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Characterization of the Biosynthetic Operon for the Antibacterial Peptide Herbicolin in *Pantoea vagans* Biocontrol Strain C9-1 and Incidence in *Pantoea* Species

Tim Kamber,[a] Theresa A. Lansdell,[b] Virginia O. Stockwell,[c] Carol A. Ishimaru,[b] Theo H. M. Smits,[a] and Brion Duffy[a]

Agroscope Changins-Wädenswil ACW, Plant Protection Division, Wädenswil, Switzerland[a]; Department of Plant Pathology, University of Minnesota, St. Paul, Minnesota, USA[b]; and Oregon State University, Department of Botany and Plant Pathology, Corvallis, Oregon, USA[c]

***Pantoea vagans* C9-1 is a biocontrol strain that produces at least two antibiotics inhibiting the growth of *Erwinia amylovora*, the causal agent of fire blight disease of pear and apple. One antibiotic, herbicolin I, was purified from culture filtrates of *P. vagans* C9-1 and determined to be 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine, also known as N_{β}-epoxysuccinamoyl-DAP-valine. A plasposon library was screened for mutants that had lost the ability to produce herbicolin I. It was shown that mutants had reduced biocontrol efficacy in immature pear assays. The biosynthetic gene cluster in *P. vagans* C9-1 was identified by sequencing the flanking regions of the plasposon insertion sites. The herbicolin I biosynthetic gene cluster consists of 10 coding sequences (CDS) and is located on the 166-kb plasmid pPag2. Sequence comparisons identified orthologous gene clusters in *Pantoea agglomerans* CU0119 and *Serratia proteamaculans* 568. A low incidence of detection of the biosynthetic cluster in a collection of 45 *Pantoea* spp. from biocontrol, environmental, and clinical origins showed that this is a rare trait among the tested strains.**

Erwinia amylovora, the causal agent of the blight disease, is a major threat to pome fruit production. The pathogen colonizes flowers and, under favorable weather conditions, enters through the nectarthodes, kills tissues, and spreads throughout the plant (34, 44). Until now, no cure for infected plants has been known, and diseased tissues have to be removed by pruning. Measures to reduce disease incidence include antibiotic and biocontrol agent application during bloom. The emerging resistance of *E. amylovora* to the most effective antibiotic, streptomycin (6, 25), raises the need for further management measures. Application of biocontrol agents of the related enterobacterial genus *Pantoea* can remarkably reduce epiphytic growth of the pathogen (2, 11, 29, 40, 41). Site exclusion, nutrient competition, and antibiotic production contribute to the effectiveness of biocontrol agents (23, 24, 28, 45, 47, 48).

Pantoea vagans C9-1, recently reassigned from the closely related *Pantoea agglomerans* (31, 32), is an effective biocontrol agent inhibiting the growth of *E. amylovora in vitro* (18) and in orchard trials (41, 42). The biosynthesis of at least two antibiotics contributes to the suppression of *E. amylovora* in immature pear assays (18). The effectiveness of *P. vagans* C9-1 was evaluated in field trials, resulting in a significant reduction of fire blight incidence (41, 42). *P. vagans* C9-1 has been commercialized as BlightBan C9-1 (NuFarm Americas, Burr Ridge, IL) for biocontrol of fire blight of pear and apple.

Many *Pantoea* species produce one or multiple antibiotics that are effective against fire blight, including a phenazine antibiotic produced by *P. agglomerans* Eh1087 (12), pantocin A, produced by *P. agglomerans* strains P10c and Eh318 and *Pantoea* sp. Eh252 (21, 31, 40, 47), and pantocin B, produced by *P. agglomerans* Eh318 (3, 49). Although the production of antibiotics is observed for many *Pantoea* species, the biosynthesis genes of only a few have been identified (12, 15, 20, 21). The identification of antibiotic biosynthetic gene clusters allows the screen for potential antibiotic producing strains and the assessment of novel biocontrol agents.

The main objective of this study was to genetically and chemically characterize the antibiotic produced by *P. vagans* C9-1 given the common name herbicolin I. A recent study indicated that herbicolin I could be identical to 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine, produced by *Pantoea agglomerans* 48b/90 (35), of which the biosynthetic genes were to date unknown. This study confirms the chemical structure of herbicolin I, while the gene cluster was identified using plasposon mutants. The analysis of the biosynthetic genes revealed a similar gene cluster in *P. agglomerans* CU0119, which produces a family of dapdiamide antibiotics (dapdiamide A to E) (7, 16). Additionally, we evaluated the incidence of homologous biosynthetic genes across a wide range of *Pantoea* spp., including plant, environmental, and clinical isolates.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. vagans* C9-1, originally isolated from apple (*Malus* × *domestica* 'Jonathan'; Michigan) (18), was used throughout. Additional bacterial strains used are listed in Table 1. *Escherichia coli* S17-1 λ*pir* was used for transformation of rescued pTn*Mod*-RKm′ plasposons (R6K *ori*, Tn*Mod*, Km^r, RP4, *oriT*, Tn5*tnp*). All strains were stored at −80°C and routinely cultured in Luria-Bertani (LB) medium. *E. coli* was cultured at 37°C, and all other strains were cultured at 26°C. Kanamycin (Km) was used at a concentration of 50 μg ml^{-1} as appropriate.

Antimicrobial production and activity assays. Antibiotic production was examined in a double diffusion assay on MGA medium (morpholinopropanesulfonic acid [MOPS], gluconate, asparagine medium) at 26°C (18). Bacteria were grown for 18 to 24 h in LB medium or LB medium with

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Address correspondence to Brion Duffy, duffy@acw.admin.ch.

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TABLE 1 *Pantoea* species collection used for prevalence screening of pantocin A and dapdiamide biosynthetic genes

Strain	Origin (reference)	Host	Properties	PCR result	
				paaABC[a]	*ddaD*, *ddaF*[b]
Pantoea vagans					
C9-1	USA (18)	*Malus* × *domestica* (apple stem)	Biocontrol agent	+	+
C9-1W	Switzerland (38)	NA[c]	Nonpigmented, pPag3 plasmid-cured variant of C9-1	+	+
LMG 24196	Argentina (31)	*Eucalyptus* sp.	Phytopathogen	−	−
LMG 24199^T	Uganda (31)	*Eucalyptus* sp.	Phytopathogen	−	−
Pantoea agglomerans					
CPA-2	Spain (27)	*Malus* × *domestica* (fruit surface)	Biocontrol agent	−	−
E325	USA (29)	*Malus* × *domestica* (flower)	Biocontrol agent	−	−
Eh1087	New Zealand (24)	*Malus* × *domestica* (flower)	Biocontrol agent	−	−
Eh239	USA (4)	*Hordeum vulgare* (barley, kernel)	Biocontrol agent	−	−
Eh318	USA (49)	*Malus* × *domestica* (stem)	Biocontrol agent	+	−
Eh454	USA (4)	*Hordeum vulgare* (kernel)	Biocontrol agent	−	−
Eh460	USA (4)	*Hordeum vulgare* (kernel)	Biocontrol agent	−	−
P10c	New Zealand (46)	*Malus* × *domestica* (flower)	Biocontrol agent	+	−
EPS125	Spain (5)	*Pyrus communis* (pear, fruit surface)	Biocontrol agent	−	−
ATCC 27987 (CDC 1400-74)	USA (31)	Human (ear)	Clinical isolate	−	−
ATCC 27998 (CDC 1741-71)	USA (31)	Human (trachea)	Clinical isolate	−	−
EM 21cb	Spain (31)	Human (bile)	Clinical isolate	−	−
EM 22cb	Spain (31)	Human (blood)	Clinical isolate	−	−
LMG 1286^T	Zimbabwe (31)	Human (knee wound)	Clinical isolate	−	−
VA21971	Spain (31)	Human (arm wound)	Clinical isolate	−	−
CIP 82.100	Canada (31)	*Triticum aestivum* (wheat)	Environmental	−	−
LMG 2557	United Kingdom (31)	*Pyrus communis*	Environmental	−	−
LMG 2595	South Africa (31)	*Allium cepa* (onion, necrotic stalk/leaf)	Environmental	−	−
LMG 2941	NA (31)	*Malus sylvestris* (leaf, epiphyte)	Environmental	−	−
P3SA	Australia (31)	*Triticum aestivum* (rhizosphere)	Environmental	−	−
Pantoea agglomerans pv. gypsophilae					
ATCC 43348	USA (31)	*Gypsophila paniculata* (baby's breath, plant gall)	Phytopathogen	−	−
CFBP 4342	The Netherlands (31)	*Gypsophila paniculata* (plant gall)	Phytopathogen	−	−
Pantoea sp.					
Eh252	USA (44)	*Malus pumila*	Biocontrol agent	+	−
EPS486	Spain (31)	*M.* × *domestica* (bud)	Environmental	−	−
EPS595	Spain (31)	*Pyrus communis* (bud)	Environmental	−	−
Pantoea septica					
LMG 5345	USA (31)	Human	Clinical isolate	−	−
Pantoea brenneri					
ATCC 29001 (CDC 164-75)	USA (31)	Human (prostate)	Clinical isolate	−	−
LMG 5343^T	USA (31)	Human (urethra)	Clinical isolate	−	−
Pantoea dispersa					
CIP 102701	France (31)	Human (ear)	Clinical isolate	−	−
LMG 2770	USA (31)	Human (blood)	Clinical isolate	−	−
LMG 2603^T	Japan (31)	Soil	Environmental	−	−
LMG 2605	Tanzania (31)	*Vigna unguiculata* (cowpea, seed)	Environmental	−	−
Pantoea ananatis					
ATCC 27995 (CDC 4854-73)	USA (31)	Human	Clinical isolate	−	−
LMG 5342	USA (31)	Human	Clinical isolate	−	−
ATCC 27996	USA (31)	Insect	Environmental	−	−
LMG 20103	South Africa (31)	*Eucalyptus* sp.	Phytopathogen	−	−
LMG 2665^T	Brazil (31)	*Ananas comosus* (pineapple)	Phytopathogen	−	−
LMG 2676	USA (31)	*Puccinia graminis* f. sp. *tritici* (cereal stem rust-black rust)	Phytopathogen	−	−
Pantoea conspicua					
EM 17cb	Spain (31)	Human (blood)	Clinical isolate	−	−
Pantoea stewartii subsp. *indologenes*					
CFBP 3614^T	India (31)	*Setaria italica* (foxtail millet, leaf spot)	Phytopathogen	−	−

(Continued on following page)

TABLE 1 (Continued)

Strain	Origin (reference)	Host	Properties	PCR result	
				paaABC[a]	*ddaD*, *ddaF*[b]
Pantoea stewartii subsp. *stewartii* CFBP 3517^T	USA (31)	*Zea mays* var. *rugosa* (maize, corn)	Phytopathogen	–	–
Erwinia oleae CFBP 6632^T	Spain (31)	*Olea europaea* (olive, plant gall)	Phytopathogen	–	–
Enterobacter sp. LMG 5339	USA (31)	*Gallus gallus* (chicken, liver)	Veterinary isolate	–	–
Tatumella citrea (ex. *Pantoea citrea*) LMG 23359	Philippines (31)	*Ananas comosus*	Environmental	–	–
Tatumella punctata (ex. *Pantoea punctata*) LMG 22097	Japan (31)	*Citrus* × *sinensis* (orange)	Environmental	–	–

[a] *paaABC*, pantocin A biosynthesis genes used as a target in PCR to identify potential pantocin A-producing strains. Data are taken from reference 31.
[b] *ddaD*, *ddaF*, dapdiamide biosynthesis genes used as a target in PCR to identify potential dapdiamide-producing strains.
[c] NA, not applicable.

Km (LB-Km) for mutants. A 10-μl cell suspension (0.1 optical density at 600 nm [OD_{600}]) was spotted onto the basal MGA layer. The colonies were grown for 48 h, exposed to chloroform, and subsequently overlaid with molten MGA agar seeded with wild-type *E. amylovora* Ea110 and pantocin A (CIR555)- and herbicolin I (CIR550)-resistant mutants (18). The overlays were incubated for 48 h and visually inspected for zones of inhibition. Antimicrobial activities of purified antibiotics were assayed similarly, except that test samples were spotted on MGA agar and dried before being overlaid with an indicator strain.

Mutational analysis. Plasposon mutants of *P. vagans* C9-1 were generated according to the method of Dennis et al. (8). Briefly, pTn*Mod*-RKm′ was introduced into *P. vagans* C9-1 cells by electroporation. Mutants containing pTn*Mod*-RKm′ were selected on LB agar containing Km. Antibiotic-deficient mutants were identified by their inability to form a zone of inhibition against the pantocin A-resistant *E. amylovora* strain CIR555. Genomic DNA of the herbicolin I mutants containing pTn*Mod*-RKm′ was isolated using the method of Desomer et al. (9). The genomic DNA was digested with the restriction enzyme PstI, self ligated overnight, and transformed into *E. coli* S17-1 λ*pir*. Replicating plasmids were recovered, and the flanking regions were sequenced using the primers TnMod_FP-1 and TnMod_RP-1 (Table 2). Sequences were identified in the published genome sequence of *P. vagans* C9-1 plasmid pPag2 (GenBank accession no. CP001894) (37).

Immature pear fruit assay. Inhibition of *E. amylovora* by *P. vagans* C9-1 and its antibiotic-deficient derivatives was tested in immature pear fruit (30). Fruit surfaces were disinfected with 70% ethanol, bisected longitudinally, and placed on sterile, moistened Whatman filters in petri dishes. A 50-μl suspension of *P. vagans* in 5 mM phosphate buffer (pH 6.5) at 5×10^5 CFU ml^{-1} or 5×10^6 CFU ml^{-1} or buffer alone was introduced into a 5-mm-deep-well in each pear half with a sterile pipette tip and left to absorb for 2 h. A 50-μl suspension of *E. amylovora* (5×10^5 CFU ml^{-1}) in 5 mM phosphate buffer (pH 6.5) was introduced into the same well. Treatments consisted of six replicate pear fruit halves, and each experiment was repeated three times.

Disease symptoms (necrosis and/or bacterial ooze) were first observed 2 days after inoculation and recorded daily over 5 days after inoculation. The incidence of disease was calculated for each treatment and day. Disease incidence was transformed into the arcsine square root for normalization prior to analysis with the analysis-of-variance (ANOVA) procedure of the software program SAS (Statistical Analysis Systems, Cary, NC). Disease incidence data did not vary significantly among experiments and were pooled. Treatment means for each day were separated by Fisher's protected least-significant-difference test at a P value of 0.05.

Sequence analysis. The BLASTN subroutine in the software program GenDB (26) was used to identify the insertion sites of the plasposons in the genome sequence of *P. vagans* C9-1 (37). Routine sequence manipulations were done using the subroutines of the Lasergene software package, version 8.1.5 (DNAstar, Madison, WI). Additional BLAST searches (1) were done at NCBI.

Antibiotic purification. Herbicolin I was purified from culture supernatants of *P. vagans* C9-1 by a combination of cation exchange chromatography, reverse-phase high-pressure liquid chromatography (HPLC), and instant thin-layer chromatography (ITLC). Cells of C9-1 were grown until late log phase in 10 liters MGA. Samples of concentrated supernatants were adjusted to pH 2.5 with 5 N H_2SO_4, and quickly added to a 5.5-cm-by-10-cm column of Dowex 50W × 4 (200-400 mesh) equilibrated in 2.5 mM ammonium acetate buffer (pH 5). After washing with 2.5 mM ammonium acetate buffer, antibiotics were eluted with 20 mM ammonium acetate buffer (pH 5), concentrated to dryness under reduced pressure at 40°C, and then resuspended in distilled water. Preparations were kept frozen at −20°C. Active fractions were applied to octydecyl (C_{18}) bonded-phase sorbent packed in a 100-ml flash chromatography column and equilibrated in 0.1% (vol/vol) trifluoroacetic acid (TFA) in water. The column was eluted with 0, 5, 10, 20, and 50% methanol in 0.1% TFA, and fractions were immediately adjusted to a pH of 3.5 to 4.0 with 2% ammonium hydroxide. Fractions with antibiotic activity were pooled and concentrated under reduced pressure. Following flash chromatography, the active component was further purified by HPLC on a C_8 semipreparative column (25 cm by 9.4 mm) eluted with acetonitrile-water-TFA (5:94.9:0.1). Peaks at absorbance at 215 nm were collected and

TABLE 2 Primers used for sequencing the flanking regions of Tn*Mod*-RKm′ and for prevalence screening

Primer name	Sequence (5′–3′)	Size (bp)	T_{anneal}[b] (°C)
TnMod_FP-1	TCCCTCACTTTCTGGCTGGA	—[a]	56
TnMod_RP-1	CCTCTCAAAGCAATTTTGAG		
ddaD_F	GGATCTTGCATCGTTCGCAC	807	58
ddaD _R	CGATCGCCTGTGCGGTAGTA		
ddaF_F	ATCCCTGCATTTCAGGCGCT	853	63
ddaF _R	ATGCCCCAGACACTCTTCGA		
paaA_fw	CTCTTGCCAAAATGGATGGT	2,398	55
paaC_rev	TTGCAAATTCTGCACTCTCG		

[a] —, different amplicon sizes.
[b] Annealing temperature.

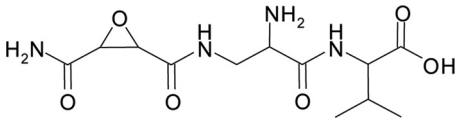

FIG 1 Chemical structure of herbicolin I [2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine].

adjusted to pH 3.5 to 4.0. After concentration, the antibacterial activity of each sample was assayed. Samples with antibacterial activity were repeatedly lyophilized to remove salts. The active compound was finally purified on instant thin-layer chromatography (ITLC-SA) papers developed in acetonitrile-water (80:20).

To determine pH stability, aliquots of samples were pH adjusted with 0.5 N H_2SO_4 and 2% NH_4OH and after 2 h were neutralized with 1 M potassium phosphate buffer (pH 7.0) and adjusted to a constant volume. Heat stability was determined by examining residual antibiotic activity in preparations heated at 95°C for 2 h in sealed vials. Beta-lactamase assays were carried out with penicillinase 1 (Sigma Chemical Co., St. Louis, MO) and were incubated for 1 h at 26°C.

The ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectra in D_2O were obtained on a Bruker WM-250 instrument (Bruker BioSpin Corporation, Billerica, MA) with suppression of the solvent HDO resonance signal. The molecular weight and chemical formula were obtained by fast atom bombardment mass spectrometry (FAB-MS) of intact, underivatized herbicolins (0.01 mg in 0.01 ml glycerol matrix). The amino acid composition and sequence of herbicolin I were determined by Edman degradations liberated as phenylthiohydantoin amino acids and analyzed by HPLC.

Sensitivities of orchard isolates of *E. amylovora* to antibiotics of *P. vagans* C9-1. Thirty-four streptomycin-sensitive isolates and 25 streptomycin-resistant isolates of *E. amylovora* from commercial orchards in the U.S. Pacific Northwest (25) were evaluated for sensitivities to the antibiotics of *P. vagans* C9-1, using the double diffusion assay on MGA medium described above. L-Histidine (10 mM) was added to the overlay medium in replicate plates for each isolate to suppress antibiosis due to production of pantocin A. Additionally, isolates of Ea153 that were recovered from 20 diseased blossom clusters on pear trees treated twice with *P. vagans* C9-1 during bloom also were tested (42). Sensitivity assays were repeated twice.

RESULTS

Chemical identification of antibiotic compound. The antibiotic referred to as herbicolin I was isolated from liquid cultures of *P. vagans* C9-1 and chemically characterized. The antibiotic was purified from culture supernatants of *P. vagans* C9-1 by a combination of cation exchange chromatography, reverse-phase HPLC, and instant thin-layer chromatography (ITLC). A different method than that of Sammer et al. (35) was required to exclude the copurification of pantocin A. The purified antibiotic was insensitive to beta-lactamase, base labile (pH > 10), and heat and acid stable. The observed $[M+H]^+$ value of the antibiotic was 317.14461, corresponding to a calculated $[M+H]^+$ value of 317.14610 and a chemical formula of $C_{12}H_{21}N_4O_6$. The antibiotic contained the amino acid valine. The proton NMR spectrum of herbicolin I was identical to that of the dapdiamide produced by *P. agglomerans* 48b/90: 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine (Fig. 1) (35). We previously proposed (37) that this compound would be identical to dapdiamide E, but now we are referring to the compound as herbicolin I to be consistent with earlier publications (7, 18) and because the stereochemistry for both compounds has not been determined.

Identification of the herbicolin I gene cluster. To identify the herbicolin I biosynthetic genes, a pTn*Mod*-RKm′ plasposon mutant library of *P. vagans* C9-1 was constructed. This library, consisting of 3,800 plasposon mutants, was screened for the loss of antibiotic production in a double agar diffusion assay using the pantocin A-resistant and dapdiamide (herbicolin I)-sensitive biosensor strain CIR555. A total of 38 mutants were potentially deficient in dapdiamide biosynthesis. The flanking regions of all mutants were sequenced and analyzed using the BLASTN subroutine of the program GenDB (26) against the fully annotated genome sequence of *P. vagans* C9-1 (37). A total of 25 plasposon insertions were located in a single locus, which was identified as the biosynthetic operon of herbicolin I (Table 3). The operon consists of 10 genes (*ddaA-ddaI*) (Fig. 2) and is located on plasmid pPag2 in a region with a low G+C content compared to the overall G+C content of the plasmid. This indicates that *P. vagans* C9-1 acquired herbicolin I biosynthesis most probably by horizontal gene transfer. However, immediately flanking the operon, no direct evidence for transposition was found, so this cluster may have been acquired together with the complete plasmid pPag2, which is described as a biocontrol-specific feature (37). The other plasposon insertions are infrequently distributed on the chromosome and the other plasmids, pPag1 and pPag3, and are most likely not involved in the biosynthesis of herbicolin I.

The most frequent insertion sites of the plasposons were identified in *ddaB*, *ddaF1*, *ddaG*, and *ddaI*. DdaB is predicted to be involved in L-2,3-diaminopropionic acid (DAP) biosynthesis, one of the central monomers linked to valine and fumaramic acid via condensation reactions by DdaF and DdaG to form the dapdiamide (16). DdaI is predicted to be involved in export of the antibiotics. The gene products of *ddaB*, *ddaF1*, *ddaG*, and *ddaI* appear to be strictly required for herbicolin I biosynthesis. We identified only a few or no plasposon integration in the genes *ddaCDE* and *ddaH*. In studies of genes cloned from *P. agglomerans* CU0119 and expressed in *E. coli*, *ddaH* and *ddaCDE* are involved in the biosynthesis of the fumaramic acid moiety and formation of the epoxide precursor of N_{β}-epoxysuccinamoyl-DAP, respectively (17). One mutant identified as containing a plasposon in-

TABLE 3 Plasposon Tn*Mod*-RKm′ mutant insertion identification

C9-1 plasposon mutant(s)	Locus tag	Gene	Putative function
CIR620, CIR621, CIR625, CIR635, CIR646	Pvag_pPag20158	*ddaB*	Ornithine cyclodeaminase
CIR613	Pvag_pPag20159	*ddaC*	Fe(II)/α-KG-dependent dioxygenase
CIR591, CIR603, CIR619, CIR633	Pvag_pPag20162	*ddaF1*	Biotin carboxylase
CIR624	Pvag_pPag20163	*ddaF2*	Biotin carboxylase
CIR592, CIR594, CIR596, CIR597, CIR600, CIR616, CIR617, CIR626	Pvag_pPag20164	*ddaG*	Phenylacetate-CoA[a] ligase
CIR599	Pvag_pPag20165	*ddaH*	Asparagine synthetase
CIR589, CIR614, CIR636, CIR641	Pvag_pPag20166	*ddaI*	Putative membrane protein

[a] CoA, coenzyme A.

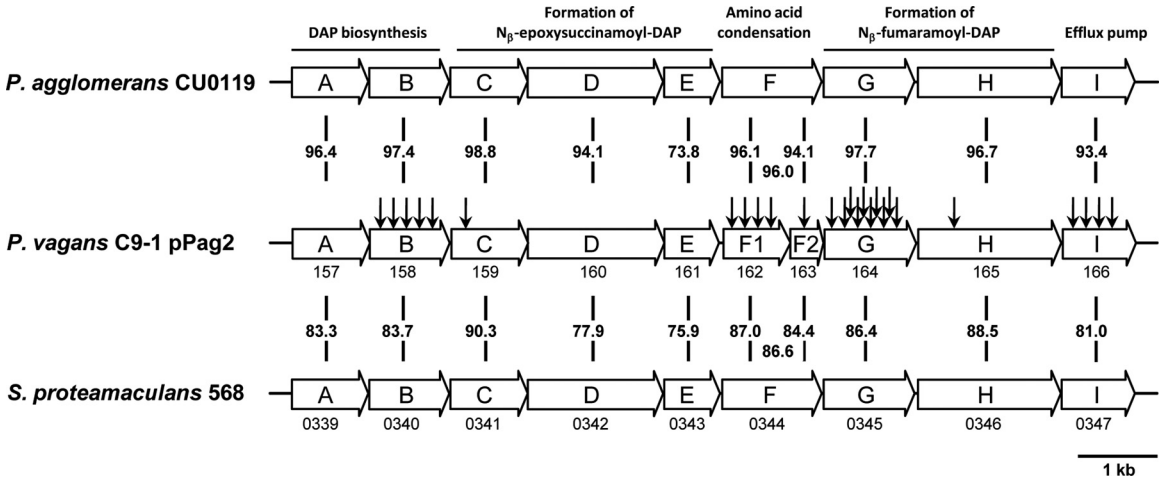

FIG 2 Dapdiamide operons of *Pantoea vagans* C9-1, *P. agglomerans* CU0119, and *S. proteamaculans* 568. The proposed biochemical functions encoded by the genes of *P. agglomerans* CU0119 (16) are indicated above. Plasposon insertion sites are indicated by arrows. Numbers between clusters indicate the identities at the amino acid level. Where available, locus tags are indicated directly below clusters: for *P. vagans* strain C9-1, the prefix is Pvag_pPag20, and for *S. proteamaculans* strain 568, it is Spro_. Note that the gene cluster of *P. vagans* C9-1 differs in the number of genes due to the naturally separated genes *ddaF1* and *ddaF2*, which together constitute the biochemical function of *ddaF* in *P. agglomerans* CU0119.

sertion in the putative self-resistance gene *ddaI* produced less antibiotic than the wild type and remained viable on the antibiotic production medium, MGA.

P. vagans C9-1 significantly reduced the incidence of disease symptoms on immature pear fruits inoculated with *E. amylovora* Ea110 at each time point compared to results with the buffer treatment (Fig. 3). Biocontrol was significantly decreased with *P. vagans* C9-1 Tn*Mod* mutants lacking production of herbicolin I (CIR591) or pantocin A (CIR638) compared to that with the wild type (Fig. 3). The mutant lacking production of herbicolin I and pantocin A (CIR600) did not suppress symptoms, and the incidence of disease was similar to that with treatment with buffer (Fig. 3). Growth rates of all mutants were unaltered in compared to that of the wild type (data not shown), and each showed reduced biocontrol activity even when applied at 5×10^6 CFU ml^{-1} (Fig. 3A).

Among 59 isolates of the pathogen *E. amylovora* from commercial orchards, 2 isolates were tolerant of herbicolin I in the double diffusion agar assay but sensitive to the histidine-reversible antibiotic pantocin A. These two orchard isolates had an inhibition pattern similar to that of the herbicolin I-resistant biosensor CIR550, with an inhibition zone distal from the *P. vagans* C9-1 colony, which was abolished when histidine was added to the overlay medium. One of the herbicolin I-tolerant isolates was resistant to 100 μg ml^{-1} streptomycin, and the other was sensitive; both caused necrosis and production of bacterial ooze in immature pear fruits. All of the isolates of strain Ea153 recovered from diseased blossom clusters on inoculated trees treated with *P. vagans* C9-1 were sensitive to both herbicolin I and pantocin A.

Sequence analysis. BLAST search (NCBI) revealed the presence of highly similar gene clusters in the genomes of *P. agglomerans* CU0119 (7) and *Serratia proteamaculans* 568 (43) and a distantly related gene cluster in *Vibrio caribbeanicus* ATCC BAA-2122 (14). The sequence of the gene cluster of *P. agglomerans* 48b/90 is not available, but the operon structure of *P. agglomerans* CU0119 (7) is very similar to that for *P. vagans* C9-1 (Fig. 2). The gene cluster of *P. agglomerans* CU0119 has been cloned and ex-

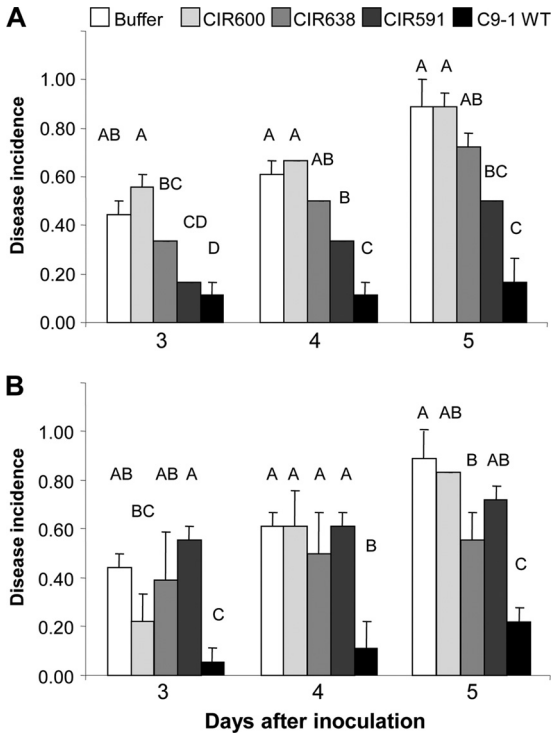

FIG 3 Reduction of incidence of disease symptoms (necrosis and/or bacterial ooze) in immature pear fruit by *P. vagans* C9-1 and antibiotic-deficient derivatives CIR591 (herbicolin I$^-$/pantocin A$^+$), CIR638 (herbicolin I$^+$/pantocin A$^-$), and CIR600 (herbicolin I$^-$/pantocin A$^-$). Immature pear fruits were treated with buffer or 5×10^6 CFU ml^{-1} wild-type C9-1 or derivatives (A) or buffer or 5×10^5 CFU ml^{-1} of wild-type C9-1 or antibiotic-deficient mutants (B) and subsequently challenged with 5×10^5 CFU ml^{-1} *E. amylovora* Ea110. Vertical lines indicate standard errors of the means. Similar letters above bars for a time point indicate that the transformed incidence of symptoms between treatments is not significantly different according to Fisher's protected least significant difference at $P = 0.05$.

pressed in *E. coli* and shown to produce a mixture of dapdiamides (7). Since the sequence of *P. agglomerans* CU0119 (7) comprises only the dapdiamide biosynthesis cluster, no evidence for horizontal transfer to *P. agglomerans* CU0119 is available.

In comparison to the 10 genes in *P. vagans* C9-1, the clusters of *P. agglomerans* CU0119 and *S. proteamaculans* 568 consist of 9 genes. Overall the gene cluster is highly conserved between *P. vagans* C9-1 and *P. agglomerans* CU0119 (above 90% amino acid identity), except for *ddaE* (Pvag_pPag20161) (73.8% identity). In the two other strains, the genes *ddaF1* and *ddaF2* are combined to only one open reading frame (ORF), a homolog of biotin carboxylase functioning in the condensation reaction between N_{β}-fumaramoyl-DAP and valine (17). A putative insertion event of 6 bp led to amino acid changes in Pvag_pPag20162. The insertion resulted in a leucine instead of a proline, an additional cysteine, and an early stop codon in Pvag_pPag20162 compared to the other two strains. The bases integrated directly in front of a methionine codon, which serves as a start codon for *ddaF2* (Pvag_pPag20163).

Screening for dapdiamide biosynthetic genes in *Pantoea* species. A broad collection of *Pantoea* spp. that includes biocontrol, clinical, and environmental isolates (31) was screened for the presence of the dapdiamide biosynthetic genes (Table 1). The collection was tested for the presence of dapdiamide biosynthetic genes using two different primer combinations targeting *ddaD* (Pvag_pPag20160) and *ddaF1* and *ddaF2* (Pvag_pPag162 and Pvag_pPag20163). The primers were generated by comparison of the three available sequences (Fig. 1). The dapdiamide genes were detected only in *P. vagans* C9-1 and its nonpigmented variant C9-1W cured of its megaplasmid pPag3 (38).

DISCUSSION

The establishment and antibiotic synthesis of antagonists in the floral court are critical for successful suppression of *E. amylovora* (13). Biocontrol strains that either naturally produce no antibiotic or are inactivated in biosynthesis of an antibiotic can still reduce the growth of *E. amylovora* (13, 40, 45). Antibiotic biosynthesis mutants inhibit the growth of the pathogen to a lesser extent than the producing strains, since site exclusion and competition for limited nutrients (e.g., nitrogen and iron) are still active. Recovered isolates of the pathogen inoculated onto trees treated with the biocontrol agent were sensitive to both antibiotics produced by *P. vagans* C9-1. Among isolates of the pathogen from commercial pear orchards, 3% were resistant to herbicolin I, and all were sensitive to pantocin A. None of the commercial orchards in the U.S. Pacific Northwest were exposed to *P. vagans* C9-1, which was isolated in Michigan, so the herbicolin I resistance was not correlated to exposure or selection pressure from this biocontrol agent. In double diffusion assays, spontaneous mutants of *E. amylovora* may arise over time that are resistant to herbicolin I or pantocin A; this was the source of the biosensor strains (18). We anticipate that a low incidence of spontaneous mutation may lead to isolates of the pathogen with resistance to herbicolin I. Nonetheless, we postulate that *P. vagans* C9-1 would continue to be an effective management tool for fire blight, even if some strains of the pathogen were resistant to herbicolin I. *P. vagans* C9-1 produces another antibiotic, pantocin A, and the isolates insensitive to herbicolin I were sensitive to pantocin A, an antibiotic demonstrated to contribute to biocontrol efficacy (40, 45, 49). Along with antibiosis, *P. vagans* C9-1 effectively competes with the pathogen for floral nutrients and colonization sites. The multiprong approach of *P. vagans* C9-1 to colonize and secure niches on flowers likely will mitigate a "breakdown" in biological control efficacy due to herbicolin I-resistant populations of the pathogen (10).

P. vagans C9-1 produces at least two antibiotics in culture that suppress growth of *E. amylovora* (18). One antibiotic of *P. vagans* C9-1 is suppressed by histidine and is presumed to be pantocin A based on the presence of a pantocin A gene cluster in the genome and preliminary characterization of the antibiotic (19, 37). In our *Pantoea* collection, the presence of pantocin A producers is much lower than that in a previous report indicating 61 of 88 *P. agglomerans* strains that produced a histidine-suppressible antibiotic were positive for pantocin A biosynthesis genes (22, 31). The other, herbicolin I, was shown here to belong to the dapdiamide family of antibiotics. The most abundant histidine-insensitive antibiotic isolated from *P. vagans* C9-1 is the epoxide of dapdiamide A, N_{β}-epoxysuccinamoyl-DAP-valine, which has been synthesized and shown to be biologically active against *E. amylovora* (17). The ^{1}H-NMR spectrum of the purified antibiotic was identical to that of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine, identified in *P. agglomerans* 48b/90, which has the same $[M+H]^{+}$ value and biochemical properties as the isolated antibiotic (35). These findings are consistent with the detection of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine in culture filtrates of *P. vagans* C9-1 (34) by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). Based on recent studies with dapdiamides and synthesized dapdiamide analogues, dapdiamides target cell wall biosynthesis by attacking glucosamine-6-phosphate synthase (17).

Given the similarities between the biosynthetic gene clusters in *P. vagans* C9-1 and *P. agglomerans* CU0119, the antibiotic produced by the gene cluster in *P. vagans* C9-1 most likely uses a similar biosynthetic pathway. The biosynthesis of the dapdiamide antibiotics comprises the linkage of L-2,3-diaminopropionic acid (DAP) to two variable units (e.g., amino acids, fumaramic acid and derivates) via amide bond formation (7, 15). Culture supernatants of *E. coli* expressing the dapdiamide biosynthetic cluster from *P. agglomerans* CU0119 contained dapdiamide A as well as the less-abundant variants dapdiamide B and C, having an isoleucine or leucine moiety instead of valine. Dapdiamide D and dapdiamide E also were present. These differ in linkage of DAP to fumaramic acid and an epoxide instead of the fumaramic acid double bond, respectively (7, 15). It is possible that *P. vagans* C9-1 produces other dapdiamides that were not detected or isolated by the protocols used for isolation of N_{β}-epoxysuccinamoyl-DAP-valine. For example, another histidine-insensitive antibiotic produced with the same antimicrobial activity spectrum as herbicolin I was detected in cultures of *P. vagans* C9-1 but due to low yield was not characterized further. Further studies of optimization of dapdiamide production and isolation from *P. vagans* C9-1 would be valuable in this respect and in future studies on the importance of N_{β}-epoxysuccinamoyl-DAP-valine or other dapdiamides in the biological control of fire blight by *P. vagans* C9-1.

DdaI, a putative membrane protein potentially involved in the export of the antibiotic, conferred resistance to dapdiamide A to sensitive *E. coli* (7). In our screen, mutants with an integrated plasposon in this gene resulted in viable cells, which formed a reduced zone of inhibition against *E. amylovora*. This suggests that different mechanisms of self-resistance exist within *P. vagans* C9-1, since the mutation in the putative exporter does not lead to lethality caused by the intracellular accumulation of dapdiamides.

The putative target, glucosamine-6-phosphate synthase, might be insensitive to dapdiamide due to enzymatic or steric protection. Alternatively, the antibiotic might not be activated before its export, therefore displaying no toxic effect on the cells. However, additional mutagenesis and complementation studies would be required to determine the function of *ddaI* in *P. vagans* C9-1.

Biosynthesis of dapdiamide antibiotics is not a common trait in *Pantoea* spp. We identified dapdiamide biosynthetic genes only in *P. vagans* C9-1 and *P. agglomerans* CU0119 after screening a wide range of biocontrol, environmental, and clinical isolates. The rarity of dapdiamide biosynthesis within *Pantoea* spp. suggests that pathogen-inhibitory activity described for biocontrol *Pantoea* strains is likely due to production of other antibiotic compounds (28, 30). Analysis of the genes revealed that the operon, consisting of nine coding sequences (CDS) in *P. agglomerans* CU0119 (7), has 10 CDS due to base insertions in *P. vagans* C9-1. Besides this, the clusters have a similar gene organization, and the gene products display high similarity to *S. proteamaculans* 568 at the amino acid level (Fig. 2). Another member of this genus, *Serratia plymuthica*, also was found to produce a dapdiamide compound (35, 36). Notably, N_{β}-epoxysuccinamoyl-DAP-valine was not isolated from culture supernatants of *E. coli* expressing the dapdiamide genes from *P. agglomerans* CU0119. Although the genes and predicted proteins share a high degree of similarity, differences in dapdiamide production between *P. agglomerans* CU0119 and wild-type *P. vagans* C9-1 might reflect slight genetic differences between dapdiamide structural genes or its regulation. It is also possible that sequences outside the dapdiamide gene cluster affect the composition of dapdiamide antibiotics produced. We identified several plasposon mutants containing insertions outside the biosynthetic cluster that were deficient or reduced in herbicolin I. Methods used to purify dapdiamide antibiotics also may have influenced the types of dapdiamides detected.

The operon is most likely acquired by horizontal gene transfer, which is evidenced by the lower G+C content (47%) of the genes in both *Pantoea* strains than the G+C content of the chromosome (53.8%) (37). The dapdiamide biosynthesis operon is located on plasmid pPag2, which comprises diverse IS elements, which indicates that many important biocontrol attributes of *P. vagans* C9-1 might be acquired traits (37). Sorbitol metabolism, indole acetic acid biosynthesis from aldoximes, and tellurite resistance are located on this plasmid and might contribute to the ecological fitness of *P. vagans* C9-1. The biosynthesis of pantocin A and dapdiamide, as well as the overlapping nutrient utilization profile (37, 39, 41) of *P. vagans* C9-1, contribute to the effectiveness of this biocontrol agent at inhibiting the growth of *E. amylovora*.

Confirmation of the chemistry and genetics of this biocontrol trait in C9-1 resolves outstanding regulatory questions regarding active-ingredient mechanisms of action. Identification of the biosynthetic genes of dapdiamide will facilitate streamlining the screening process for new biocontrol agents by rapid selection of environmental isolates that produce dapdiamide antibiotics and have higher potential for effective pathogen suppression (33). The genetic characterization presented here also provides a foundation for discovery of novel compounds produced by similar pathways or by a subset of the biosynthetic genes using different substrates for synthesis.

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