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ARTICLE

Simple and Rapid Capillary Zone Electrophoresis Method for the Detection of Coronamic Acid, a Precursor to the *Pseudomonas syringae* Phytotoxin Coronatine

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The phytotoxin coronatine (COR) is produced by various pathovars of the plant pathogen *Pseudomonas syringae*, which infects a wide variety of crops. COR consists of two distinct moieties, coronafacic acid (CFA) and coronamic acid (CMA), which are derived from a modified polyketide pathway and isoleucine, respectively. Mutants defective in the CMA or CFA structural gene clusters have been used to study COR biosynthesis, and these mutants are commonly characterized using high-performance liquid chromatography (HPLC). Although the same extraction and HPLC method can be used for detection and quantification of COR and CFA, the detection of CMA by HPLC requires different fractionation and HPLC separation procedures, which are tedious and labor intensive. In this study, we used capillary zone electrophoresis (CZE) as a fast and accurate detection method for the quantification of CMA present in the culture supernatant of *P. syringae* pv. *glycinea* (*Psg*) PG4180 and *P. syringae* pv. *tomato* (*Pst*) DC3000. Analysis was performed by CZE using 100 mM phosphate buffer (pH 2.5) as a separating buffer, an applied voltage of 12 kV, and UV detection at 214 nm. Selected mutants defective in COR biosynthesis were used to validate CZE as a detection method. CMA production by *Psg* strain 18a/90, which lacks the COR gene cluster, and derivatives of 18a/90 was also evaluated. Furthermore, a procedure for the extraction and detection of CMA present inside the cells of *Psg* 18a/90 was developed. In conclusion, CZE was shown to be a rapid and sensitive method for the detection and quantification of CMA in *P. syringae*.

KEYWORDS: Capillary zone electrophoresis; coronatine; cornonamic acid; *Pseudomonas syringae*

INTRODUCTION

Coronatine (COR) is a nonhost specific phytotoxin produced by various pathovars of *Pseudomonas syringae* including *atropurpurea*, *glycinea*, *maculicola*, *morsprunorum*, and *tomato*, which infect ryegrass, soybean, crucifers, *Prunus* spp., and tomato, respectively. Depending on the plant host, this toxin is known to elicit chlorosis, inducing hypertrophy, inhibit root elongation, and stimulate ethylene production (*1*, *2*). COR shares structural and functional similarities with jasmonic acid, an endogenous signaling molecule in plants that functions as a growth regulator (*3*−*5*). COR impacts the outcome of the plant stress response associated with pathogens and herbivory and also modulates plant responses to environmental stresses (*6*). In recent years, COR has attracted considerable interest because of its role in the plant defense response and its potential use in various commercial applications. COR has been shown to alleviate salinity stress in cotton (*7*) and has potential use as an abscission agent in the harvest of mature citrus fruits (*8*).

COR consists of two distinct moieties, coronamic acid (CMA) and coronafacic acid (CFA) (**Figure 1A**) (*9*), which are derived from two entirely different biosynthetic pathways. CFA is biosynthesized via a modified polyketide pathway (*10*), whereas CMA is an ethylcyclopropyl amino acid that originates from isoleucine (*11*). CMA is generated from L-*allo*-isoleucine by a nonribosomal peptide synthetase (*12*). CFA and CMA are coupled by an amide bond to form COR (*11*, *13*), and the enzyme involved in this reaction lacks rigid specificity for the amino acid substrate. Hence, in addition to COR, various other CFA−amino acid complexes are biosynthesized including coronafacoylisoleucine, coronafacoylalloisoleucine, and coronafacoylvaline (*14*−*17*). Among the analogues, COR is the most toxic coronafacoyl compound made by COR-producing organisms (*15*). However, studies have shown that both CFA and CMA are important in the biological activity of COR (*5*, *18*).

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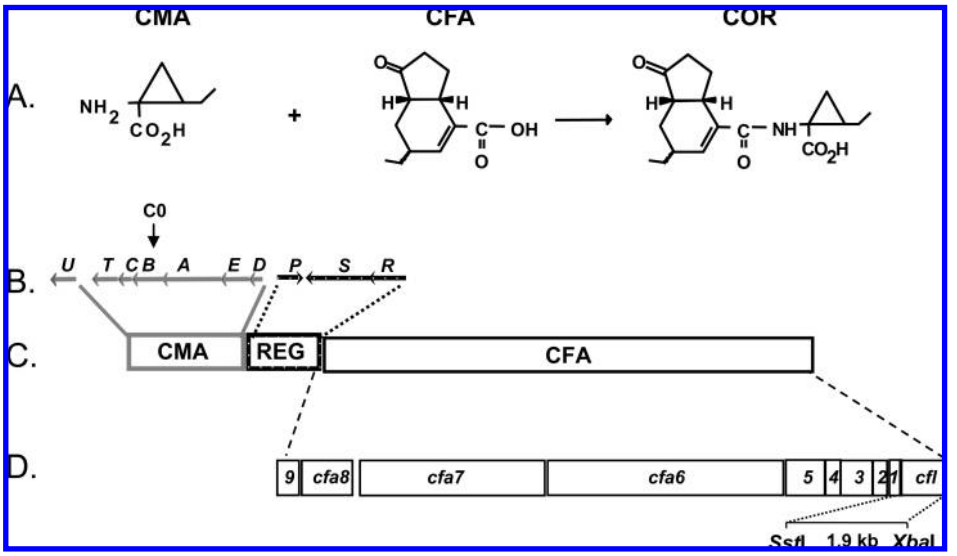

Figure 1. Phytotoxin coronatine (COR) and cloning of the COR gene cluster from *Psg* PG4180. (**A**) Structure of COR, coronafacic acid (CFA), and coronamic acid (CMA). (**B**) Genes encoded by the CMA biosynthetic and regulatory regions of the COR gene cluster. Abbreviations: R, *corR*; S, *corS*, P, *corP*; D, *cmaD*; E, *cmaE*; A, *cmaA*, B, *cmaB*; C, *cmaC*; T, *cmaT*; U, *cmaU*. Horizontal lines with arrowheads indicate the transcriptional organization. Vertical arrow indicates the location of the Tn*5* insertion in PG4180.C0 (*cmaB*). (**C**) Functional map of the CMA biosynthetic cluster, regulatory (REG) region, and CFA biosynthetic cluster. (**D**) Genes encoded by the CFA biosynthetic region of the COR gene cluster. Abbreviations: 1, *cfa1*; 2, *cfa2*; 3, *cfa3*; 4, *cfa4*; 5, *cfa5*; and 9, *cfa9*.

COR biosynthesis in *P. syringae* pv. *glycinea* (*Psg*) PG4180, a pathogen of soybean, has been extensively studied (*19*). In this strain, the genes required for COR biosynthesis are plasmid-borne. The structural genes for CFA and CMA biosynthesis are located on opposing ends of the COR gene cluster in *Psg* PG4180 (**Figure 1C**) and in a separate regulatory region consisting of three genes (**Figure 1B**): *corR* and *corP* encode response regulators, and *corS* encodes a histidine protein kinase (*20*−*22*). The CFA gene cluster in *Psg* PG4180 consists of 10 discrete ORFs, which are designated *cfl* and *cfa1*−*cfa9* (*23*) (**Figure 1D**). The CMA gene cluster of *Psg* PG4180 contains an operon consisting of six genes *cmaA*−*E* and *cmaT* (*24*, *25*) (**Figure 1B**), and roles for each of these genes in CMA biosynthesis have been described (*12*, *24*, *26*). A function for *cmaU*, which is not transcribed with the CMA gene cluster (*27*) (**Figure 1B**), was not apparent from the original sequence analysis (*25*). However, more recent BLASTX analysis revealed that the protein product of *cmaU* is related to threonine efflux proteins, suggesting a potential role for CmaU in the transport of CMA, CFA, and/or COR to the extracellular environment.

In addition to *Psg* PG4180, two more strains of *Pseudomonas*, *Pst* DC3000, which is now considered a model plant pathogen (*28*), and *Psg* 18a/90 were also used in this study. Much of our knowledge regarding COR biosynthesis in strains of *P. syringae* has been obtained using mutants defective in the CFA or CMA structural gene clusters (*29*). These mutants have been analyzed for CFA, CMA, and COR production and further characterized in exogenous feeding or cocultivation studies for the restoration of COR production (*13*, *22*, *27*, *30*−*32*). It is critical, therefore, to have sensitive and accurate methods for the detection of CFA, CMA, COR, and related compounds. High-performance liquid chromatography (HPLC) (*33*) and monoclonal antibodies have been used to detect COR in vitro (*34*, *35*). A modified form of an indirect competitive ELISA, which is at least 5 times more sensitive than detection by HPLC, was used to detect very low amounts (5−40 ng/mL) of COR in plants (*36*). However, unlike HPLC, this method cannot distinguish between COR and CFA−amide conjugates (*35*, *36*).

In addition to COR and CFA, CMA can also be detected by HPLC using reverse-phase chromatography with a C-18 column; however, this requires purification of the bacterial supernatant with an ion-exchange column and derivatization with phenylisothiocyanate, extra steps that are time-consuming. Although this HPLC method was shown to be quite sensitive in detecting low levels of CMA (10 pmol), very large culture volumes of *Psg* PG4180 (600 mL) were needed for accurate detection (*27*). Thus, a method for the rapid detection and quantification of CMA is currently unavailable.

Capillary electrophoresis (CE) is a highly sensitive method that can be used for the detection of various biological compounds including nonderivatized amino acids (*37*, *38*). Analysis of metabolites using CE is much faster than HPLC since gradient elution is not required (*38*−*40*). In addition, small sample volumes can be injected, and the amount of solvent waste produced is negligible in CE when compared to that in HPLC (*39*). Capillary zone electrophoresis (CZE), which is the most commonly used form of capillary electrophoresis, was used in this study for the detection of nonderivatized CMA. The aim of this study was to develop a valid CZE method for CMA production by *P. syringae*.

MATERIALS AND METHODS

Reagents. All reagents used were of analytical grade purity. Sodium hydroxide was purchased from Mallinckrodt Chemical, Inc. (Paris, KY, USA). Zeta buffers were from MicroSolv Technology Co., Eatontown, NJ, USA, and consisted of 100 mM sodium tetraborate (pH 9.2 or 8.2) and 100 mM sodium phosphate (pH 6.2, 4.3, or 2.5). Sodium dihydrogen phosphate monohydrate and acetonitrile were purchased from EMD Chemicals, Inc., Gibbstown, NJ, USA. Glycine sodium and acetic acid were obtained from Pharmco Science, Brookfield, CT, USA. Hydoxypropyl methyl cellulose (HPMC) and *n*-propanol were from Sigma Aldrich, St. Louis, MO, USA. Mannitol, glutamic acid, glucose, sucrose, rifampicin, kanamycin, pectinomycin, tetracycline, and chloramphenicol were also purchased from Sigma Aldrich.

Bacterial Strains, Plasmids, and Media. The bacterial strains and plasmids used in this study are described in **Table 1**. *P. syringae* strains were grown on mannitol−glutamate medium at 28 °C (*41*). Antibiotics used for the selection of *P. syringae* strains included (in μg/mL) rifampicin,

Table 1. Bacterial Strains and Plasmids Used in This Study

strain or plasmid	relevant characteristics[a]	reference or source
	P. syringae pv. *glycinea*	
Psg PG4180	CFA^+ CMA^+ COR^+	*13*
PG4180.D5	Km^R; *cfa8*::Tn*5*; CFA^- CMA^+COR^-	*13*
PG4180.C0	Km^R; *cmaB*::Tn*5*; CFA^+CMA^- COR^{-+}	*32*
Psg 18a/90	pathogenic to soybean, COR^-	*49*
	P. syringae pv. *tomato*	
Pst DC3000	Rif^R derivative of NCPPB1106	*50*
DB4G3	Rif^R Km^R; *cfa6*::Tn*5 uidA*; CFA^- CMA^+ COR^-	*30*
AK7E2	Rif^R Sm/Sp^R; *cmaA*::Tn*5 uidA*; CFA^- CMA^+ COR^-	*30*
AS1	Rif^R Km^R; *Pst* DC3000 containing Km^R cassette in *corR*	*46*
	Plasmids	
pYZ19A1	Tc^r; cosmid clone from p4180A in pRK7813	*36*
pYZ8H3	Tc^r; cosmid clone from p4180A in pRK7813	*36*

[a] Rif^R, Km^R, Sm^R, Tc^R, and Sp^R indicate resistance to rifampicin, kanamycin, streptomycin, tetracycline, and spectinomycin, respectively. COR, CMA, and CFA are abbreviations for coronatine, coronamic acid, and coronafacic acid, respectively.

100; kanamycin, 25; spectinomycin, 10 or 25; tetracycline, 25; and chloramphenicol, 25. *P. syringae* derivatives were incubated in Hoitink−Sinden medium supplemented with either 1 M glucose (HSC) (*33*) or 100 mM sucrose (HSS) (*42*).

Isolation of CMA. *Psg* PG4180 and *Pst* DC3000 were grown at 18 °C in 5, 10, 20 (2 × 10 mL), or 50 mL (5 × 10 mL) of HSC or HSS medium for 24, 48, 120, or 168 h. Derivatives of *Psg* PG4180 [PG4180.D5, (*cfa8* mutant); PG4180.C0 (*cmaB*)], *Pst* DC3000 [AK7E2 (*cmaA*); DB4G3 (*cfa6*)]; and *Psg* 18a/90 (containing pYZ19A1 or pYZ8H3) were grown in 20 mL (2 × 10 mL) of HSS medium at 18 °C for 3 days. The cells were pelleted by centrifugation (3450*g* for 10 min), and the supernatant was stored at 4 °C. COR, CFA, and coronafacoyl analogues were extracted from the culture supernatant with ethyl acetate at pH 2.0 as described previously (*33*). The aqueous phase was lyophilized and used for the detection of CMA. For the isolation of internal CMA from *Psg* 18a/90 containing pYZ19A1 or pYZ8H3, the cells were first pelleted by centrifugation. The pelletted cells were then suspended in 20 mL of sterile distilled H_2O and disrupted by sonication (*43*). The organic acid fraction and aqueous phase were extracted from the lysate using ethyl acetate as described above.

Sample Preparation and Capillary Zone Electorphoresis (CZE). Lyophilized bacterial samples and purified CMA standards (kindly provided by Dr. Robin E. Mitchell, HortResearch, New Zealand) were resuspended in 2 mL of 50% HPLC grade acetonitrile, vortexed for 20 min, and allowed to stand for 5 min to enable separation of three layers, namely, precipitate, emulsion, and a clear upper phase. A 50−100 μL aliquot from the upper phase of the resuspended samples, which contained CMA, was filtered with a 0.45 μm nylon membrane (SUNSri, Wilmington, NC) prior to analysis by CZE.

MicroSolv Zeta buffers at pH 2.5, 4.3, 6.2, 8.2, and 9.2 were used to determine the optimum pH for the analysis of CMA. The samples were resuspended in 1:1 (v/v) *n*-propanol and each of the Zeta buffers. After determining the optimum pH for the resolution of CMA, a phosphate buffer (100 mM phosphate, pH 2.5) containing 20% (v/v) acetonitrile, 0.4% (w/v) glycine, and 0.05% (w/v) hydoxypropyl methyl cellulose was used as the separation buffer in subsequent experiments.

CZE was performed on a Beckman P/ACE System (MDQ instrument from Beckman Instruments, Fullerton, CA) equipped with a UV/vis detector. A 30 cm fused-silica capillary column (20 cm to the detector; 50 μm internal diameter) from Polymicro Technologies Inc. (Phoenix, AZ) was used. The column for CZE was rinsed for 1 min at 20 psi with 0.1 N NaOH and 0.5 M acetic acid. The column was washed with nanopure water prior to rinsing with each solvent and then saturated with phosphate separating buffer. The samples were injected at 0.5 psi for 5 s and analyzed using a voltage of 12 kV and detection at 214 nm.

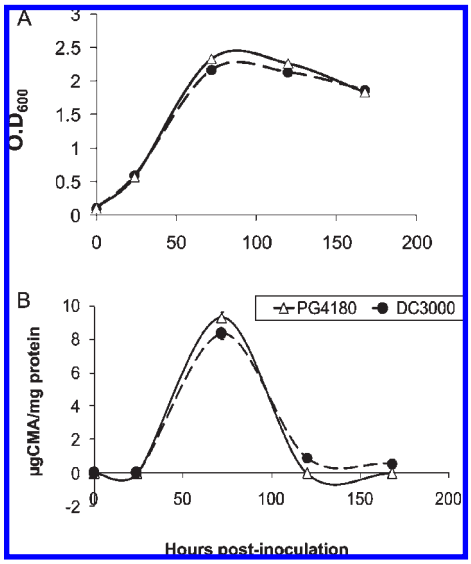

Figure 2. Time course comparing OD_{600} values and levels of CMA produced by *Psg* PG4180 and *Pst* DC3000. The strains were grown in HSS medium at 18 °C, and the samples were removed 0, 24, 72, 120, and 168 h postinoculation as described in Materials and Methods. (**A**) Growth (OD_{600}) of the strains *Psg* PG4180 and *Pst* DC3000. (**B**) CMA production by *Psg* PG4180 and *Pst* DC3000. Ethyl acetate (pH 2.5) was added to culture supernatants, and the aqueous phase was lyophilized and used for CMA quantification by capillary zone electrophoresis. The organic phase was used for the quantification of CFA and COR production by HPLC.

Quantification of CMA. A calibration curve for CMA was constructed by injecting a dilution series of CMA ranging from 0.03125 to 2.5 μg/μL (five calibration levels). Quantitative information on CMA was then obtained from the calibration curve ($y = 19723x$). To confirm the identity of the CMA peak in the electropherogram, a stock solution of pure crystalline CMA was prepared and used for spiking the sample extracts prior to analysis. A percentage increase in the area and height of the peak of interest and similar electrophoretic mobility of the CMA standard and sample were used to positively identify CMA in the samples. All analyses were performed on three replicates with three subsamples, and the average yields were reported.

Recombinant DNA Methods. Plasmid DNA was isolated from *P. syringae* (*44*) and *Escherichia coli* using standard procedures (*45*). The construction of cosmids pYZ19A1 and pYZ8H3 have been described elsewhere (*36*). ^{32}P-labeled probes were prepared using Rad Prime DNA Labeling System (Gibco BRL, Gaithersburg, MD). Plasmids were mobilized into recipient bacterial strains and mutants by electroporation or transformation (*E. coli*) (*45*).

RESULTS AND DISCUSSION

Optimization of Culture Volume for CMA Extraction. HSC and HSS media were evaluated for growing bacterial cells prior to CZE analysis. Preliminary results using *Pst* DC3000 and *Psg* PG4180 indicated that the higher amount of sugar present in HSC medium interfered with the quantification of CMA by CZE; thus, cultures were grown in HSS medium.

P. syringae cells were grown in HSS medium at 18 °C for 3 days. Different volumes (5, 10, 20, and 50 mL) of *Psg* PG4180 and *Pst* DC3000 cultures were used to determine the optimum volume of bacterial supernatant required for CMA detection. For both strains, the extract from a 50 mL culture was extremely viscous and not used for further studies. A volume of 20 mL was optimal

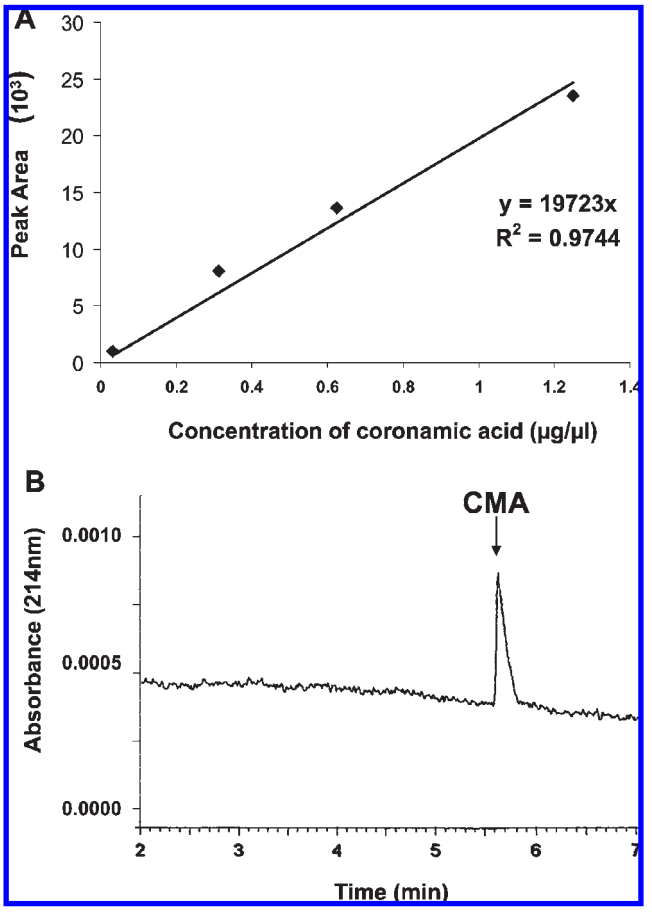

Figure 3. Detection and quantification of CMA using capillary zone electrophoresis. (**A**) Calibration curve for CMA quantification. (**B**) Electropherogram showing the resolution of the CMA peak in the CMA standard.

for the detection of CMA in both *Pst* DC3000 and *Psg* PG4180 and was chosen for further experiments.

Optimization of Time for CMA Detection. The kinetics of CMA production by *Psg* PG4180 and *Pst* DC3000 was evaluated by growing fermentations in HSS medium at 18 °C for different lengths of time (24, 72, 120, and 168 h). Culture supernatants were analyzed for CMA as described above. Growth of *Pst* DC3000 and *Psg* PG4180 was similar throughout the 168 h sampling period (**Figure 2A**). Neither strain produced detectable CMA at 24 h (**Figure 2B**). The highest level of CMA in both strains was detected at 72 h (**Figure 2B**); thus, this time point was chosen for detection of CMA in *Psg* PG4180, *Pst* DC3000, and derivative strains. In *Psg* PG4180, CMA was not detected at 120 or 168 h postinoculation; however, in DC3000, detectable levels of CMA were present at 120 h (0.9 ± 0.04 µg/mL) and 168 h (0.56 ± 0.01 µg/mL) (**Figure 2B**). These results suggest that the kinetics of CMA production by *Psg* PG4180 and *Pst* DC3000 are different. Thus, it is important to evaluate individual strains at various incubation times before selecting the time for CMA detection since this may vary with the genetic background of the bacterium.

Detection of CMA by CZE. A distinct peak for CMA was observed when both the CMA standard and extracts from *Psg* PG4180 and *Pst* DC3000 were analyzed using HPMC phosphate buffer at pH 2.5 (214 nm). The calibration curve for CMA was linear from 0.031 to 1.2 µg/µL (**Figure 3A**), and the lower limit for CMA detection was 0.031 ng/µL. The apparent electrophoretic mobility (1.46×10^{-4} cm^2/V·s) (**Table 2**) and migration time for CMA (5.69 ± 0.05 min) (**Figure 3B**) were reproducible. The presence of CMA in the samples was confirmed by spiking the sample with the CMA standard (**Figure 4**). **Table 2** shows the peak area in DC3000 compared with that of the extract spiked with the CMA standard. The relative larger standard deviation on the spiked sample area suggests that variations in the incorporation of purified CMA in the spiked samples are probably a source of error. Representative electropherograms of extracts from *Pst* DC3000 and *Pst* DC3000 spiked with the CMA standard are shown in **Figure 4**. The electropherograms suggest a good separation of CMA from other components in the extract.

Table 2. Representative Peak Migration Time, Electrophoretic Mobility, and Area of DC3000 and DC3000 Spiked with CMA Standard Obtained from CZE

sample	migration time (min)[a]	μ_{app}[b] cm^2/(V·s)	area[a] (arbitrary units)
CMA standard	5.69 ± 0.05	1.46×10^{-4}	3734 ± 314
DC 3000	5.77 ± 0.05	1.44×10^{-4}	1153 ± 143
DC3000 spiked w/ CMA	5.60 ± 0.01	1.49×10^{-4}	1958 ± 519

[a] Mean ± standard deviation ($n = 6$). [b] Electrophoretic mobility (apparent).

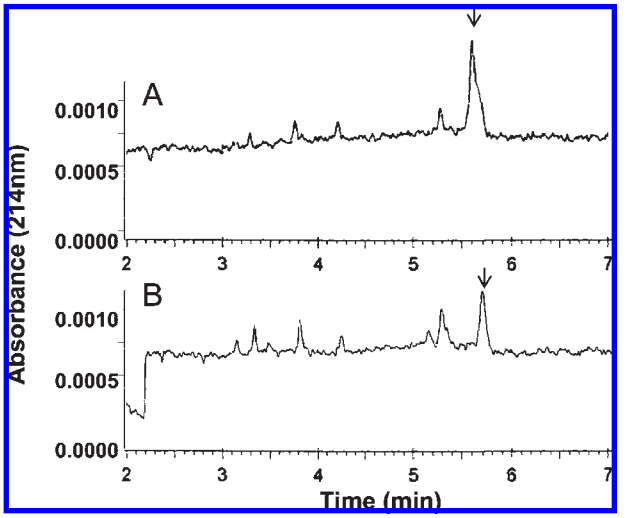

Figure 4. Electropherograms showing the resolution of the CMA peak in DC3000 spiked with the CMA standard (**A**) and DC3000 not spiked (**B**).

Detection of CMA in Selected Strains of *P. syringae*. Derivatives of *Psg* PG4180, *Pst* DC3000, and *Psg* 18a/90 were used for validating the CZE method. As expected, CMA-defective mutants AK7E2 (*cmaA*) and PG4180.C0 (*cmaB*) (**Figure 1B**), did not produce detectable levels of CMA (**Table 3**). However, the CFA$^-$ CMA$^+$ mutants, PG4180.D5 (*cfa8*) and DB4G3 (*cfa6*) produced 12.9 and 4 µg/mL of CMA, respectively (**Table 3**).

In addition to the mutants of *Pst* DC3000 defective in genes encoding CFA and CMA biosynthesis (DB4G3 and AK7E2), a regulatory mutant of *Pst* DC3000, AS1 (*corR*) (*46*), was also analyzed for CMA production. Preliminary studies using indirect assays (e.g., exogenous feeding and cocultivation experiments) suggested that the *corR* mutant was defective in CMA biosynthesis (*46*). Furthermore, transcriptional activity of the *cmaB* gene was significantly impaired (approximately 100-fold lower) in the *corR* mutant when compared to that in *Pst* DC3000 (*46*). In this study, CZE analysis of AS1 confirmed that this mutant was defective in CMA production (**Table 3**), which indicates a regulatory role for *corR* in CMA biosynthesis by *Pst* DC3000.

Psg 18a/90 and derivatives were also analyzed for CMA production. Two clones designated pYZ19A1 and pYZ8H3 were introduced into *Psg* 18a/90, which lacks the COR gene cluster. Clone pYZ19A1 contains the intact COR gene cluster from *Psg* PG4180 and has been described elsewhere (*36*). Sequence analysis of pYZ8H3 indicated that this clone contained all COR genes

Table 3. Production of CMA (Present in the Supernatant) by *P. syringae* pv. *glycinea* PG4180, *P. syringae* pv. *tomato* DC3000, *Psg* 18a/90, and selected mutants[a]

strain	expected CMA production in culture supernatant	CMA (μg/mL)
PG4180	CMA^+	9.3
PG4180.C0	CMA^-	ND[b]
PG4180.D5	CMA^+	12.9
DC3000	CMA^+	8.3
AK7E2	CMA^+	ND
DB4G3	CMA^-	4.0
AS1	CMA^-	ND
Psg 18a/90	CMA^-	ND
Psg 18a/90 (pYZ19A1)	CMA^+	3.2
Psg 18a/90 (pYZ8H3)	CMA^-	ND

[a] Production of CMA was analyzed by growing the cultures in HSS medium at 18 °C for 72 h and removing the organic acids using ethyl acetate extraction at pH 2.5. The aqueous phase was lyophilized and used for the detection of CMA by capillary zone electrophoresis. This experiment was repeated twice. [b] ND = not detected.

Table 4. Production of CMA (Present in the Cell Lysate) by *P. syringae* pv. *glycinea* 18a/90 and Transconjugants[a]

strain	expected CMA production in cell lysate	CMA (μg/mL)
Psg 18a/90	CMA^+	ND[b]
Psg 18a/90 (pYZ19A1)	CMA^+	10
Psg 18a/90 (pYZ8H3)	CMA^+/CMA^-	ND

[a] Production of CMA was analyzed by growing the cultures in HSS medium at 18 °C for 72 h, pelleting and lysing the cells using sonication, and removing the organic acids from the cell lysate using ethyl acetate extraction at pH 2.5. CMA was detected and quantified after freeze drying the remaining aqueous phase using capillary zone electrophoresis. This experiment was repeated twice. [b] ND = not detected.

contained in pYZ19A1 with the exception of a 276 nucleotide sequence located at the 3′ end of *cmaU*. As expected, the culture supernatant of *Psg* 18a/90, which lacks the COR gene cluster, did not contain CMA (**Table 3**). However, *Psg* 18a/90(pYZ19A1) produced 3.2 μg CMA/mg protein (**Table 3**). *Psg* 18a/90-(pYZ8H3), which lacks an intact copy of *cmaU*, failed to produce CMA (**Table 3**).

Detection of CMA in the Cellular Lysates of *Psg* 18a/90 and Transconjugants. Bioinformatic analysis of *cmaU* (GenBank AY391839) showed amino acid relatedness to threonine efflux proteins; thus, we speculated that CmaU could be involved in the export of CMA (*47*, *48*). Hence, the absence of CMA in the culture supernatant of 18a/90(pYZ8H3) further suggested that CmaU might be involved in the biosynthesis or export of CMA. If CmaU is involved in the export of CMA, we predicted that CMA would accumulate inside cells of *Psg* 18a/90(pYZ8H3). Thus, CMA would be absent from culture supernatants of *Psg* 18a/90(pYZ8H3) but present in the cellular lysate. To test this hypothesis, we standardized a procedure for the extraction and detection of the CMA contained inside bacterial cells. *Psg* 18a/90(pYZ19A1) and 18a/90(pYZ8H3) were grown in HSS medium for 3 days; cells were then harvested by centrifugation, lysed, and analyzed for CMA as described above. The lysate of *Psg* 18a/90(pYZ8H3) did not contain detectable levels of CMA (**Table 4**); however, cellular lysates of *Psg* 18a/90(pYZ19A1) contained 10 μg CMA/mg protein (**Table 4**). Thus, our results indicate that CmaU has no obvious role in the export of CMA but could be involved in CMA biosynthesis.

Detection of CMA by CZE and HPLC. In a previous study, amino acid extracts containing CMA were partially purified by column chromatography, derivatized using phenylisothiocyanate, and separated by HPLC (*27*). In the present study, culture supernatants were extracted with ethyl acetate (pH 2.5), and the aqueous phase was lyophilized and used for CMA detection. Using the method described in the present study, organic phases from the same extract can also be used for the detection of COR and CFA. The CZE method required fewer than half the steps for CMA isolation as compared to the HPLC method (*27*), and the total time for the separation and detection of CMA using CZE was only half the time required for HPLC detection. Thus, one advantage of CZE included rapid and efficient sample preparation since one sample could be simultaneously analyzed for CMA (by CZE) and CFA and COR (by HPLC). Furthermore, only 20 mL of cell culture was needed for CMA detection using CZE as compared to the 600 mL required for the HPLC method previously described (*27*).

CMA detection using CZE is a fast, easy, and cost-effective alternative to HPLC-based methods. This simple and highly efficient method was successfully applied for the analysis of CMA present in the culture supernatants and inside the cells of various *P. syringae* strains. CZE is a valuable method for the detection of CMA and will be useful in future studies to more clearly define the roles of genes in the CMA biosynthetic gene cluster.

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LITERATURE CITED

(1) Kenyon, J. S.; Turner, J. G. The stimulation of ethylene synthesis in *Nicotiana tabacum* leaves by the phytotoxin coronatine. *Plant Physiol.* **1992**, *100*, 219–224.

(2) Sakai, R.; Nishiyama, K.; Ichihara, A.; Shiraishi, K.; Sakamura, S. The Relation between Bacterial Toxic Action and Plant Growth Regulation. In *Recognition and Specificity in Plant Host-Parasite Interactions*; Daly, J. M., Uritani, I., Eds.; University Park Press: Baltimore, MD, 1979; pp 165−179.

(3) Weiler, E. W.; Kutchan, T. M.; Gorba, T.; Brodschelm, W.; Niesel, U.; Bublitz, F. The *Pseudomonas* phytotoxin coronatine mimics octadecanoid signalling molecules of higher plants. *FEBS Lett.* **1994**, *345*, 9–13.

(4) Feys, B.; Benedetti, C. E.; Penfold, C. N.; Turner, J. G. Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial phytotoxin. *Plant Cell* **1994**, *6*, 751–759.

(5) Uppalapati, S. R.; Ayoubi, P.; Weng, H.; Palmer, D. A.; Mitchell, R. E.; Jones, W.; Bender, C. L. The phytotoxin coronatine and methyl jasmonate impact multiple phytohormone pathways in tomato. *Plant J.* **2005**, *42*, 207–217.

(6) Lauchli, R.; Boland, W. Indanoyl amino acid conjugates: tunable elicitors of plant secondary metabolism. *Chem. Rec.* **2003**, *3*, 12–21.

(7) Xie, Z.; Duan, L.; Tian, X.; Wang, B.; Egrinya Eneji, A.; Li, Z. Coronatine alleviates salinity stress in cotton by improving the antioxidative defense system and radical-scavenging activity. *J. Plant Physiol.* **2008**, *165*, 375–384.

(8) Burns, J. K.; Pozo, L. V.; Arias, C. R.; Hockema, B.; Rangaswamy, V.; Bender, C. L. Coronatine and abscission in citrus. *J. Am. Soc. Hortic. Sci.* **2003**, *128*, 309–315.

(9) Ichihara, A.; Shiraishi, K.; Sato, H.; Sakamura, S.; Nishiyama, K.; Sakai, R.; Furusaki, A.; Matsumoto, T. The structure of coronatine. *J. Am. Chem. Soc.* **1977**, *99*, 636–637.

(10) Jiralerspong, S.; Rangaswamy, V.; Bender, C. L.; Parry, R. J. Analysis of the enzymatic domains in the modular portion of the coronafacic acid polyketide synthase. *Gene* **2001**, *270*, 191–200.

(11) Parry, R. J.; Mhaskar, S. V.; Lin, M. T.; Walker, A. E.; Mafoti, R. Investigations of the biosynthesis of the phytotoxin coronatine. *Can. J. Chem.* **1994**, *72*, 86–99.

(12) Vaillancourt, F. H.; Yeh, E.; Vosburg, D. A.; O'Connor, S. E.; Walsh, C. T. Cryptic chlorination by a non-haem iron enzyme during cyclopropyl amino acid biosynthesis. *Nature* **2005**, *436*, 1191–1194.

(13) Bender, C. L.; Liyanage, H.; Palmer, D.; Ullrich, M.; Young, S.; Mitchell, R. Characterization of the genes controlling biosynthesis of the polyketide phytotoxin coronatine including conjugation between coronafacic and coronamic acid. *Gene* **1993**, *133*, 31–38.
(14) Mitchell, R. E. Norcoronatine and *N*-coronafacoyl-L-valine, phytotoxic analogues of coronatine produced by a strain of *Pseudomonas syringae* pv. *glycinea*. *Phytochemistry* **1985**, *24*, 1485–1487.
(15) Mitchell, R. E. Implications of toxins in the ecology and evolution of plant pathogenic microorganisms: Bacteria. *Experientia* **1991**, *47*, 791–803.
(16) Mitchell, R. E.; Ford, K. L. Chlorosis-inducing products from *Pseudomonas syringae* pathovars: new *N*-coronafacoyl compounds. *Phytochemistry* **1998**, *49*, 1579–1583.
(17) Mitchell, R. E.; Young, H. *N*-coronafacoyl-L-isoleucine and *N*-coronafacoyl-L-alloisoleucine, potential biosynthetic intermediates of the phytotoxin coronatine. *Phytochemistry* **1985**, *24*, 2716–2717.
(18) Palmer, D. A.; Bender, C. L. Ultrastructure of tomato leaf tissue treated with the Pseudomonad phytotoxin coronatine and comparison with methyl jasmonate. *Mol. Plant-Microbe Interact*. **1995**, *8*, 683–692.
(19) Bender, C. L.; Young, S. A.; Mitchell, R. E. Conservation of plasmid DNA sequences in coronatine-producing pathovars of *Pseudomonas syringae*. *Appl. Environ. Microbiol*. **1991**, *57*, 993–999.
(20) Penaloza-Vazquez, A.; Bender, C. L. Characterization of CorR, a transcriptional activator which is required for biosynthesis of the phytotoxin coronatine. *J. Bacteriol*. **1998**, *180*, 6252–6259.
(21) Rangaswamy, V.; Bender, C. L. Phosphorylation of CorS and CorR, regulatory proteins that modulate production of the phytotoxin coronatine in *Pseudomonas syringae*. *FEMS Microbiol. Lett*. **2000**, *193*, 13–18.
(22) Ullrich, M.; Penaloza-Vazquez, A.; Bailey, A. M.; Bender, C. L. A modified two-component regulatory system is involved in temperature-dependent biosynthesis of the *Pseudomonas syringae* phytotoxin coronatine. *J. Bacteriol*. **1995**, *177*, 6160–6169.
(23) Rangaswamy, V.; Mitchell, R.; Ullrich, M.; Bender, C. L. Analysis of genes involved in biosynthesis of coronafacic acid, the polyketide component of the phytotoxin coronatine. *J. Bacteriol*. **1998**, *180*, 3330–3338.
(24) Couch, R.; O'Connor, S. E.; Seidle, H.; Walsh, C. T.; Parry, R. Characterization of CmaA, an adenylation-thiolation didomain enzyme involved in the biosynthesis of coronatine. *J. Bacteriol*. **2004**, *186*, 35–42.
(25) Ullrich, M.; Bender, C. L. The biosynthetic gene cluster for coronamic acid, an ethylcyclopropyl amino acid, contains genes homologous to amino acid activating enzymes and thioesterases. *J. Bacteriol*. **1994**, *176*, 7574–7586.
(26) Patel, J.; Hoyt, J. C.; Parry, R. J. Investigations of coronatine biosynthesis. Overexpression and assay of CmaT, a thioesterase involved in coronamic acid biosynthesis. *Tetrahedron* **1998**, *54*, 15927–15936.
(27) Ullrich, M.; Guenzi, A. C.; Mitchell, R. E.; Bender, C. L. 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*. **1994**, *60*, 2890–2897.
(28) Moore, R. A.; Starratt, A. N.; Ma, S. W.; Morris, V. L.; Cuppels, D. A. Identification of a chromosomal region required for biosynthesis of the phytotoxin coronatine by *Pseudomonas syringae* pv. *tomato*. *Can. J. Microbiol*. **1989**, *35*, 910–917.
(29) Bender, C. L.; Alarcon-Chaidez, F.; Gross, D. C. *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiol. Mol. Biol. Rev*. **1999**, *63*, 266–292.
(30) Brooks, D. M.; Hernandez-Guzman, G.; Kloek, A. P.; Alarcon-Chaidez, F.; Sreedharan, A.; Rangaswamy, V.; Penaloza-Vazquez, A.; Bender, C. L.; Kunkel, B. N. Identification and characterization of a well-defined series of coronatine biosynthetic mutants of *Pseudomonas syringae* pv. *tomato* DC3000. *Mol. Plant-Microbe Interact*. **2004**, *17*, 162–174.
(31) Liyanage, H.; Palmer, D. A.; Ullrich, M.; Bender, C. L. Characterization and transcriptional analysis of the gene cluster for coronafacic acid, the polyketide component of the phytotoxin coronatine. *Appl. Environ. Microbiol*. **1995**, *61*, 3843–3848.
(32) Young, S. A.; Park, S. K.; Rodgers, C.; Mitchell, R. E.; Bender, C. L. Physical and functional characterization of the gene cluster encoding the polyketide phytotoxin coronatine in *Pseudomonas syringae* pv. *glycinea*. *J. Bacteriol*. **1992**, *174*, 1837–1843.
(33) Palmer, D. A.; Bender, C. L. Effects of environmental and nutritional factors on production of the polyketide phytotoxin coronatine by *Pseudomonas syringae* pv. *glycinea*. *Appl. Environ. Microbiol*. **1993**, *59*, 1619–1626.
(34) Jones, W. T.; Harvey, D.; Mitchell, R. E.; Ryan, G. B.; Bender, C. L.; Reynolds, P. H. S. Competitive ELISA employing monoclonal antibodies specific for coronafacoyl amino acid conjugates. *Food Agric. Immunol*. **1997**, *9*, 67–76.
(35) Jones, W. T.; Harvey, D.; Zhao, Y.; Mitchell, R. E.; Bender, C. L.; Reynolds, P. H. S. Monoclonal antibody-based immunoassays for the phytotoxin coronatine. *Food Agric. Immunol*. **2001**, *13*, 19–32.
(36) Zhao, Y. Biology, Genetics, And Coronatine Production of Bacterial Pathogens of Leafy Green Crucifers. Ph.D. Thesis, Oklahoma State University, Stillwater, OK, **2001**.
(37) Coufal, P.; Zuska, J.; van de Goor, T.; Smith, V.; Gas, B. Separation of twenty underivatized essential amino acids by capillary zone electrophoresis with contactless conductivity detection. *Electrophoresis* **2003**, *24*, 671–677.
(38) Smith, J. T. Recent advancements in amino acid analysis using capillary electrophoresis. *Electrophoresis* **1999**, *20*, 3078–3083.
(39) Dong, Q.; Jin, W.; Shan, J. Analysis of amino acids by capillary zone electrophoresis with electrochemical detection. *Electrophoresis* **2002**, *23*, 559–564.
(40) Smith, J. T. Developments in amino acid analysis using capillary electrophoresis. *Electrophoresis* **1997**, *18*, 2377–2392.
(41) Keane, P. J.; Kerr, A.; New, P. B. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. *Aust. J. Biol. Sci*. **1970**, *23*, 585–595.
(42) Penaloza-Vazquez, A.; Preston, G. M.; Collmer, A.; Bender, C. L. Regulatory interactions between the Hrp type III protein secretion system and coronatine biosynthesis in *Pseudomonas syringae* pv. *tomato* DC3000. *Microbiology* **2000**, *146*, 2447–2456.
(43) Riggs, P. Expression and Purification of Maltose-Binding Protein Fusions. In *Current Protocols in Molecular Biology*; Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K., Eds.; Wiley Interscience: New York, 1994; pp 16.6.1−16.6.13.
(44) Kado, C. I.; Liu, S. T. Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol*. **1981**, *145*, 1365–1373.
(45) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning, A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.
(46) Sreedharan, A.; Penaloza-Vazquez, A.; Kunkel, N. K.; Bender, C. L. CorR regulates multiple components of virulence in *Pseudomonas syringae* pv. *tomato* DC3000. *Mol. Plant-Microbe Interact*. **2006**, *19*, 768–779.
(47) Aleshin, V. V.; Zakataeva, N. P.; Livshits, V. A. A new family of amino-acid-efflux proteins. *Trends Biochem. Sci*. **1999**, *24* (4), 133–135.
(48) Bender, C. L.; Scholz-Schroeder, B. K. New Insights into the Biosynthesis, Mode of Action, And Regulation of Syringomycin, Syringopeptin and Coronatine. In *The Pseudomonads*; Ramos, J. L., Ed.; Kluwer Academic Press: Dordrecht, The Netherlands, 2004; Vol. II, pp 125−158.
(49) Ullrich, M.; Bereswill, S.; Völksch, B.; Fritsche, W.; Geider, K. Molecular characterization of field isolates of *Pseudomonas syringae* pv. *glycinea* differing in coronatine production. *J. Gen. Microbiol*. **1993**, *139*, 1927–1937.
(50) Cuppels, D. A. Generation and characterization of Tn*5* insertion mutations in *Pseudomonas syringae* pv. *tomato*. *Appl. Environ. Microbiol*. **1986**, *51*, 323–327.

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