Km^r	59
PG4180.N7	COR$^-$ CMA$^-$ CFA$^+$ CPL$^+$ Km^r	59
PG4180.N13	COR$^-$ CMA$^-$ CFA$^+$ CPL$^+$ Km^r	59
PG4180.C14	COR$^-$ CMA$^-$ CFA$^+$ CPL$^+$ Km^r	4
PG4180.C0	COR$^-$ CMA$^-$ CFA$^+$ CPL$^+$ Km^r	67
PG4180.C1	COR$^-$ CMA$^-$ CFA$^+$ CPL$^+$ Km^r	4
PG4180.C14	COR$^-$ CMA$^-$ CFA$^+$ CPL$^+$ Km^r	4
p4180A	Contains COR genes	4
pBluescript SK$^+$	Ap^r; ColE1 origin; cloning vehicle	Stratagene
pRG960sd	Sm^r Sp^r; contains promoterless *uidA* with start codon and Shine-Dalgarno sequence	60
pRK2013	Km^r Mob$^+$ Tra$^+$	16
pMU2	Tc^r; contains a 6.45-kb insert derived from p4180A; required for complementation of COR$^-$ CMA$^-$ CFA$^+$ CPL$^+$ PG4180 mutants	59
pKTX30	Sm^r; contains a 3.0-kb *Xho*I insert derived from p4180A; contains part of the CMA biosynthetic gene cluster	59
pRGMU1	Sm^r Sp^r; contains a 2.9-kb *Pst*I insert derived from pMU2 in pRG960sd	This study
pRGMU2	Sm^r Sp^r; contains a 2.9-kb *Pst*I insert derived from pMU2 in pRG960sd; insert has orientation opposite to that in pRGMU1	This study
pRGMU3	Sm^r Sp^r; contains a 1.4-kb *Sal*I-*Eco*RV insert derived from pMU2 in pRG960sd (*Sal*I-*Eco*RV-*uidA*)	This study
pRGMU4	Sm^r Sp^r; contains a 1.4-kb *Sal*I-*Eco*RV insert derived from pMU2 in pRG960sd (*Eco*RV-*Sal*I-*uidA*)	This study
pRGMU5	Sm^r Sp^r; contains a 1.9-kb *Sal*I insert derived from pMU2 in pRG960sd	This study
pRGMU6	Sm^r Sp^r contains a 1.9-kb *Sal*I insert derived from pMU2 in pRG960sd; insert has orientation opposite to that in pRGMU5	This study
pRGMU7	Sm^r Sp^r; contains a blunt-ended 1.5-kb *Pst*I-*Aat*I fragment derived from pMU2 and cloned into the *Sma*I site of pRG960sd (*Pst*I-*Aat*I-*uidA*)	This study
pRGMU8	Sm^r Sp^r; contains a 1.3-kb *Eco*RV-*Aat*I insert derived from pMU2 in pRG960sd (*Eco*RV-*Aat*I-*uidA*)	This study
pRGMU9	Sm^r Sp^r; contains a 1.1-kb *Sma*I-*Aat*I insert derived from pMU2 in pRG960sd (*Sma*I-*Aat*I-*uidA*)	This study

[a] CPL$^+$, CFA and CMA coupled via amide bond formation.

ing ladders used to size the primer extension products were derived by using the oligonucleotide primers Pr1 and Pr2 in nucleotide sequencing reaction mixtures containing the appropriate DNA templates.

Construction of promoter probes and glucuronidase assay. Plasmid pRG960sd contains a promoterless glucuronidase gene (*uidA*) downstream of a multiple cloning site (60) and was used to identify promoter sequences upstream of *cmaA* and *cmaU*. Subcloned DNA fragments were ligated into pRG 960sd, orientations were determined by restriction digests, and constructs were mobilized into PG4180. Promoter activities were initially screened by spotting bacterial suspensions (A_{590} of 0.05) onto MG agar plates containing 20 μg of X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) and incubating them at 18 or 28°C for 5 to 7 days. Promoter strength was quantified by fluorometric analysis of glucuronidase activity (66) after cells were incubated in 10 ml of HSC broth at 18 or 28°C for 5 days. Fluorescence in 96-well microtiter plates was monitored with a Fluoroscan II version 4.0 microplate reader (K&M Company, Torrance, Calif.). The protein content in cell lysates was determined with the Bio-Rad (Richmond, Calif.) protein assay kit as recommended by the manufacturer.

Isolation, detection, and quantitation of CMA. Conditions for bacterial incubations, subsequent isolation of amino compounds, chemical modification procedures, and quantitative analysis of CMA have been described previously (59).

Nucleotide accession number. The reported nucleotide sequence was deposited with GenBank/EMBL under accession no. U14657.

RESULTS

Nucleotide sequence of the CMA biosynthetic region. Approximately 6.95 kb was previously shown to be required for genetic complementation of CMA$^-$ mutants and for CMA biosynthesis in another bacterium (59). The complete nucleotide sequence of the CMA biosynthetic region is shown (see Fig. 3). Three open reading frames (ORFs) sharing a common orientation for transcription were found and designated *cmaA* (2,705 bp), *cmaT* (1,364 bp), and *cmaU* (915 bp) (Fig. 2 and 3). The existence of three ORFs was validated by codon preference analyses using the entire sequenced region, the UWGCG program CODONPREFENCE in default mode, and a *Pseudomonas* codon usage table provided by J. M. Cherry (8). To validate the location of the CMA biosynthetic gene cluster, DNAs flanking the 5′ end of *cmaA* and the 3′ end of *cmaU* were also sequenced. Sequence analysis indicated the location of another ORF immediately downstream of *cmaU* and ori-

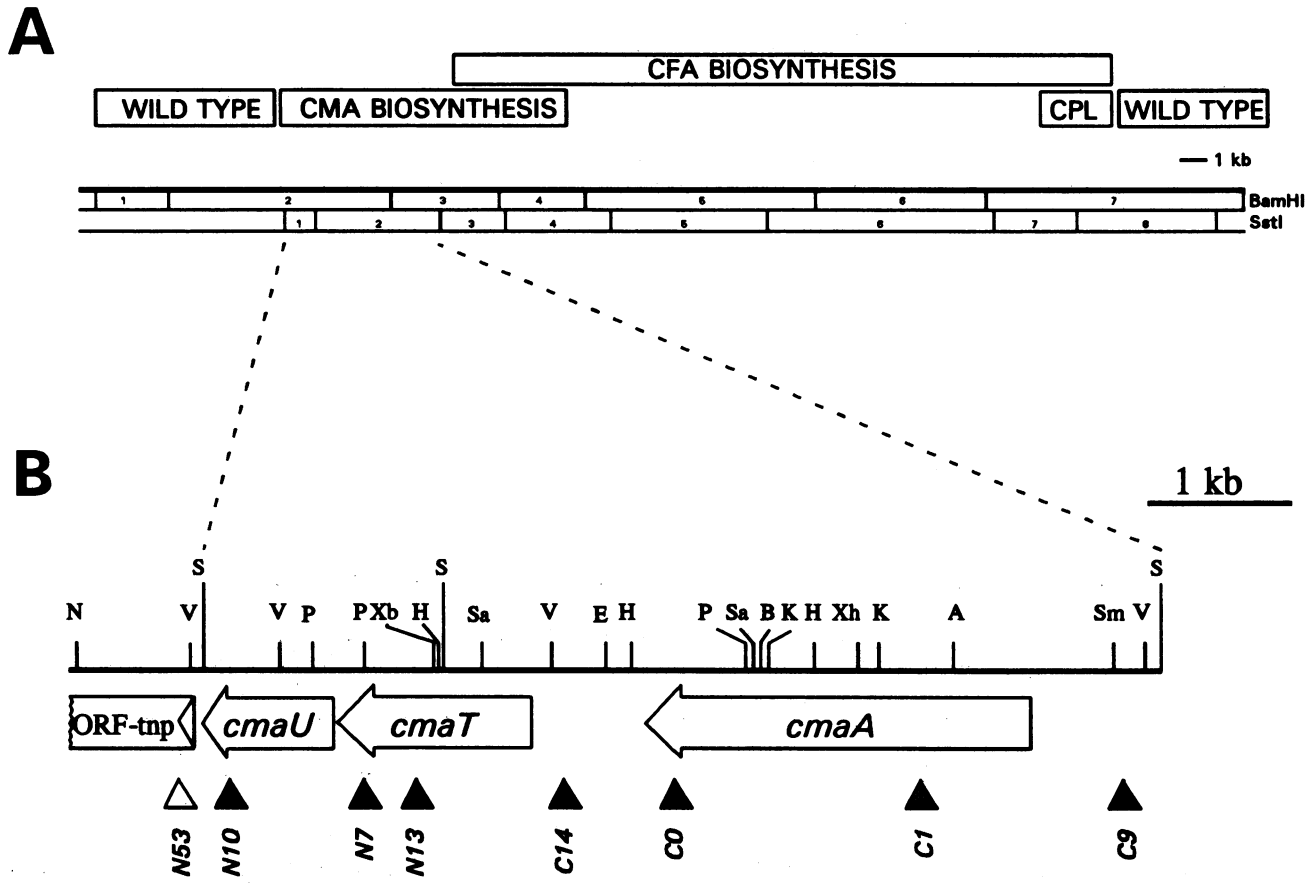

FIG. 2. (A) Partial restriction map of plasmid p4180A from *P. syringae* pv. glycinea PG4180 showing the COR biosynthetic cluster and functional regions. (B) Physical map of the CMA biosynthetic region and location of *cmaA*, *cmaT*, and *cmaU*. The direction of translation (arrows) and locations of Tn*5* insertions in CMA$^-$ (filled triangles) and CMA$^+$ (open triangle) mutants (59) are indicated. The locations of Tn*5* insertions were determined by nucleotide sequencing. CPL, amide bond formation. Restriction enzymes used: A, *Aat*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nde*I; P, *Pst*I; S, *Sst*I; Sa, *Sal*I; Sm, *Sma*I; V, *Eco*RV; Xb, *Xba*I; Xh, *Xho*I.

ented in the same direction (Fig. 2 and 3). The sequence of this ORF, designated ORF-*tnp*, shared striking DNA and amino acid sequence homology with a variety of previously characterized transposases from gram-negative bacteria (58), indicating an unlikely role in CMA biosynthesis. No distinct ORF was detected in the region upstream of *cmaA* and extending to the *Sst*I site.

Location of Tn*5* insertions in CMA$^-$ PG4180 mutants. Nucleotide sequencing of subclones containing Tn*5* insertions was used to determine the exact locations of the insertions in selected mutants (Fig. 2 and 3). The Tn*5* insertion in PG 4180.N53, a CMA$^+$ mutant (59), established a left border for the CMA biosynthetic cluster and was located in ORF-*tnp* 185 bp downstream of the *cmaU* translational stop (Fig. 2). All other Tn*5* mutants localized in the present study resulted in the CMA$^-$ phenotype. Mutant PG4180.N10 carried a Tn*5* insertion in *cmaU*, whereas mutants PG4180.N7 and PG4180.N13 contained Tn*5* insertions in *cmaT*. The Tn*5* insertions in PG4180.C1 and PG4180.C0 were located in *cmaA*. The mutation in PG4180.C14 occupied an intergenic position between *cmaT* and *cmaA*, whereas PG4180.C9 was located upstream of the *cmaA* translational start site (Fig. 2 and 3).

Sequence homology analysis. To identify enzymes with homology to the proteins encoded by *cmaA*, *cmaT*, and *cmaU*, nucleotide sequence data were compared with GenBank, EMBL, SWISSPROT, and PIR database entries by using the FASTEMBL and BLASTX programs. A region within CmaA encompassing 500 amino acid residues showed significant homology to various enzymes which activate amino acids by adenylation and bind them as thioesters (Table 2; Fig. 4A). Homology within CmaA to these enzymes corresponded to the functional domains of 500 to 600 amino acids which are conserved in the family of amino acid-activating enzymes (1, 32). CmaA showed the highest degree of homology to functional domains in surfactin synthetase (10) and gramicidin synthetase GrsB (57), which specifically activate the branched-chain amino acids valine and leucine, respectively. Within CmaA, we observed the conserved spatial arrangement of six core sequences previously identified in enzymes which activate amino acids by adenylation (18, 32, 57) (Fig. 4). Core sequence 2, SGTTGKPKG, and core sequence 4, YRTGD (Fig. 4B), have presumed roles in ATP binding and hydrolysis, respectively (57), and were more strongly conserved in CmaA than core sequences 1, 3, 5, and 6. Core sequence 6, LGGHSL (32), was previously shown to be the site for thioester formation in various nonribosomal peptide synthetases; the serine residue within this motif was reported to function as a phosphopantetheine binding site (52). Interestingly, CmaA contains a serine residue in the spatial arrangement reported for core sequence 6, suggesting the possible involvement of 4′-phos-

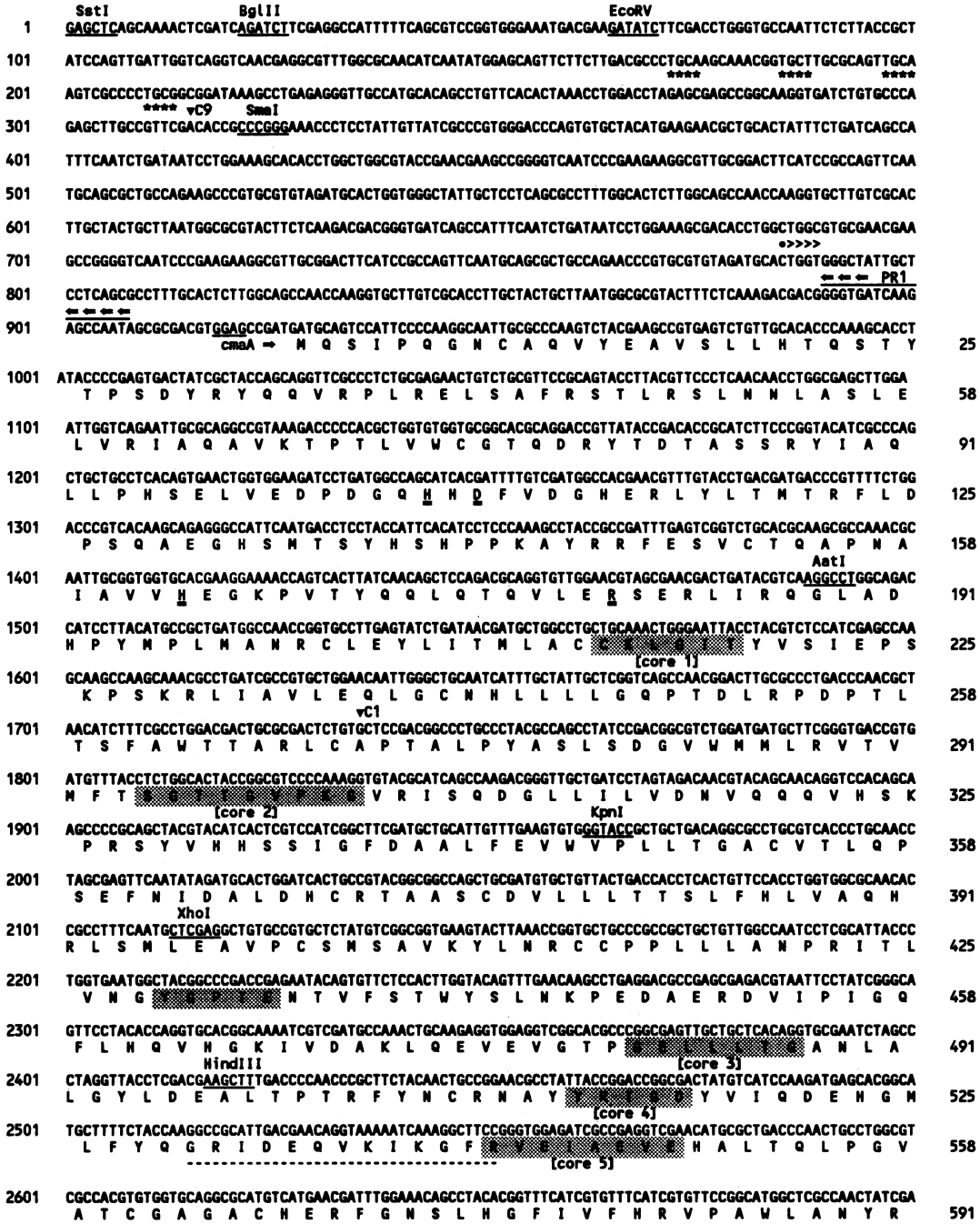

FIG. 3. Nucleotide sequence of a 6.95-kb region required for CMA biosynthesis containing genes *cmaA*, *cmaT*, and *cmaU* with deduced amino acid sequences indicated below the nucleotides. Nucleotides and amino acid residues are numbered on the left and right, respectively. Restriction sites and putative Shine-Dalgarno sequences (underlined) are indicated. The locations of Pr1 and Pr2, the oligonucleotide primers used to determine the transcriptional start sites, are indicated. Transcriptional start sites for TUC1 and TUC2 (solid and open circles, respectively, above the appropriate nucleotide), the orientation of transcription (arrows), locations of Tn*5* insertions described in the text (inverted triangles), and the locations of six core sequences found in amino acid-activating enzymes (32) (shaded areas in CmaA) are shown. The location of a potential substrate-binding region (YGPTE) (10) in CmaA (shaded), the location within CmaA of a conserved sequence previously implicated in aminoacyl adenylation (56) (underlined), the locations of two core sequences common to thioesterases (11, 65) (shaded areas in CmaT), the putative iron-binding motif of nonheme iron(II) enzymes (6) (double-underlined amino acids in CmaA), and NtrC-like activator binding sites upstream of *cmaA* (asterisks) are indicated. ORF-*tnp* is a transposase-like ORF flanking the left side of the CMA biosynthetic region.

phopantetheine in CMA biosynthesis. A conserved cysteine residue in core sequence 3 (Fig. 4B) was previously reported in domains of nonribosomal peptide synthetases (57) but was not detected in core sequence 3 of CmaA. The amino acid sequence YGPTE within CmaA (residues 429 to 433; Fig. 3) is identical to a conserved motif contained in the domains of nonribosomal peptide synthetases specific for the activation of the branched-chain amino acids L-valine, L-leucine, and D-

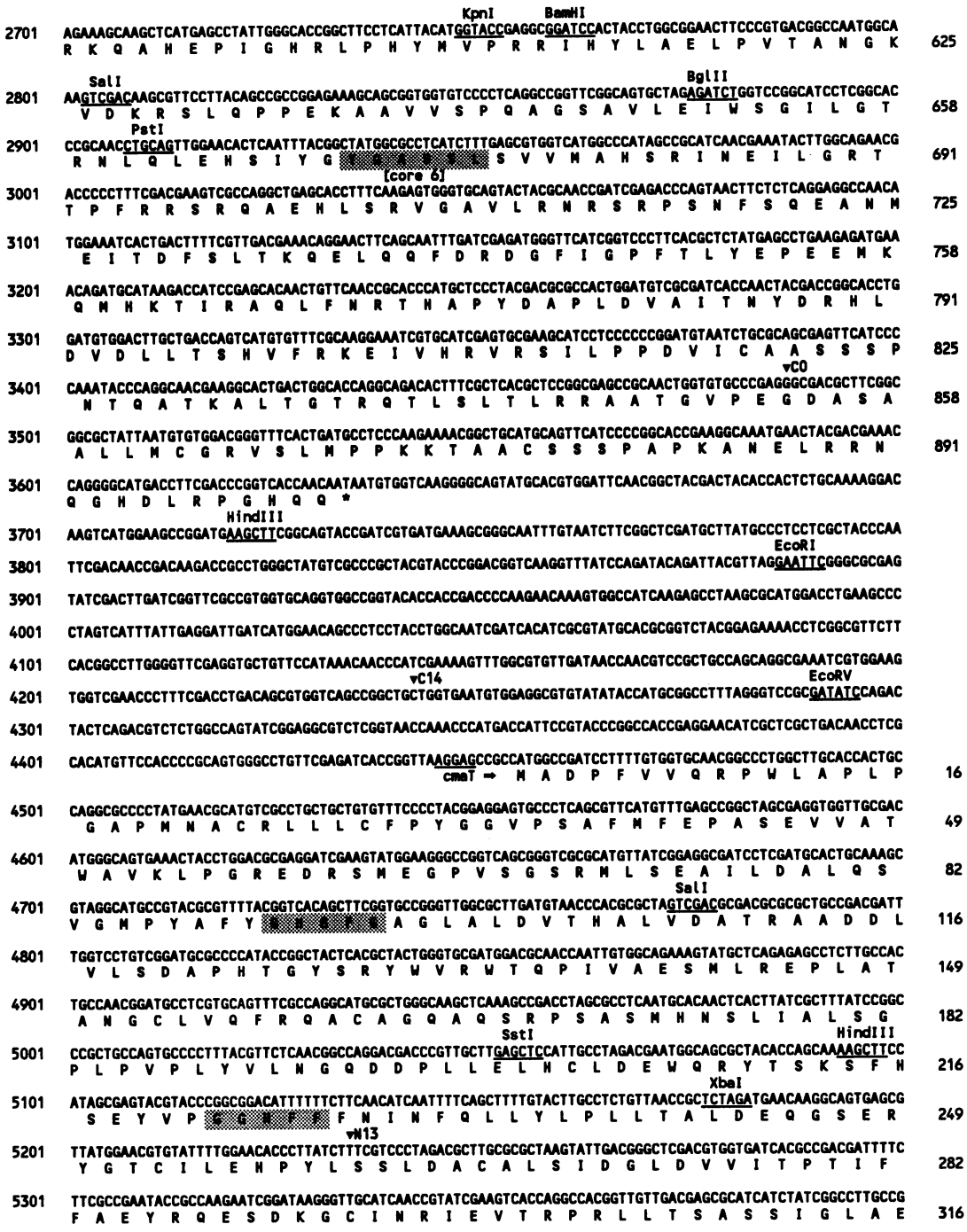

FIG. 3—*Continued.*

leucine (10). Furthermore, CmaA contained a sequence, GRI DEQVKIKGFR (Fig. 3, CmaA residues 530 to 542), which shows similarity to a conserved motif, GRXDXQXKXXGXR, in the proline-activating domain of GrsB; this motif was found essential for the activity of enzymes which adenylate amino acids (56).

In the N-terminal region of CmaA, the spatial arrangement of amino acid residues His-106, Asp-108, His-163, and Arg-180 (Fig. 3) resembled the ferrous active site of various nonheme iron(II) proteins, including hydroxylases, oxidases, and iso penicillin N synthetases (6, 33, 39). The arrangement of these amino acids was recently shown to be essential for the oxidative cyclization activity of isopenicillin N synthetase (9). This possible activity within CmaA was further investigated by subjecting the amino acid sequence to hydropathic index calculations by using PEPPLOT (UWGCG software) and the method of Kyte and Doolittle (27). Although the central and C-terminal regions of CmaA showed a uniform distribution of hydrophilic and hydrophobic regions, the N terminus contained more hydrophilic zones (Fig. 5). The histidine, aspar-

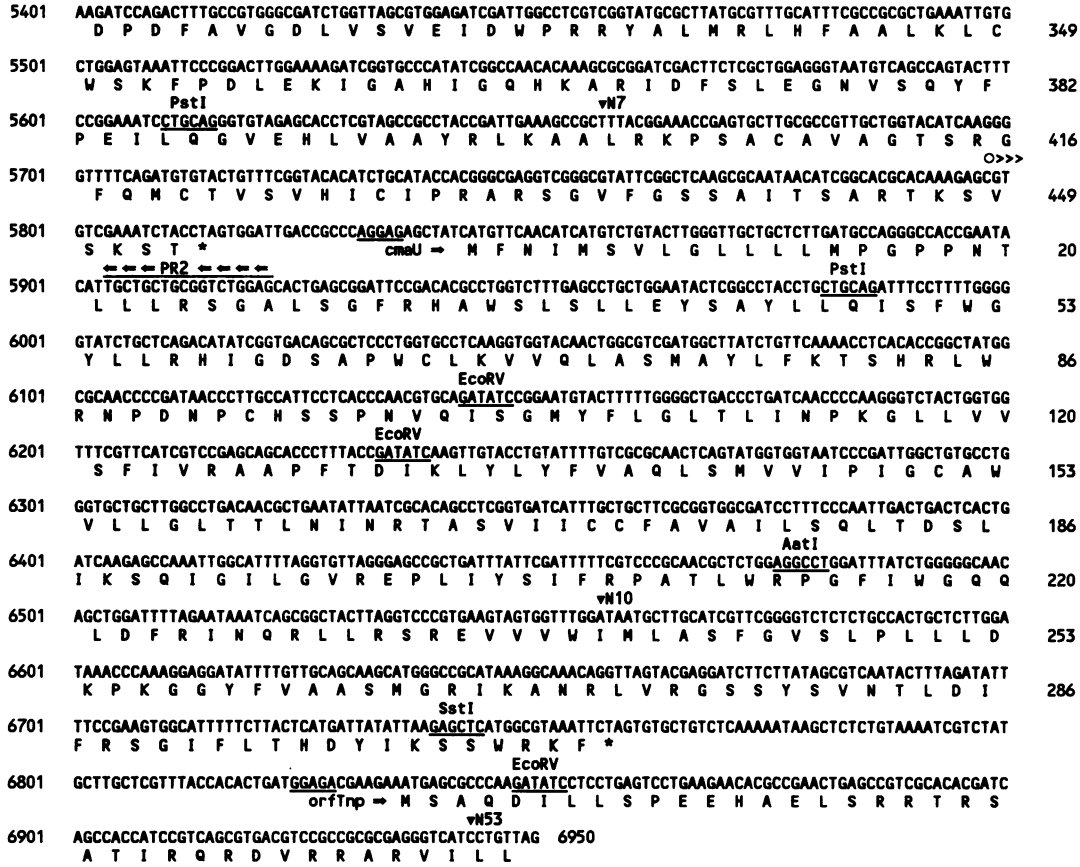

FIGURE 3—*Continued.*

tate, and arginine residues mentioned above were positioned within these hydrophilic zones, suggesting a location on the protein surface or within hydrophilic pockets, thereby allowing access to the water and ions needed for proper formation of the iron binding site (6) (Fig. 5).

The deduced protein sequence encoded by *cmaT* shared striking sequence homology with thioesterases of procaryotic and eucaryotic origins (Table 3). Four conserved regions located between amino acid residues 10 and 240 of CmaT were also found in other thioesterases (Fig. 6A). GHSFG and GGHFF, two conserved motifs previously shown to be essential for thioesterase function, were also present in CmaT in the conserved spatial arrangement (Fig. 6B) (11, 54, 65). The C terminus of CmaT (amino acid residues 250 to 454) showed no similarities to any existing database entries.

No significant similarities between the nucleotide and deduced amino acid sequences of *cmaU* and current entries in the DNA and protein databases were found. Calculation of hydropathy plots for CmaU by the method described above revealed a hydrophobic region of approximately 100 amino acids, suggesting a possible membrane association (data not shown).

Transcriptional organization of the CMA biosynthetic region. Previous genetic complementation experiments using CMA$^-$ mutants indicated the existence of a large transcriptional unit spanning *Sst*I fragment 2 and part of *Sst*I fragment 1 (59) (Fig. 2). Data obtained in the same study suggested that a second, smaller transcript originated in *Sst*I fragment 1 and extended leftward. To test these hypotheses, selected DNA fragments were subcloned into plasmid

TABLE 2. Proteins with sequence homology to CmaA

Gene	Protein (portion and function)	Organism	Reference	% Identity[a]	% Similarity[a]
acvA	AcvA synthase (domain B)	*Aspergillus nidulans*	30	28.0	50.5
grsB	Gramicidin S synthetase B (domain 4; leucine activating)	*Bacillus brevis*	57	27.5	50.4
srfA	Surfactin synthetase (domain 4; valine activating)	*Bacillus subtilis*	17	27.2	49.7
dae	D-Alanine-activating enzyme	*Lactobacillus casei*	22	26.6	49.5
tycA	Tyrocidine synthase I	*Bacillus brevis*	62	26.1	49.6
pvdD	Pyoverdine synthetase D	*Pseudomonas aeruginosa*	28	27.4	47.8
entF	Enterobactin synthetase F	*Escherichia coli*	48	25.1	45.5
acsA	Acetyl coenzyme A synthetase	*Bacillus subtilis*	20	19.4	40.3

[a] Determined by using BESTFIT analysis and the entire sequence of CmaA.

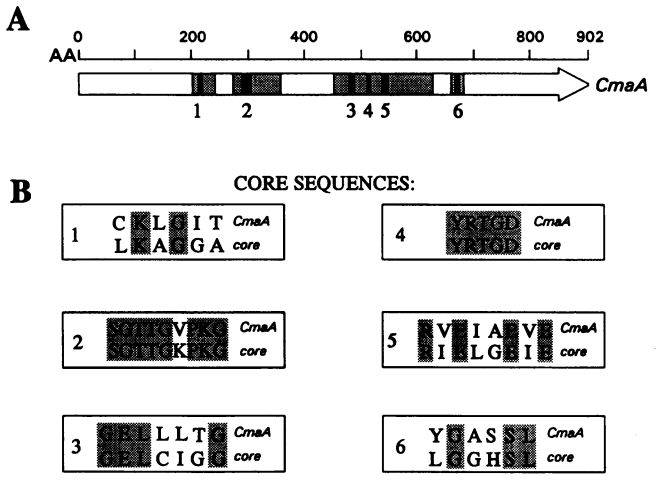

FIG. 4. (A) Organization of CmaA. Regions with homology to enzymes which activate amino acids by adenylation (shaded bars) and the locations of six core sequences in CmaA which are conserved in adenylate-forming enzymes that activate amino acids (black bars) are indicated. AA, amino acid. (B) Alignment of amino acids in CmaA with six core sequences which are conserved in enzymes that activate amino acids by adenylation and bind them as thioesters (32).

pRG960sd, which contains a promoterless glucuronidase gene. On the basis of previous information regarding transcriptional units (59) and nucleotide sequence data, three restriction fragments were subcloned in both orientations into pRG960sd, generating the subclones pRGMU1, pRGMU2, pRGMU3, pRGMU4, pRGMU5, and pRGMU6 (Fig. 7). The six promoter probe plasmids were conjugated into PG4180, and transconjugants were assayed for glucuronidase activity after incubation in HSC at 18 or 28°C for 5 days (Table 4, experiment 1). These two temperatures were chosen because prior results indicated that temperature has a significant effect on COR biosynthesis (40). Plasmids pRGMU1 and pRGMU3 showed strong promoter activity at 18°C and substantially less activity at 28°C (Table 4, experiment 1). The four remaining constructs (pRGMU2, pRGMU4, pRGMU5, and pRGMU6) did not promote transcription of the glucuronidase gene, regardless of temperature. Therefore, temperature-dependent transcription of CMA biosynthesis genes is likely to proceed from two independent promoter regions, PRO1 (subcloned on pRGMU1) and PRO2 (subcloned on pRGMU3). DNA sequences within these two promoter regions participate in the activation of two transcriptional units, TUC1 and TUC2, respectively, which share a common orientation (Fig. 7). Nucleotide sequence data indicate that *cmaA* and *cmaT* are cotranscribed on TUC1, whereas *cmaU* is situated on the second transcript, TUC2.

Determination of transcriptional start sites. Primer extension analyses were performed to define the transcriptional start sites for TUC1 and TUC2. For transcript TUC1, primer Pr1 (Fig. 3) was used for primer extension and generation of a sequencing ladder. A single extension product (Fig. 8) was detected when total RNA of strain PG4180 was used as a template, indicating an mRNA start site 133 nucleotides upstream of the predicted Shine-Dalgarno sequence and 143 nucleotides upstream of the translational start codon for *cmaA* (Fig. 3). The start site for TUC2 was defined by using primer Pr2 (Fig. 3); primer extension (data not shown) revealed a single product mapping 33 nucleotides upstream of the putative Shine-Dalgarno sequence and 45 nucleotides upstream of the translational start codon of *cmaU* (Fig. 3). These results confirm the initiation sites for the two transcripts involved in CMA biosynthesis. Careful examination of the nucleotide sequences upstream of the two transcriptional start sites did not reveal any significant homologies to known promoter sequences.

Upstream activating sequences in promoter region PRO1. To further define the promoter region designated PRO1, several additional subclones were created in pRG960sd. These new constructs, pRGMU7, pRGMU8, and pRGMU9 (Fig. 7), were conjugated into PG4180, and promoter activities were measured after growth in HSC at 18 and 28°C for 5 days (Table 4, experiment 2). Strains containing pRGMU7 (1.5-kb insert) and pRGMU8 (1.3-kb insert) produced significantly more glucuronidase than pRGMU9, which contained a 1.167-kb *Sma*I-*Aat*I fragment. These results indicate an essential role in transcriptional activation for the DNA sequence located between the *Eco*RV and *Sma*I sites (Fig. 3 and 7). A search for homologies to known transcription factor or enhancer binding sites in this region revealed the sequences TGCA-9 bp-TGCT and TGCA-9 bp-TGCG, which were 613 and 588 bp upstream of the TUC1 transcriptional start site, respectively (Fig. 3). Both motifs resemble the consensus sequence of the NtrC binding site in gram-negative bacteria, TGCA-9 bp-TGCA (14), and are absent in pRGMU9. These two regions could

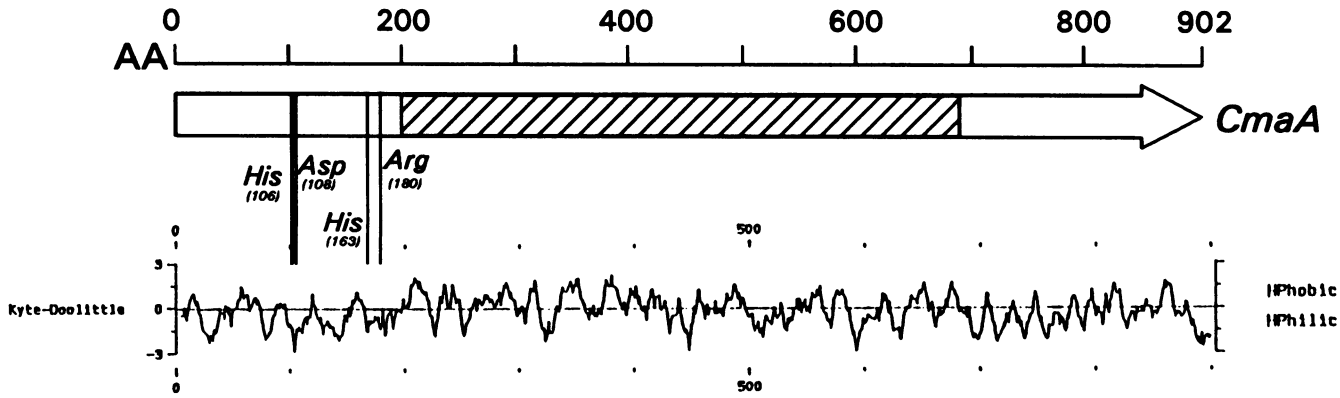

FIG. 5. Hydropathy plot of CmaA made by using the program PEPPLOT (UWGCG) and the method of Kyte and Doolittle (27). The direction of translation (arrow), the region of CmaA with a possible role in amino acid activation (hatched region), and the locations of individual amino acids in hydrophilic regions of CmaA which form a putative iron binding site (6) (vertical lines) are shown. AA, amino acid.

function as putative binding sites for a transcriptional activator similar to NtrC.

Effect of temperature on CMA biosynthesis. Strain PG4180 produces maximal amounts of COR at 18°C but significantly less at 24°C and negligible amounts at 30°C (40). In the present study, PG4180 was incubated at 18 and 28°C in 500 ml of HSC for 5 days and CMA was quantified as described previously (59). PG4180 produced 1.2 to 1.9 mg of CMA per liter at 18°C but virtually no CMA when incubated at 28°C (three replicates per temperature). These data were consistent with those obtained for transcriptional activity in response to temperature as described above.

DISCUSSION

Nucleotide sequence analysis of the CMA biosynthetic gene cluster revealed three genes, *cmaA*, *cmaT*, and *cmaU*, sharing a common orientation on two different transcripts. Physical mapping of Tn*5* insertion sites in CMA^- mutants indicated their location within the three genes or at intergenic positions. The Tn*5* insertion in CMA^+ strain PG4180.N53 was located in ORF-*tnp* and thus delimited the left border of the CMA gene cluster (Fig. 2 and 3). This confirmed previous findings regarding the dimensions of the CMA biosynthetic region (59). The intergenic location of Tn*5* insertions C9 and C14 probably interfered with the transcription of downstream genes (*cmaA* and *cmaT*, respectively) because of the polar effects of Tn*5* (46). Therefore, our data indicate that *cmaA* and *cmaT* are organized on a 5-kb transcript, whereas the size of the transcript containing *cmaU* is unknown.

Sequence analyses performed in the present study revealed valuable insights into the possible enzymatic mechanisms that lead to CMA formation. The deduced protein product of *cmaA* showed significant homology to functional domains of nonribosomal peptide synthetases (10, 32, 57, 61). Common to multifunctional peptide synthetases are the adenylation of amino acid substrates and formation of a thioester bond between enzyme and substrate. A functional domain usually contains highly conserved core sequences of 3 to 10 amino acid residues which are thought to be essential for amino acid activation and thioester formation (32). Three of these core sequences were highly conserved in CmaA: the ATP binding sequence (core 2), an ATP hydrolysis site (core 4), and the core sequence containing a serine (core 6) previously shown to be the 4′-phosphopantetheine binding site (52). The latter motif has been reported only for amino acid-adenylating enzymes that utilize the thiotemplate mechanism and not for similar enzymes which activate carboxylic acids (57). The importance of specific amino acid residues within several core sequences was recently demonstrated by site-directed mutagenesis of *tycA*, the gene encoding tyrocidine synthetase I, a multifunctional enzyme which both activates and epimerizes L-phenylalanine (18). Furthermore, Tokita and coworkers (56) recently described a conserved glycine residue within a motif common to all antibiotic peptide synthetases; this was shown to be essential for aminoacyl adenylation in the proline-activating domain of gramicidin S synthetase. Interestingly, this motif and a conserved glycine residue were found in CmaA in the present study but were not detected in adenylate-forming enzymes which specifically activate carboxylic acids (56).

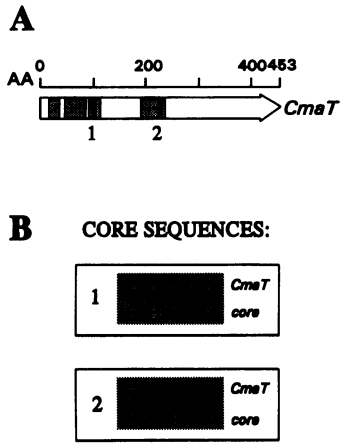

FIG. 6. (A) Organization of conserved sequences in CmaT. Regions with homology to known thioesterases (shaded bars) and the locations of motifs which are highly conserved among thioesterases (black bars) are shown. AA, amino acid. (B) Sequences found in CmaT and conserved motifs present in several thioesterases (11, 65). The two motifs and their spatial arrangement are believed to be essential for thioesterase function.

Conservation of three essential core sequences (sequences 2, 4, and 6) in CmaA suggests that a substrate amino acid could be adenylated and bound to CmaA as a thioester utilizing the cofactor 4′-phosphopantetheine. This cofactor of multifunctional peptide synthetases is assumed to facilitate transport of amino acid substrates between domains to facilitate amide bond formation between two substrate amino acids or between the progressing peptide and a new amino acid (32). However, we suspect that a putative 4′-phosphopantetheine arm attached to CmaA might function to transport an intermediate in the CMA biosynthetic pathway or CMA itself to the putative thioesterase encoded by *cmaT*. Subsequently, CmaT might function to release an intermediate or the final product (CMA) from CmaA. Support for these hypotheses results primarily from the fact that CMA should not require activation prior to ligation to CFA; instead, existing data suggest that the carboxyl group of CFA is likely to be activated prior to the ligation of CFA and CMA (29). Similar functions for the cofactor 4′-phosphopantetheine have been discussed for δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase (30). Thus, the central region of CmaA could represent a functional domain that activates and binds a specific amino acid. Although the nature of the substrate amino acid remains to be determined, it is tempting to speculate that the substrate might be either

TABLE 3. Proteins with sequence homology to CmaT

Gene	Protein (function)	Organism	Reference	% Identity[a]	% Similarity[a]
grsT	Gramicidin S synthase T	*Bacillus brevis*	26	26.8	51.6
ORF5	Thioesterase (erythromycin biosynthesis)	*Saccharopolyspora erythrea*	21	26.8	49.6
ORF-1	Thioesterase (candicidin biosynthesis)	*Streptomyces griseus*	11	28.4	49.3
ORF1	Thioesterase (3′ of *bah*) (bialaphos biosynthesis)	*Streptomyces hygroscopicus*	45	28.8	48.7
sasT	Thioesterase (*S*-acyl fatty acid synthetase)	*Anas platyrhynchos*	44	23.6	48.9

[a] Determined by using BESTFIT analysis and the entire sequence of CmaT.

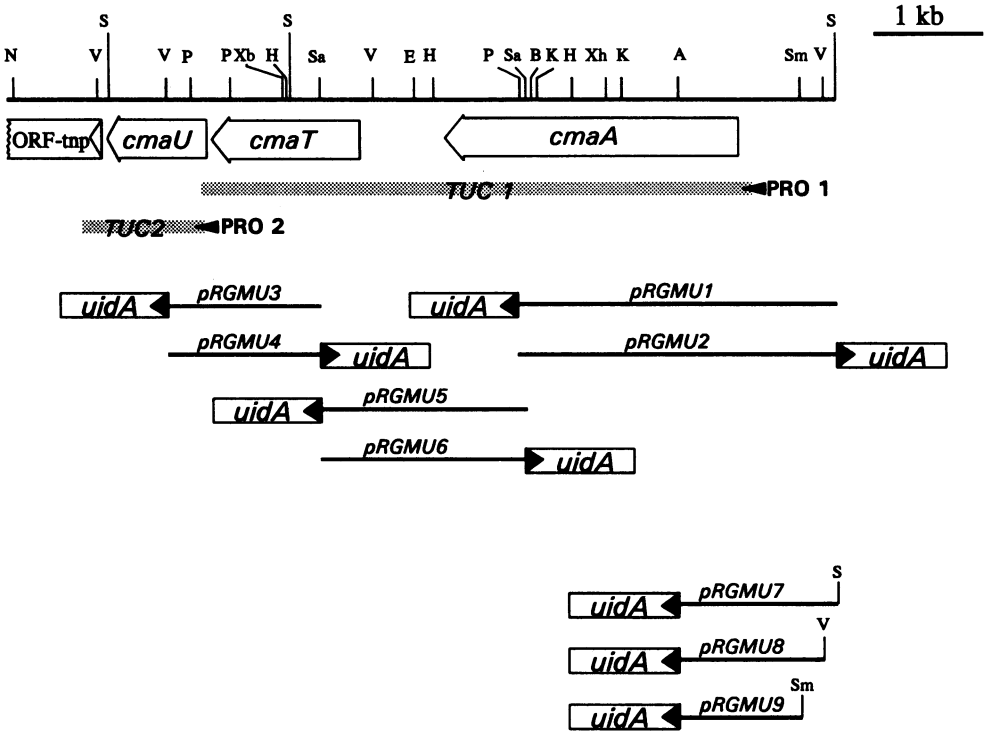

FIG. 7. Promoter analysis of the CMA biosynthetic gene cluster. The physical map and organization of CMA biosynthetic genes are outlined at the top. The locations of promoter probe constructs used in this study and their transcriptional orientations with respect to *uidA*, a promoterless glucuronidase gene, are shown (open rectangles and adjacent arrows). Promoter activities are presented in Table 4. TUC1 and TUC2, transcriptional units 1 and 2; PRO1 and PRO2, promoter regions 1 and 2. Abbreviations for restriction enzymes are defined in the legend to Fig. 2.

L-isoleucine or L-alloisoleucine, one of the two precursors for CMA (42). Furthermore, it is significant that CmaA is most similar to peptide synthetase domains which bind and activate the branched-chain amino acids valine and leucine (10).

The N-terminal region of CmaA contains a pattern of solitary amino acid residues which was recently found conserved in several nonheme iron(II) enzymes (6, 33, 39). Our hydropathy analysis indicated that these amino acid residues form a hydrophilic environment believed to be required for iron binding (6). Although the existence of an iron-binding motif in CmaA and its possible involvement in oxidative cyclization remain speculative, Parry and coworkers (42) previously predicted an oxidative cyclization reaction for CMA biosynthesis utilizing an active ferrous site. In the same study, they proposed that CMA synthesis might resemble the oxidative cyclization reactions utilized in β-lactam ring formation. Interestingly, iron was previously shown to significantly increase COR yields when amended to PG4180 cultures in vitro (40), possibly because iron is required for CMA biosynthesis.

Biochemical investigations have also indicated the isomerization of L-isoleucine to L-alloisoleucine as an essential reaction in CMA biosynthesis (41, 42). Known mechanisms for the conversion of L-isoleucine to L-alloisoleucine are thought to employ pyridoxal 5′-phosphate (PLP)-dependent enzymes (31). Two possible reactions would be characterized either by (i) an intermediacy of PLP bound to L-isoleucine followed by nonenzymatic tautomerization and release of L-alloisoleucine or by (ii) deamination of L-isoleucine to form 2-keto-(3*S*)-methylvaleric acid followed by keto-enol tautomerization, subsequent amination, and release of L-alloisoleucine (31). The cofactor PLP is known to play an important role in a variety of amino acid-modifying enzymes and is bound to them via a conserved lysine residue (2, 34). Although our efforts to identify a putative PLP binding site in the three CMA genes have not yet succeeded, such a site may not be readily detected by conventional homology searches.

Like COR biosynthesis (40), CMA biosynthesis is also temperature dependent, and reporter gene studies further

TABLE 4. Glucuronidase activity in response to temperature

Expt and PG4180 transconjugant[a]	Mean GUS activity[b] (U/mg of protein) at:	
	18°C	28°C
1		
pRG960sd	1.3	0.9
pRGMU1	412.7	99.5
pRGMU2	1.1	0.2
pRGMU3	304.6	135.7
pRGMU4	0.2	0.9
pRGMU5	0.5	1.2
pRGMU6	1.2	0.6
2		
pRG960sd	0.4	1.1
pRGMU1	396.8	86.7
pRGMU7	402.1	112.6
pRGMU8	360.0	101.2
pRGMU9	42.5	56.8

[a] Locations and sizes of inserts are indicated in Fig. 7 and Table 1.
[b] Average of two experiments with three replicates per temperature. One unit equals 1 pmol of methylumbelliferone formed per min. GUS, glucuronidase.

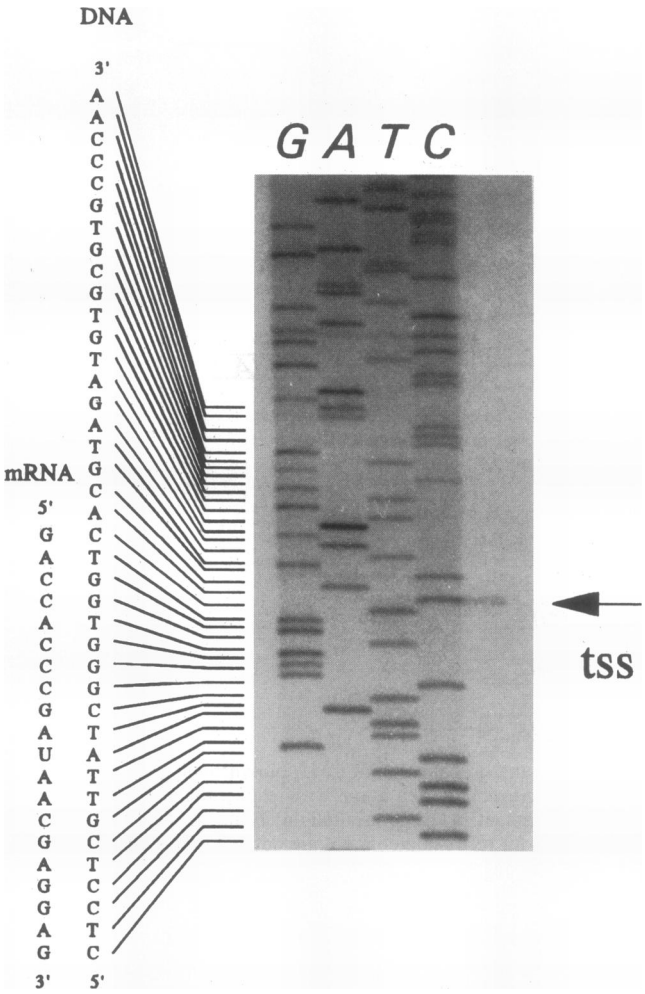

FIG. 8. Mapping of the TUC1 transcriptional start site (tss) by primer extension analysis and use of oligonucleotide Pr1. DNA and corresponding mRNA sequences are shown. The primer extension product is indicated (arrow).

indicated regulation of CMA biosynthesis at the transcriptional level by temperature. Although transcription of TUC1 was shown to be temperature dependent, further studies are needed to clarify its relatedness to the obvious effects of temperature on COR and CMA biosynthesis. Deletion analysis for one of the promoter regions revealed putative upstream activating sequences with homologies to NtrC binding sites (7, 14, 43). Binding sites of this type are usually found associated with σ^{54}-dependent promoter sequences (43, 55). However, we did not find a conserved -24(GG)/-12(GC) motif upstream of the transcriptional start site as would be presumed for a σ^{54}-dependent promoter (35, 55). Therefore, transcriptional regulation of CMA genes by an activator or enhancer system remains highly speculative. Interestingly, the temperature dependence of PRO1 was abolished when putative upstream activation sequences were removed, suggesting that temperature effects on transcription might occur indirectly, perhaps through putative regulatory genes recently identified within the COR biosynthetic cluster (58). Nucleotide sequencing of the region presumed to be involved in regulation of the COR biosynthetic cluster is under way (58) and should provide new insights into the temperature dependence of COR and CMA biosynthesis in *P. syringae*.

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REFERENCES

1. **Aharonowitz, Y., J. Bergmeyer, J. M. Cantoral, G. Cohen, A. L. Demain, U. Fink, J. Kinghorn, H. Kleinkauf, A. MacCabe, H. Palissa, E. Pfeifer, T. Schwecke, H. van Liempt, H. von Döhren, S. Wolfe, and J. Zhang.** 1992. δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase, the multienzyme integrating the four primary reactions in β-lactam biosynthesis, as a model peptide synthetase. Bio/Technology **11**:807–811.
2. **Alexander, F. W., E. Sandmeier, P. K. Mehta, and P. Christen.** 1994. Evolutionary relationships among pyridoxal-5′-phosphate-dependent enzymes. Eur. J. Biochem. **219**:953–960.
3. **Bender, C. L., and D. A. Cooksey.** 1986. Indigenous plasmids in *Pseudomonas syringae* pv. tomato: conjugative transfer and role in copper resistance. J. Bacteriol. **165**:534–541.
4. **Bender, C., H. Liyanage, D. Palmer, M. Ullrich, S. Young, and R. Mitchell.** 1993. Characterization of the genes controlling biosynthesis of the polyketide phytotoxin coronatine including conjugation between coronafacic and coronamic acid. Gene **133**:31–38.
5. **Bender, C. L., S. A. Young, and R. E. Mitchell.** 1991. Conservation of plasmid DNA sequences in coronatine-producing pathovars of *Pseudomonas syringae*. Appl. Environ. Microbiol. **57**:993–999.
6. **Britsch, L., J. Dedio, H. Saedler, and G. Forkmann.** 1993. Molecular characterization of flavone 3β-hydroxylases. Consensus sequence, comparison with relevant enzymes and the role of conserved histidine residues. Eur. J. Biochem. **217**:745–754.
7. **Calogero, S., R. Gardan, P. Glaser, J. Schweizer, G. Rapoport, and M. Debarbouille.** 1994. RocR, a novel regulatory protein controlling arginine utilization in *Bacillus subtilis*, belongs to the NtrC/NifA family of transcriptional activators. J. Bacteriol. **176**:1234–1241.
8. **Cherry, J. M. (Stanford University).** 1994. Personal communication.
9. **Cohen, G., O. Landman, I. Borovok, D. Arad, R. Kreisberg, and Y. Aharonowitz.** 1994. Genetic studies of the ferrous active site of isopenicillin N synthase, abstr. P-274, p. 188. Abstr. 7th Int. Symp. Genet. Ind. Microorg. 1994.
10. **Cosmina, P., F. Rodriguez, F. de Ferra, G. Grandi, M. Perego, G. Venema, and D. van Sinderen.** Sequence and analysis of the genetic locus responsible for surfactin synthesis in *Bacillus subtilis*. Mol. Microbiol. **8**:821–831.
11. **Criado, L. M., J. F. Martin, and J. A. Gill.** 1993. The *pab* gene of *Streptomyces griseus*, encoding *p*-aminobenzoic acid synthase, is located between genes possibly involved in candicidin biosynthesis. Gene **126**:135–139.
12. **Crosa, J. H., and S. Falkow.** 1981. Plasmids, p. 266–282. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
13. **Ferguson, I. B., and R. E. Mitchell.** 1985. Stimulation of ethylene production in bean leaf discs by the pseudomonad phytotoxin coronatine. Plant Physiol. **77**:969–973.
14. **Ferro-Luzzi Ames, G., and K. Nikaido.** 1985. Nitrogen regulation in *Salmonella typhimurium*. Identification of an *ntrC* protein-binding site and definition of a consensus binding sequence. EMBO J. **4**:539–547.
15. **Feys, B. J. F., C. E. Benedetti, C. N. Penfold, and J. G. Turner.** 1994. *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell **6**:751–759.
16. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc. Natl. Acad. Sci. USA **79**: 1648–1652.
17. **Fuma, S., Y. Fujishima, N. Corbell, C. D'Souza, M. M. Nakano, P. Zuber, and K. Yamane.** 1993. Nucleotide sequence of 5′ portion of *srfA* that contains the region required for competence establish-

ment in *Bacillus subtilis*. Nucleic Acids Res. **21**:93–97.
18. **Gocht, M., and M. A. Marahiel.** 1994. Analysis of core sequences in the D-Phe-activating domain of the multifunctional peptide synthetase TycA by site-directed mutagenesis. J. Bacteriol. **176**: 2654–2662.
19. **Greulich, F., T. Yoshihara, H. Toshima, and A. Ichihara.** 1993. Coronatine, a bacterial toxin, affects the regulation of ethylene biosynthesis in the same way as methyl (+)-epijasmonate, abstr. 4154, p. 388. Abstr. XVth Int. Bot. Congr. 1993.
20. **Grundy, F. J., D. A. Waters, T. Y. Takova, and T. M. Henkin.** 1993. Identification of genes involved in utilization of acetate and acetoin in *Bacillus subtilis*. Mol. Microbiol. **10**:259–271.
21. **Haydock, S. F., J. A. Dowson, N. Dhillon, G. A. Roberts, J. Cortes, and P. F. Leadlay.** 1991. Cloning and sequence analysis of genes involved in erythromycin biosynthesis in *Saccharopolyspora erythrea*: sequence similarities between EryG and a family of S-adenosylmethionine-dependent methyltransferases. Mol. Gen. Genet. **230**:120–128.
22. **Heaton, M. P., and F. C. Neuhaus.** 1992. Biosynthesis of D-alanyllipoteichoic acid: cloning, nucleotide sequence, and expression of the *Lactobacillus casei* gene for the D-alanine-activating enzyme. J. Bacteriol. **174**:4707–4717.
23. **Keane, P. J., A. Kerr, and P. B. New.** 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. Sci. **23**:585–595.
24. **Kenyon, J. S., and J. G. Turner.** 1992. The stimulation of ethylene synthesis in *Nicotiana tabacum* leaves by the phytotoxin coronatine. Plant Physiol. **100**:219–224.
25. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. **44**:301–307.
26. **Krätschmar, J., M. Krause, and M. A. Marahiel.** 1989. Gramicidin S biosynthesis operon containing the structural genes *grsA* and *grsB* has an open reading frame encoding a protein homologous to fatty acid thioesterases. J. Bacteriol. **171**:5422–5429.
27. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
28. **Lamont, I.** GenBank accession no. U07359.
29. **Liyanage, H., C. N. Penfold, J. G. Turner, and C. L. Bender.** Sequence, expression and transcriptional analysis of the coronafacate ligase-encoding gene required for coronatine biosynthesis by *Pseudomonas syringae*. Gene, in press.
30. **MacCabe, A. P., H. van Liempt, H. Palissa, S. E. Unkles, M. B. R. Riach, E. Pfeifer, H. von Döhren, and J. R. Kinghorn.** 1991. δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase from *Aspergillus nidulans*. J. Biol. Chem. **266**:12646–12654.
31. **Mamer, O. A., and M. L. J. Reimer.** 1992. On the mechanisms of the formation of L-alloisoleucine and the 2-hydroxy-3-methylvaleric acid stereoisomers from L-isoleucine in maple syrup urine disease patients and in normal humans. J. Biol. Chem. **267**:22141–22147.
32. **Marahiel, M. A.** 1992. Multidomain enzymes involved in peptide synthesis. FEBS Lett. **307**:40–43.
33. **Matsuda, J., S. Okabe, T. Hashimoto, and Y. Yamada.** 1991. Molecular cloning of hyoscyamine 6β-hydroxylase, a 2-oxoglutarate-dependent dioxygenase, from cultured roots of *Hyoscyamus niger*. J. Biol. Chem. **266**:9460–9464.
34. **Mehta, P. K., and P. Christen.** 1993. Homology of pyridoxal-5′-phosphate-dependent aminotransferases with the *cobC* (cobalamin synthesis), *nifS* (nitrogen fixation), *pabC* (*p*-aminobenzoate synthesis), and *malY* (abolishing endogenous induction of the maltose system) gene products. Eur. J. Biochem. **211**:373–376.
35. **Merrick, M. J.** 1993. In a class of its own—the RNA polymerase sigma factor σ^{54} (σ^{N}). Mol. Microbiol. **10**:903–909.
36. **Mitchell, R. E.** 1982. Coronatine production by some phytopathogenic pseudomonads. Physiol. Plant. Pathol. **20**:83–89.
37. **Mitchell, R. E., C. N. Hale, and J. C. Shanks.** 1983. Production of different pathogenic symptoms and different toxins by strains of *Pseudomonas syringae* pv. tomato not distinguishable by gel-immunodiffusion assay. Physiol. Plant Pathol. **23**:315–322.
38. **Mitchell, R. E., S. A. Young, and C. L. Bender.** 1994. Coronamic acid, an intermediate in coronatine biosynthesis by *Pseudomonas syringae*. Phytochemistry **35**:343–348.
39. **Myllylä, R., V. Günzler, K. I. Kivirikko, and D. D. Kaska.** 1992. Modification of vertebrate and algal prolyl 4-hydroxylases and vertebrate lysyl hydroxylase by diethyl pyrocarbonate. Biochem. J. **286**:923–927.
40. **Palmer, D. A., and C. L. Bender.** 1993. Effects of environmental and nutritional factors on production of the polyketide phytotoxin coronatine by *Pseudomonas syringae* pv. glycinea. Appl. Environ. Microbiol. **59**:1619–1623.
41. **Parry, R. J., M. T. Lin, A. E. Walker, and S. Mhaskar.** 1991. Biosynthesis of coronatine: investigations of the biosynthesis of coronamic acid. J. Am. Chem. Soc. **113**:1849–1850.
42. **Parry, R. J., S. V. Mhaskar, M.-T. Lin, A. E. Walker, and R. Mafoti.** 1994. Investigations of the biosynthesis of the phytotoxin coronatine. Can. J. Chem. **72**:86–99.
43. **Patriarca, E. J., M. Chiurazzi, G. Manco, A. Riccio, A. Lamberti, A. De Paolis, M. Rossi, R. Defez, and M. Iaccarino.** 1992. Activation of the *Rhizobium leguminosarum glnII* gene by NtrC is dependent on upstream DNA sequences. Mol. Gen. Genet. **234**: 337–345.
44. **Poulose, A. J., L. Rogers, T. M. Cheesbrough, and P. E. Kolattukudy.** 1985. Cloning and sequencing of the cDNA for S-acyl fatty acid synthase thioesterase from the uropygial gland of mallard duck. J. Biol. Chem. **260**:15953–15958.
45. **Raibaud, A., M. Zalacain, T. G. Holst, R. Tizard, and C. J. Thompson.** 1991. Nucleotide sequence analysis reveals linked *N*-acetyl hydrolase, thioesterase, transport, and regulatory genes encoded by the bialaphos biosynthetic gene cluster of *Streptomyces hygroscopicus*. J. Bacteriol. **173**:4454–4463.
46. **Reznikoff, W. S.** 1993. The Tn*5* transposon. Annu. Rev. Microbiol. **47**:945–963.
47. **Rich, J. J., and D. K. Willis.** 1990. A single oligonucleotide can be used to rapidly isolate DNA sequences flanking a transposon Tn*5* insertion by the polymerase chain reaction. Nucleic Acids Res. **18**: 6673–6676.
48. **Rusnak, F., M. Sakaitani, D. Drueckhammer, J. Reichert, and C. T. Walsh.** 1991. Biosynthesis of the *Escherichia coli* siderophore enterobactin: sequence of the *entF* gene, expression and purification of EntF, and analysis of covalent phosphopantetheine. Biochemistry **30**:2916–2927.
49. **Sakai, R., K. Nishiyama, A. Ichihara, K. Shiraishi, and S. Sakamura.** 1979. The relation between bacterial toxic action and plant growth regulation, p. 165–179. *In* J. M. Daly and I. Uritani (ed.), Recognition and specificity in plant host-parasite interactions. University Park Press, Baltimore.
50. **Salmeron, J. M., and B. J. Staskawicz.** 1993. Molecular characterization and *hrp* dependence of the avirulence gene *avrPto* from *Pseudomonas syringae* pv. tomato. Mol. Gen. Genet. **239**:6–16.
51. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
52. **Schlumbohm, W., T. Stein, C. Ullrich, J. Vater, M. Krause, M. A. Marahiel, V. Kruft, and B. Wittmann-Liebold.** 1991. An active serine is involved in covalent substrate amino acid binding at each reaction center of gramicidin S synthetase. J. Biol. Chem. **266**: 23135–23141.
53. **Shirashi, K., K. Konoma, H. Sato, A. Ichihara, S. Sakamura, K. Nishiyama, and R. Sakai.** 1979. The structure-activity relationship in coronatine analogs and amino compounds derived from (+)-coronafacic acid. Agric. Biol. Chem. **43**:1753–1757.
54. **Tai, M.-H., S. S. Chirala, and S. J. Wakil.** 1993. Roles of Ser101, Asp236, and His237 in catalysis of thioesterase II and the C-terminal region of the enzyme in its interaction with fatty acid synthase. Proc. Natl. Acad. Sci. USA **90**:1852–1856.
55. **Thöny, B., and H. Hennecke.** 1989. The −24/−12 promoter comes of age. FEMS Microbiol. Rev. **63**:341–358.
56. **Tokita, K., K. Hori, T. Kurotsu, M. Kanda, and Y. Saito.** 1993. Effect of single base substitutions at glycine-870 codon of gramicidin S synthetase 2 gene on proline activation. J. Biochem. **114**: 522–527.
57. **Turgay, K., M. Krause, and M. A. Marahiel.** 1992. Four homologous domains in the primary structure of GrsB are related to domains in a superfamily of adenylate-forming enzymes. Mol. Microbiol. **6**:529–546.

58. **Ullrich, M., and C. L. Bender.** Unpublished data.
59. **Ullrich, M., A. C. Guenzi, R. E. Mitchell, and C. L. Bender.** 1994. 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. Appl. Environ. Microbiol. **60:**2890–2897.
60. **Van den Eede, G., R. Deblaere, K. Goethals, M. V. Montagu, and M. Holsters.** 1992. Broad host range and promoter selection vectors for bacteria that interact with plants. Mol. Plant-Microbe Interact. **5:**228–234.
61. **von Döhren, H.** 1993. The organization of multifunctional peptide and depsipeptide synthetases. Biochem. Soc. Trans. **21:**214–218.
62. **Weckermann, R., R. Fürbass, and M. A. Marahiel.** 1988. Complete nucleotide sequence of the *tycA* gene coding the tyrocidine synthetase I from *Bacillus brevis*. Nucleic Acids Res. **16:**11841.
63. **Weiler, E. W., T. M. Kutchan, T. Gorba, W. Brodschelm, U. Niesel, and F. Bublitz.** 1994. The *Pseudomonas* phytotoxin coronatine mimics octadecanoid signalling molecules of higher plants. FEBS Lett. **345:**9–13.
64. **Wiebe, W. L., and R. N. Campbell.** 1993. Characterization of *Pseudomonas syringae* pv. maculicola and comparison with *P.s.* tomato. Plant Dis. **77:**414–419.
65. **Witkowski, A., J. Naggert, H. E. Witkowska, Z. I. Randhawa, and S. Smith.** 1992. Utilization of an active serine 101 → cysteine mutant to demonstrate the proximity of the catalytic serine 101 and histidine 237 residues in thioesterase II. J. Biol. Chem. **267:** 18488–18492.
66. **Xiao, Y., Y. Lu, S. Heu, and S. W. Hutcheson.** 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. syringae 61 *hrp* cluster. J. Bacteriol. **174:**1734–1741.
67. **Young, S. A., S. K. Park, C. Rodgers, R. E. Mitchell, and C. L. Bender.** 1992. Physical and functional characterization of the gene cluster encoding the polyketide phytotoxin coronatine in *Pseudomonas syringae* pv. glycinea. J. Bacteriol. **174:**1837–1843.