

Characterization and Transcriptional Analysis of the Gene Cluster for Coronafacic Acid, the Polyketide Component of the Phytotoxin Coronatine

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Coronafacic acid (CFA), the polyketide component of the phytotoxin coronatine (COR), is activated and coupled to coronamic acid via amide bond formation, a biosynthetic step presumably catalyzed by the CFA ligase (*cfl*) gene product. The COR biosynthetic gene cluster in *Pseudomonas syringae* pv. *glycinea* PG4180 is located within a 32-kb region of a 90-kb plasmid designated p4180A. In the present study, a cloned region of p4180A complemented all CFA⁻ mutants spanning an 18.8-kb region of the COR biosynthetic cluster. The genetic evidence presented in this study indicates that *cfl* and the CFA biosynthetic gene cluster are encoded by a single transcript and that transcription of all of the genes in this operon is directed by the *cfl* promoter. The *cfl* promoter was localized to a 0.37-kb region upstream of the transcriptional start site by progressive subcloning in pRG960sd, a vector containing a promoterless glucuronidase gene. Transcription of the *cfl*/CFA operon was temperature sensitive and showed maximal glucuronidase activity at 18°C. Furthermore, transcription of the *cfl*/CFA operon was dependent on the functional activity of a modified two-component regulatory system located within the COR biosynthetic gene cluster. Thermoregulation of the *cfl*/CFA operon and the coronamic acid biosynthetic gene cluster via the modified two-component regulatory system is discussed.

Biosynthesis of the phytotoxin coronatine (COR) in the phytopathogen *Pseudomonas syringae* involves amide bond formation between two distinct intermediates in the COR pathway, coronafacic acid (CFA), a bicyclic hydrindanone carboxylic acid, and coronamic acid (CMA), which is 2-ethyl-1-aminocyclopropane 1-carboxylic acid (15, 20, 21). CFA is derived from the polyketide pathway and originates from three acetate units and one unit each of butyrate and pyruvate (21). 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 (19, 21).

In *P. syringae* pv. *glycinea* PG4180, the COR biosynthetic cluster is located within a 32-kb contiguous region of a 90-kb plasmid designated p4180A (1). Extensive mutational analysis, as well as the cloning, expression, nucleotide sequencing, and transcriptional mapping of several genes required for COR production (1, 13, 25, 27), has resulted in the development of a functional map of the COR biosynthetic gene cluster (Fig. 1A). These studies have indicated a clustering of the genes for CMA and CFA biosynthesis at opposing ends of the COR biosynthetic gene cluster (Fig. 1A). Transposon insertions in the CMA and CFA biosynthetic regions resulted in mutants with CMA⁻ CFA⁺ and CMA⁻ CFA⁺ phenotypes; these could be complemented when exogenous CMA or CFA, respectively, was supplied to the bacterial cultures (1, 27). Recently, the DNA region believed to be involved in the coupling (CPL) of CFA and CMA via amide bond formation was cloned, sequenced, and overexpressed in *Escherichia coli* (1, 13). Sequence analysis of this 2.37-kb region revealed the presence of a single gene encoding coronafacate ligase (*cfl*). The predicted protein product of *cfl* showed relatedness to enzymes which

activate substrates for ligation via adenylation (adenylate-forming enzymes) (13).

The CMA and CFA biosynthetic gene clusters are separated by a 4.3-kb region in which mutations interrupt the production of both intermediates, suggesting a possible role for this region in the regulation of COR biosynthesis. Nucleotide sequencing of this region revealed the presence of three genes, *corS*, *corR*, and *corP*, which encode a modified two-component regulatory system consisting of one sensor protein, CorS, and two response regulator proteins, CorR and CorP (28). Transcriptional fusions to a promoterless glucuronidase (*GUS*) gene (*uidA*) indicated that *cmaA* and *cmaT*, which are cotranscribed from the *cmaA* promoter and required for CMA biosynthesis, were not expressed in *corS*, *corR*, or *corP* mutants (28).

COR production in *P. syringae* pv. *glycinea* PG4180 is temperature dependent with maximal amounts of COR, CFA, and CMA produced at 18°C and negligible amounts produced at 28 to 30°C (16, 17, 25). A *cmaA::uidA* transcriptional fusion indicated that 18°C was an optimal temperature for transcription of the CMA biosynthetic cluster (25). Furthermore, the modified two-component regulatory system consisting of *corS*, *corR*, and *corP* was shown to be required for the temperature-dependent activation of the *cmaA::uidA* transcriptional fusion (28).

The present study focused on transcriptional activation of the *cfl* gene and the extent of DNA transcribed from the *cfl* promoter. Our specific objectives included the following: (i) determination of the minimal size of the *cfl* promoter, (ii) investigation of whether expression of the *cfl* promoter is temperature dependent and controlled by the modified two-component regulatory system, and (iii) investigation of whether *cfl* is cotranscribed with genes encoded by the CFA biosynthetic gene cluster.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* HB101 and DH5 α (23) were used as hosts in cloning experiments and were cultured in

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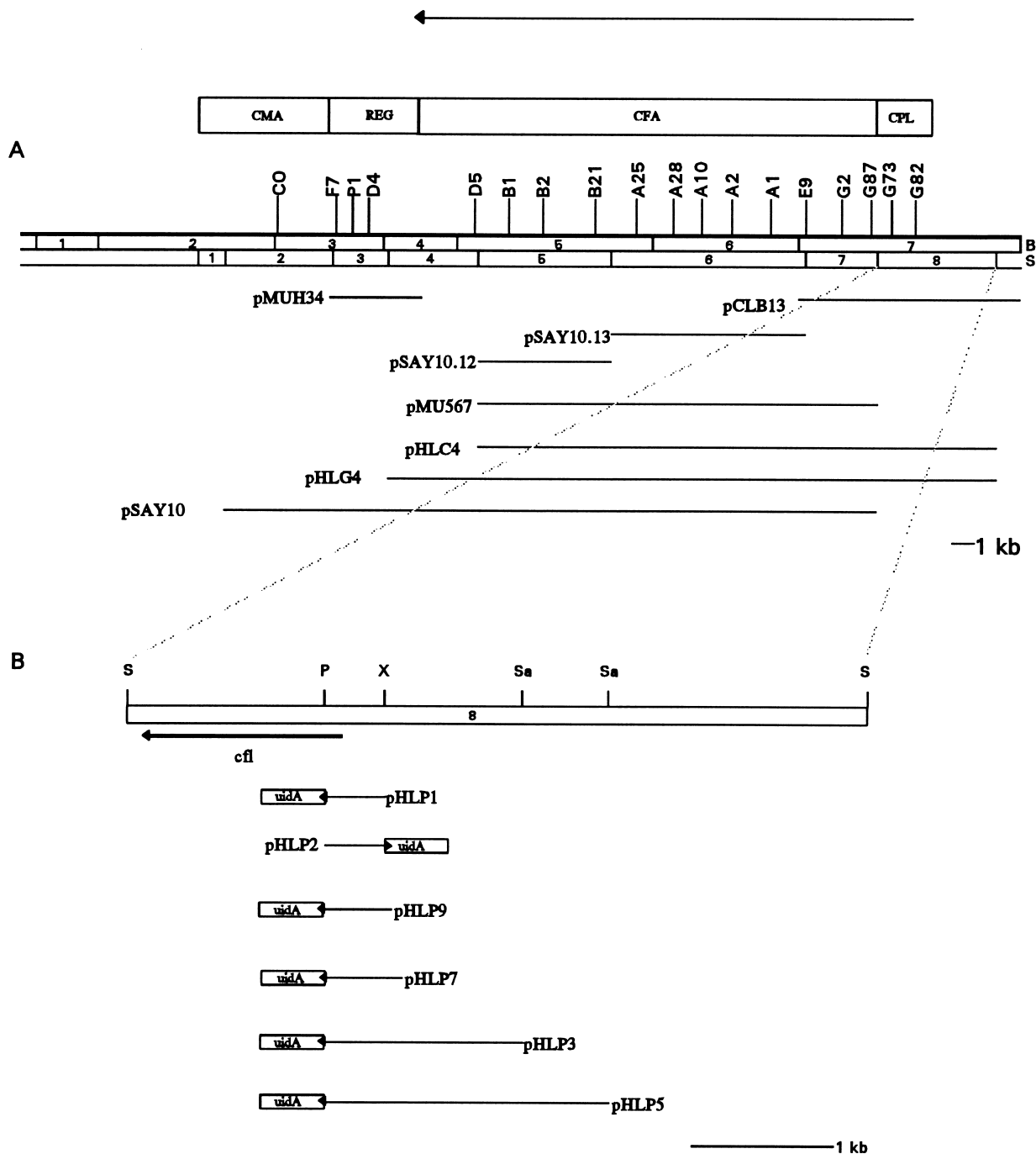


FIG. 1. (A) Physical and functional map of the COR biosynthetic gene cluster in *P. syringae* pv. *glycinea* PG4180. Vertical lines above the physical map with letter-number designations represent the locations of mutations generated with Tn5 or antibiotic cassettes. Functional regions of the COR biosynthetic cluster are shown in the rectangles above the physical map and are abbreviated as follows: CMA, CMA biosynthetic gene cluster; REG, regulatory region; CFA, CFA biosynthetic gene cluster; CPL, CPL region (coupling of CFA and CMA via amide bond formation). The horizontal arrow above the functional map indicates the location of the *cfl*/CFA transcript described in this report. The horizontal lines below the physical map show the subclones used for complementation analysis. (B) Physical map of *Sst*I fragment 8 showing the location of the gene encoding CFA ligase (*cfl*) from the transcriptional start site to the translational stop (arrowhead). The locations and orientations of promoter probe constructs used in this study are indicated. Restriction enzymes: B, *Bam*HI; P, *Pst*I; S, *Sst*I; Sa, *Sal*I; X, *Xba*I.

Luria-Bertani medium (23) at 37°C. *P. syringae* pv. *glycinea* was routinely cultured on mannitol-glutamate medium (10) at 24 to 26°C. Derivatives of PG4180 containing transposon or antibiotic cassette insertions in the COR biosynthetic cluster are designated by letter-number combinations (Fig. 1A and Table 1). COR production and GUS activity were assayed in broth cultures of PG4180 grown in Hoitink-Sinden medium optimized for COR production (HSC) (16) on a rotary shaker (250 rpm) at 18 or 28°C. Antibiotics were added to media in the

following concentrations (micrograms per milliliter): ampicillin, 50; kanamycin, 25; streptomycin, 25; spectinomycin, 25; and tetracycline, 12.5. All experiments were repeated at least once.

Molecular and genetic techniques. Agarose gel electrophoresis, restriction digests, PCRs, and small-scale plasmid isolations from *E. coli* were performed by standard procedures (23). Plasmid DNA was isolated from *Pseudomonas* strains as described previously (2, 5). DNA fragments were excised from agarose gels,

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference(s) or source
<i>Escherichia coli</i>		
DH5 α		23
HB101		23
<i>Pseudomonas syringae</i>		
pv. <i>glycinea</i>		
PG4180	COR ⁺ ; contains p4180A	3
PG4180.A2	Km ^r COR ⁻ CFA ⁻ CMA ⁺ CPL ⁺	31
PG4180.A10	Km ^r COR ⁻ CFA ⁻ CMA ⁺ CPL ⁺	31
PG4180.B21	Km ^r COR ⁻ CFA ⁻ CMA ⁺ CPL ⁺	31
PG4180.C0	Km ^r COR ⁻ CFA ⁺ CMA ⁻ CPL ⁺ ; <i>cmA</i> ::Tn5	25, 31
PG4180.D4	Km ^r COR ⁻ CFA ⁻ CMA ⁻ CPL ^{+/-} ; <i>corR</i> ::Tn5	28
PG4180.D5	Km ^r COR ⁻ CFA ⁻ CMA ⁺ CPL ⁺	27
PG4180.E9	Km ^r COR ⁻ CFA ⁻ CMA ⁺ CPL ⁺	31
PG4180.F7	Km ^r COR ⁻ CFA ⁻ CMA ⁻ CPL ^{+/-} ; <i>corP</i> ::Tn5	3, 28
PG4180.G73	Km ^r COR ⁻ CFA ⁻ CMA ⁺ CPL ⁻ ; <i>cfl</i> ::Tn5	3, 13
PG4180.G82	Km ^r COR ⁻ CFA ⁻ CMA ⁺ CPL ⁺	3
PG4180.G87	Km ^r COR ⁻ CFA ⁻ CMA ⁺ CPL ⁺	3
PG4180.P1	Gm ^r COR ⁻ CFA ⁻ CMA ⁻ CPL ^{+/-} ; <i>corS</i> ::Tn5	28
Plasmids		
pRK415	Tc ^r ; RK2-derived cloning vector	11
pLAFR3	Tc ^r ; RK2-derived cosmid vector	24
p4180A	90-kb plasmid in <i>P. syringae</i> pv. <i>glycinea</i> PG4180; contains COR biosynthetic gene cluster	3
pRG960sd	Sm ^r Sp ^r ; 17.0 kb; contains promoterless <i>uidA</i> with start codon and Shine-Dalgarno sequence	29
pHL1	Tc ^r ; 5.2-kb <i>SstI</i> fragment in pRK415; contains <i>cfl</i> and 3.4 kb of upstream DNA	3
pHLC4	Tc ^r ; cosmid clone containing 22 kb of p4180A DNA	This study
pHLG4	Tc ^r ; cosmid clone containing 27 kb of p4180A DNA	This study
pHLP1	Sm ^r Sp ^r ; 0.46-kb <i>XbaI-PstI</i> fragment from pHL1 in pRG960sd (<i>XbaI-PstI-uidA</i>)	13
pHLP2	Sm ^r Sp ^r ; 0.46-kb <i>XbaI-PstI</i> fragment from pHL1 in pRG960sd (<i>PstI-XbaI-uidA</i>)	13
pHLP3	Sm ^r Sp ^r ; 1.4-kb <i>SalI-PstI</i> fragment from pHL1 in pRG960sd	This study
pHLP5	Sm ^r Sp ^r ; 2.5-kb <i>SalI-PstI</i> fragment of pHL1 in pRG960sd	This study
pHLP7	Sm ^r Sp ^r ; 0.52-kb <i>PstI</i> fragment derived by PCR in pRG960sd	This study
pHLP9	Sm ^r Sp ^r ; 0.47-kb <i>PstI</i> fragment derived by PCR in pRG960sd	This study
pCLB13	Tc ^r ; 10.1-kb <i>BamHI</i> fragment of p4180A in pRK415	3
pSAY10	Tc ^r ; pLAFR3 containing 29-kb insert derived from p4180A	31
pSAY10.13	Tc ^r ; 8.7-kb <i>SstI</i> fragment cloned in pRK415	31
pSAY10.12	Tc ^r ; 6.0-kb <i>SstI</i> fragment cloned in pRK415	31
pMU567	Tc ^r ; 17.9-kb insert derived from pSAY10 in pLAFR3	26
pMUH34	Tc ^r ; 3.4-kb <i>HindIII-EcoRI</i> fragment containing regulatory genes	28

and residual agarose was removed with the GeneClean DNA extraction kit (Bio 101, La Jolla, Calif.). Nonradioactive labeling of DNA fragments and Southern hybridizations were performed as specified in the Genius Labeling and Detection Kit (Boehringer Mannheim, Indianapolis, Ind.). A cosmid library of PG4180 plasmid DNA was constructed in pLAFR3 as described previously (3). Selected plasmids and cosmids were mobilized from *E. coli* to *P. syringae* by a triparental mating method (4), and transconjugants were verified by agarose gel electrophoresis.

pHLP7 and pHLP9 (Table 1) are 0.52- and 0.47-kb PCR products, respectively, cloned into the *PstI* sites of pRG960sd. The template in each amplification was pHL1. The forward 5' primers (shown 5' to 3') were as follows: (i) primer 1 was TAGCTGCAGCGTCCCGATGATAATACTTTGGCG and contained a *PstI* recognition site and nucleotides 386 to 411 as reported previously (13); (ii) primer 2 was GTCCTGCAGCGTTACAGAACCCTTCTAGAAAAC and contained a *PstI* recognition site and nucleotides 443 to 466 (13). The reverse primer (primer 3; also shown 5' to 3') was GATCGCCCGGCTGCAGGCCTTGCA and was the complement of nucleotides 902 to 925 in the previously reported sequence (13). Primers 1 and 3 were used to amplify the 0.52-kb product cloned in pHLP7; primers 2 and 3 were used to amplify the 0.47-kb sequence in pHLP9. All oligonucleotide primers were synthesized by the Oklahoma State University Recombinant DNA/Protein Resource Facility.

Construction of transcriptional fusions and GUS assays. Plasmid pRG960sd (29), which contains a promoterless GUS gene (*uidA*) downstream of a multiple cloning site, was used to identify and characterize promoter sequences upstream of *cfl*. Subcloned DNA fragments were ligated into pRG960sd, orientations were determined by restriction digests, and constructs were mobilized into *Pseudomonas* strains via triparental matings. Transcriptional activity was quantified by

fluorometric analysis of GUS activity (30) of cells grown for 5 days in 10 ml of HSC broth at 18 or 28°C. Fluorescence was monitored with a Fluoroskan II version 4.0 microplate reader (ICN Biomedicals, Inc., Costa Mesa, Calif.) in 96-well microtiter plates. Total protein content of cell lysates was determined with the Bio-Rad (Richmond, Calif.) protein assay kit in accordance with the manufacturer's instructions.

Detection of COR. *Pseudomonas* strains were incubated in 10-ml aliquots of HSC medium at 18 or 28°C as described previously (16). Organic acids were extracted from bacterial supernatants, and extracts were analyzed by high-pressure liquid chromatography as described elsewhere (16).

RESULTS

Cloning and genetic complementation of the CFA biosynthetic region. pCLB13, pSAY10.13, pSAY10.12, pMU567, and pSAY10 are constructs from previous studies (Table 1) which contain various portions of the CFA biosynthetic cluster (Fig. 1A). Strains PG4180.G82, PG4180.G87, PG4180.G2, PG4180.E9, PG4180.A1, PG4180.A2, PG4180.A28, PG4180.A25, PG4180.B21, PG4180.B2, PG4180.B1, and PG4180.D5 (Fig. 1A) are COR⁻ CFA⁻ CMA⁺ CPL⁺ mutants which produce COR when supplied with exogenous CFA (7, 27, 31). G73 is a COR⁻ CFA⁻ CMA⁺ CPL⁻ mutant which produces COR when genetically complemented with the CPL region and supplied with

TABLE 2. COR production by various CFA⁻ mutants containing cloned regions from the CFA biosynthetic cluster^a

Mutant	COR production (mg/liter)						
	pCLB13	pSAY10.13	pSAY10.12	pMU567	pHLC4	pHLG4	pSAY10
G82	— ^b	NT ^c	NT	NT	—	24.6	NT
G73	—	NT	NT	NT	—	23.1	NT
G87	—	NT	NT	—	NT	22.6	NT
G2	—	NT	NT	<1.0 ^d	—	NT	NT
E9	—	NT	NT	<1.0	—	11.6	—
A1	NT	NT	NT	<1.0	NT	NT	<1.0
A2	NT	—	NT	<1.0	—	19.0	<1.0
A28	NT	—	NT	<1.0	NT	NT	<1.0
A25	NT	NT	NT	<1.0	NT	NT	<1.0
B21	NT	NT	—	NT	—	18.6	<1.0
B2	NT	NT	—	—	NT	NT	<1.0
B1	NT	NT	—	—	NT	NT	<1.0
D5	NT	NT	NT	NT	—	28.1	NT

^a Values are averages of three replicates.

^b —, COR not produced at a detectable level.

^c NT, COR production not assessed for this mutant-plasmid combination.

^d COR production below 1.0 mg/liter was not considered significant (3).

CFA. When pCLB13, pSAY10.13, pSAY10.12, pMU567, and pSAY10 were introduced into the various CFA⁻ mutants listed in Table 2, COR production either was not observed or occurred at insignificant levels (equal to or less than 2% of the quantity produced by PG4180). On the basis of these results, we suspected that the CFA biosynthetic cluster might be co-transcribed with the CPL region from a common promoter. Since the *cfl* gene was previously localized within the CPL region and DNA upstream of the *cfl* transcriptional start site was shown to have promoter activity (13), we hypothesized that the CFA biosynthetic cluster was transcribed from the *cfl* promoter. If this assumption was correct, the *cfl*/CFA transcript would initiate in *Sst*I-8 and extend throughout the CFA biosynthetic region.

To test this hypothesis, a library of p4180A DNA was constructed in the *Bam*HI site of pLAFR3 as previously described (3). The following fragments were labeled with digoxigenin and hybridized to cosmid clones from the library: (i) the 1.9-kb *Xba*I-*Sst*I fragment located on the left side of *Sst*I fragment 8 (Fig. 1B), (ii) *Sst*I fragment 4 (Fig. 1A), and (iii) *Sst*I fragment 5 (Fig. 1A). Southern blot hybridizations to *Sst*I-digested cosmid DNA indicated that pHLC4 contained *Sst*I fragments 5 to 8 and pHLG4 contained *Sst*I fragments 4 to 8 (Fig. 1A). These two cosmids were evaluated for the ability to restore COR production to mutants defective in CFA production. Although pHLC4 failed to restore COR production to the CFA⁻ mutants tested, introduction of pHLG4 into these mutants resulted in significant COR production, at levels ranging from 11.6 to 28.1 mg/liter (Table 2). These levels approached the quantity of COR produced by wild-type PG4180 in these experiments (approximately 31.9 mg/liter).

Characterization of the *cfl* promoter region. We previously reported that the 311-bp region upstream of the *cfl* transcriptional start site contained promoter activity (13). This was shown with a construct designated pHLP1 (Fig. 1B), which contained the 311-bp region upstream of a promoterless GUS gene. pHLP2, a construct containing this region in the inverse orientation relative to pHLP1 (Fig. 1B), did not promote *uidA* transcription (13), thereby establishing the transcriptional direction as right to left, as indicated for pHLP1 (Fig. 1B).

In the present study, we further delimited the *cfl* promoter by fusing various amounts of DNA upstream of *cfl* to the

promoterless *uidA* gene in pRG960sd (Fig. 1B). Interestingly, PG4180 transconjugants containing pHLP3, pHLP5, and pHLP7 were 25 to 30 times more active at 18°C in the GUS assay than was PG4180(pHLP1) (Table 3). However, GUS activity in PG4180(pHLP9) was not significantly different from that expressed by PG4180(pHLP1) (Table 3). pHLP9 lacks an additional 57 bp of upstream DNA which is present in pHLP7, emphasizing the importance of this region in transcriptional activation of the *cfl* promoter.

Previous studies indicated that the *cmaA* and *corS* promoter regions are regulated at the transcriptional level by temperature, showing maximal activity at 18°C and significantly lower activity at 28°C (25, 28). In the present study, we incubated PG4180 transconjugants containing pHLP3, pHLP5, and pHLP7 at 18 and 28°C and measured GUS activity. Transcriptional activity was approximately fivefold higher when cells were incubated at 18°C than when they were incubated at 28°C (Table 3), demonstrating that the *cfl* promoter resembles the *cmaA* and *corS* promoters in its response to temperature.

Transcriptional activation of the *cfl*/CFA operon. pMUH34, which contains all of *Sst*I-3 and a portion of *Sst*I-4 (Fig. 1A), contains three genes (*corR*, *corS*, and *corP*) which presumably function as a modified two-component regulatory system (28). PG4180 derivatives containing mutations in these genes were CFA⁻ CMA⁻ and produced low levels of COR when supplied with exogenous CFA and CMA (1), indicating that CPL activity was not completely abolished. However, the level of COR synthesized in these feeding experiments was extremely low (1), indicating that coupling activity was also reduced in the regulatory mutants.

Recently, Ullrich et al. (28) showed that *corR*, *corS*, and *corP* are required for transcriptional activation of the *cmaA* promoter, thereby explaining why CMA biosynthesis was inactivated in the regulatory mutants. The elimination of CFA biosynthesis and reduced CPL activity in these mutants suggested that the regulatory region might also function in transcriptional activation of the *cfl* promoter. To explore this possibility, expression of GUS activity in transconjugants containing pHLP3 (*cfl::uidA*) was investigated. GUS activities for wild-type PG4180 and three regulatory mutants (PG4180.F7, PG4180.P1, and PG4180.D4) containing pHLP3 were compared at 18 and 28°C (Fig. 2A). GUS activity was quite high in PG4180(pHLP3) incubated at 18°C and approximately fourfold lower when this construct was incubated at 28°C (Fig. 2A). GUS activity was negligible in PG4180.F7(pHLP3), PG4180.P1(pHLP3), and PG4180.D4(pHLP3) at both temperatures (Fig. 2A), suggesting that these mutants are defective in transcriptional activa-

TABLE 3. GUS activity in PG4180 transconjugants containing different *cfl::uidA* transcriptional fusions

PG4180 transconjugant	Mean GUS activity ^a (U/mg of protein)	
	18°C	28°C
pHLP1	4.7	2.6
pHLP3	146.1	26.5
pHLP5	150.8	28.2
pHLP7	125.2	23.2
pHLP9	10.6	6.4
pHLP2 ^b	0.7	1.0

^a Mean of three replicates per temperature. One unit equals 1 μmol of methylumbelliferone formed per min.

^b PG4180 containing pHLP2 was included as a negative control. In pHLP2, *uidA* was oriented in the transcriptionally inactive direction relative to the *cfl* promoter (Fig. 1B).

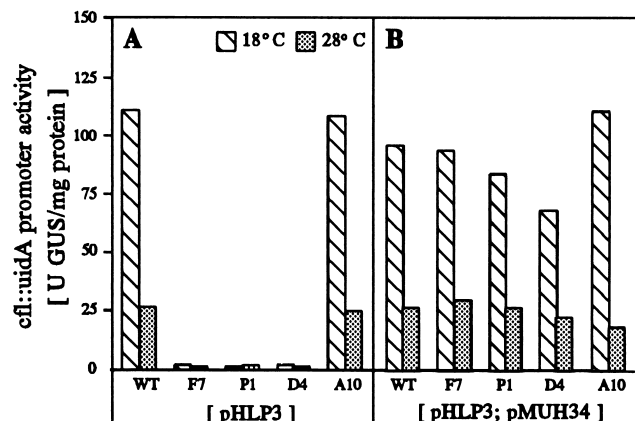


FIG. 2. Effects of temperature and genetic background on *cfl::uidA* promoter activity in *P. syringae*. GUS activity is shown for transconjugants harboring pHLP3 (A) and pHLP3 and pMUH34 (B). WT, wild-type PG4180. Strains PG4180.F7, PG4180.P1, and PG4180.D4 are defective in the regulation of the COR biosynthetic cluster and contain mutations in *corP*, *corS*, and *corR*, respectively. Strain PG4180.A10 contains a Tn5 insertion in the CFA biosynthetic cluster. The quantities shown are averages of three replicates.

tion of the *cfl::uidA* promoter. As controls, we investigated the GUS activity of pHLP3 in PG4180.C0 and PG4180.A10, which contain Tn5 insertions in the CMA and CFA biosynthetic clusters, respectively (3). Unlike the results obtained with the three regulatory mutants, GUS activity in strains PG4180.A10 (pHLP3) (Fig. 1A) and PG4180.C0(pHLP3) (data not shown) was similar to that observed for strain PG4180(pHLP3).

To investigate whether pMUH34 could restore activation of the *cfl* promoter to the regulatory mutants, this clone was introduced into mutants containing pHLP3 (Fig. 2B). Introduction of pMUH34 into PG4180.F7(pHLP3), PG4180.P1 (pHLP3), and PG4180.D4(pHLP3) resulted in GUS activity comparable to that of PG4180(pHLP3) at both temperatures (Fig. 2B), indicating that the regulatory genes encoded by pMUH34 are definitely involved in transcriptional activation of the *cfl* promoter.

DISCUSSION

Genetic complementation of CFA-defective mutants revealed that only pHLG4 contained all of the genes necessary for CFA biosynthesis. The 27-kb region cloned in pHLG4 includes the CFA biosynthetic region (localized in *SstI* fragments 4 to 7), *cfl*, and the upstream promoter region. Although both pHLC4 and pSAY10 contain the structural information required for CFA biosynthesis, only pHLG4 complemented the CFA⁻ mutants. Therefore, the failure of pSAY10 to complement CFA⁻ mutants is likely due to the absence of the promoter region in this construct since Cfl presumably functions to adenylate CFA for subsequent ligation to CMA and has no known role in CFA biosynthesis (13). The data presented in this report indicate that the CFA biosynthetic gene cluster is organized with *cfl* in a polycistronic operon. By using a common promoter to transcribe genes for synthesis of CFA and its activation and ligation to CMA, the bacterium has optimized the production of the phytotoxic end product, COR, and minimized production of the nontoxic intermediate CFA. This genetic organization is consistent with the observation that COR is the most abundant coronafacoyl compound produced by PG4180 (31).

The sequence of events in CFA biosynthesis remains fairly obscure, and sequence data within the CFA biosynthetic clus-

ter are limited. However, nucleotide sequence analysis of *SstI* fragment 7 has revealed the presence of two discrete open reading frames with a probable role in CFA biosynthesis. One open reading frame showed relatedness to acyl carrier proteins, and the second open reading frame resembled β -ketoacyl synthases (22). Polyketide synthetases are generally classified into type I and II systems and consist of protein complexes that act on covalently bound substrates that are attached as thioesters to an acyl carrier protein (9). The identification of two distinct open reading frames within the CFA biosynthetic gene cluster with a probable role in CFA biosynthesis suggests that the CFA polyketide synthase more closely resembles the type II system, in which discrete polypeptides associate in a complex (9). This is a bit surprising, since most type II polyketide synthases catalyze the synthesis of aromatic polyketides (9) and CFA has a fairly reduced structure. This anomaly may arise from the fact that most published polyketide synthase sequences are for antibiotics produced by streptomycetes and other gram-positive organisms. Although pseudomonads make a number of antifungal compounds from the polyketide pathway (6, 7), we are unaware of any published sequence information for these compounds.

The results obtained in the present study with *cfl::uidA* transcriptional fusions clearly showed that *cfl* is regulated at the transcriptional level by temperature and requires functional copies of *corR*, *corS*, and *corP*. The *cmaA* and *corS* promoters also share these characteristics (25, 28). Several schemes were recently proposed to explain how CorS, CorR, and CorP might function in the transcriptional activation of the COR biosynthetic gene cluster. The scenarios proposed by Ullrich et al. (28) each involve the autophosphorylation of CorS, a putative histidine protein kinase, at the inducing temperature (18°C). CorS would then phosphorylate CorR and CorP, which both show relatedness to response regulators in the RO_{III} group (18). Phosphorylated CorR and CorP would then function in concert or sequentially to activate transcription of the *corS*, *cmaA*, and *cfl* promoters. CorR contains a helix-turn-helix motif (28) and could conceivably bind and activate transcription of promoter regions in the COR biosynthetic gene cluster. Therefore, the three temperature-regulated promoters might contain a consensus sequence for recognition of a shared transcriptional activator, such as CorR. To investigate this possibility, the *cfl*, *cmaA*, and *corS* promoter regions were compared by BESTFIT analysis (University of Wisconsin Genetics Computer Group). Alignment of the three sequences revealed a series of conserved bases in the three promoter regions, including a 5-bp sequence (CAGCG) (Fig. 3). Furthermore, the 60-bp sequence shown in Fig. 3 for *cfl* is present in pHLP7 but

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cfl -366 TGGCAGCCCGATGATAATACTTTGGCGAAGGTTACCTCATTGCGATCAGTCATTACGGCT
cmaA -798 GGCATGTAATCCATGAGCTCAGCAAAACTCGATCAGATCTTCGAGGCATTTTTTCAGCGT
corS AGCCAGCCCGACCCGAGGACTATCGGCGCCACCCTGTCGGTCACTTCAGCCACGGC

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FIG. 3. Alignment of 60-bp regions from the *cfl*, *cmaA*, and *corS* promoter regions. The 60-bp sequence shown for *cfl* was previously published (13) and is present in pHLP3, pHLP5, and pHLP7, three *cfl::uidA* fusions which showed high levels of GUS activity in PG4180 transconjugants incubated at 18°C (Table 3). This sequence was not present in pHLP1 and pHLP9, which were significantly less active than the former three constructs in GUS assays. The activity of the *cfl*, *cmaA*, and *corS* promoters requires functional copies of *corP*, *corR* (putative response regulators [28]), and *corS* (putative histidine protein kinase [28]). The transcriptional start sites for *cfl* and *cmaA* have been established by primer extension (13, 25), and the numbers on the left indicate the locations of the sequences relative to the transcriptional start site. The transcriptional start site for *corS* has not been determined. The sequence cited above is located approximately 250 bp upstream of the *corS* translational start and within the promoter region previously defined by progressive subcloning in pRG960sd (28). Shaded areas indicate identical bases in the three promoter regions.

absent from pHLP9. PG4180(pHLP7) was approximately 12.5 times more active in GUS assays than was PG4180(pHLP9) when incubated at 18°C (Table 3), providing additional support for the role of this conserved region in transcriptional activation of the *cfl* promoter. We are currently evaluating whether CorR binds to the regions shown in Fig. 3 by using gel retardation and footprinting assays with purified CorR.

Optimal production of COR, CFA, and CMA occurs at 18°C, and data presented in this and other studies (28) suggest that the underlying mechanism for temperature regulation is the modified two-component regulatory system. Although the role of temperature in the expression of virulence genes in human and animal pathogens is well established (14), thermo-regulated gene expression in plant-associated bacteria is not well understood. In *Agrobacterium tumefaciens*, elevated temperatures were shown to decrease autophosphorylation of VirA (a histidine protein kinase) and subsequent phosphorylation of VirG (transcriptional activator of *vir* genes), thereby resulting in decreased tumor formation (8). It is possible that temperature might function in a similar manner to modulate the proposed phosphorylation activities of CorS. Alternatively, temperature might modulate the activity of the putative response regulators, CorR and CorP. For example, in *Klebsiella pneumoniae*, the transcriptional activator NifA is heat labile and unable to catalyze transcriptional activation of the *nifHDK* promoter (12). Efforts to obtain CorP, CorR, and CorS in purified form are currently under way, and these proteins will be used to elucidate the mechanisms involved in thermoregulation of the COR biosynthetic cluster.

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