THE HISTIDINE-REVERSIBLE ANTIBIOTIC HERBICOLIN O PRODUCED BY *PANTOEA VAGANS* C9-1 IS PANTOCIN A

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SUMMARY

Pantoea vagans C9-1 is one of the most effective and reliable biocontrol agents against fire blight, and has been commercialized as Blight Ban C9-1. Production of multiple antibiotics contributes to its antagonism of *Erwinia amylovora*. Here we describe the genetics, chemical isolation and structure of herbicolin O, the histidine-reversible antibiotic produced by *P. vagans* C9-1. Mutational analyses indicated that biosynthesis of herbicolin O depends on *paaAB* and a sequence encoding the peptide precursor of pantocin A. The *paaABC* gene cluster encoding biosynthesis and autoresistance was located within a 28-kb chromosomal genomic island of the complete genome sequence of *P. vagans* C9-1. The cluster was cloned and expressed in *E. coli* and purified antibiotic was isolated using improved methods for small peptides. The ^{1}H NMR spectra of the C9-1 antibiotic closely resembled those of pantocin A produced by *P. agglomerans* Eh318. Detailed analysis of the proton spin systems showed that the chemical shift values and coupling constants of the protons in C9-1 herbicolin O correspond exactly to those of pantocin A. Based on these genetic and chemical analyses, herbicolin O and pantocin A are confirmed to be the same antibiotic.

Keywords: fire blight, biocontrol, herbicolin O, peptide antibiotic.

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INTRODUCTION

The genus *Pantoea* has proven to be a significant source for excellent biological control agents of fire blight, caused by *Erwinia amylovora*. Some of these have been developed into commercially available products for use in the USA and Canada. Representatives of *Pantoea* species produce a range of potent antimicrobial agents, including the peptide antibiotics herbicolin A and B, which inhibit fungi and sterol-requiring mycoplasmas (Freund, 1984, Winkelmann, 1980), novel β-lactam antibiotics (Kearns, 1998, Parker et al., 1982), pyrrolnitrin (Chernin *et al.*, 1996), and a novel phenazine (Giddens *et al.*, 2002). In addition, there is a large body of literature describing antimicrobial activity of antibiotics produced by *Pantoea agglomerans* strains selected as biological control agents for agriculture. Some affect only closely related species, while others inhibit the growth of a wide range of bacteria outside of the *Enterobacteriaceae*, including Gram-positive bacteria. Antibiotics produced by plant-associated strains have been broadly classified on the basis of inactivation by certain amino acids (Wodzinski and Paulin, 1994). For example, several strains of *P. agglomerans* produce antibiotics that are inactivated in the presence of histidine (Ishimaru *et al.*, 1988; Wodzinski and Paulin, 1994; Wright et al., 2001). One of these, pantocin A, was isolated from *P. agglomerans* Eh318 (Jin *et al.*, 2003). Pantocin A is a novel peptide-derived antibiotic inhibiting histidine biosynthesis. The complete synthesis and characterization of pantocin B, another antibiotic from *P. agglomerans* Eh318, has also been reported (Brady *et al.*, 1999; Sutton and Clardy, 1999). Pantocin B activity is suppressed in the presence of arginine and its mode of action targets arginine biosynthesis. Pantocins A and B are structurally unique from one another and represent two classes of low molecular weight antibiotics. Both pantocin A and B are taken up by cells through the tripeptide transporter system and inhibit amino acid biosynthesis in sensitive organisms (Jin *et al.*, 2003).

One of the first and most effective biological control agents described for the control of fire blight is *Pantoea vagans* C9-1 (formerly *E. herbicola* and *P. agglomerans*)

(Gavini *et al*., 1989; Rezzonico *et al*., 2010; Smits *et al*., 2011; Brady *et al*., 2009; Ishimaru *et al*., 1988). *P. vagans* C9-1 produces at least two antibiotics, originally referred to as herbicolin I and O which are active *in vitro* and in immature pear assays against *E. amylovora* (Ishimaru *et al*., 1988, Kamber *et al*., 2012). Another antibiotic, herbicolin 2C, was detected during the isolation of herbicolin I and herbicolin O but has not been characterized further. Structurally, herbicolin I is identical to 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine (APV), which is produced by *P. agglomerans* 48b/90 (Sammer *et al*., 2009; Kamber *et al*., 2012). APV contributes to growth inhibition of *E. amylovora* in flowers in a temperature-dependent manner (Sammer *et al*., 2012). Plasposon mutagenesis and complete genome sequencing of *P. vagans* C9-1 located the biosynthetic genes for herbicolin I on the 166-kb plasmid pPag2, (Smits *et al*., 2011; Kamber *et al*., 2012; Smits *et al*., 2010). Orthologous gene clusters for herbicolin I biosynthesis are present in *P. agglomerans* CU0119, which produces a family of dapdiamide antibiotics (dapdiamide A to E) and in *Serratia proteamaculans* (Dawlaty *et al*., 2010; Kamber *et al*., 2012). Overall, the herbicolin I/APV biosynthetic cluster is uncommon among *Pantoea* spp. from biocontrol, environmental, and clinical origins (Palmer *et al*., 2016).

In contrast to herbicolin I, a concise summary of the chemical structure and genetics of herbicolin O from *P. vagans* C9-1 is lacking. Like many of the antimicrobial activities described from large scale *in vitro* screens for fire blight biocontrol agents, the activity of herbicolin O is reversed in the presence of histidine (Ishimaru *et al*., 1988; Vanneste *et al*., 2008; Wodzinski and Paulin, 1994). Herbicolin O is less stable than herbicolin I and is destroyed by acidic and basic conditions and high temperatures (Ishimaru *et al*., 1988). These features are also general characteristics of pantocin A, the histidine-reversed antibiotic produced by *P. agglomerans* Eh318 (Jin *et al*., 2003; Wright *et al*., 2001). Moreover, the genome of *P. vagans* C9-1 contains an orthologous cluster of genes identified as important in the biosynthesis of pantocin A by *P. agglomerans* Eh318 and another histidine-sensitive antibiotic producing strain, MccEh252 (Vanneste *et al*., 2008). While it is possible that herbicolin O and pantocin A are the same antibiotics, direct structural and genetic evidence in support of this has not been provided. The purpose of this study was to identify the chemical structure of the histidine-sensitive antibiotic produced by *P. vagans* C9-1 and to identify the genes required for herbicolin O biosynthesis. Based on our findings we conclude that the antibiotic herbicolin O is the same as pantocin A.

MATERIALS AND METHODS

Bacterial strains and media. *Pantoea vagans* strain C9-1 was isolated previously from *Malus pumila* cv. Jonathan stem tissue (Ishimaru *et al*., 1988). *Erwinia amylovora* strains resistant to one or more of the antibiotics produced by *P. vagans* C9-1 were previously described (Ishimaru *et al*., 1988). *E. amylovora* CIR556 is a spontaneous rifampicin resistant mutant of *E. amylovora* Ea110 (Ishimaru *et al*., 1988). Bacteria were maintained as stored stocks in glycerol at −80°C. All bacteria were inoculated directly from frozen stocks onto Luria-Bertani (LB) agar plates. *P. vagans* C9-1 and *E. amylovora* strains were incubated at 26°C for 24 h. *Escherichia coli* strains were incubated on LB agar and incubated at 37°C for 24 h. Media were supplemented as appropriate with antibiotics at the following final concentrations: kanamycin (Km) 50 µg/ml, rifampicin (Rf) 25 µg/ml, tetracycline (Tc) 20 µg/ml. Antibiotic production assays were performed on MGA (morpholino-propane sulfonic acid buffered medium containing 10 mM potassium gluconate, 10 mM L-asparagine, 10 mM thiamine and 10 mM nicotinic acid) (Ishimaru *et al*., 1988).

Antibiotic bioassay. Antimicrobial activities of cells of wild type and mutants of *P. vagans* C9-1 and of *E. coli* (pCIE85) were assayed in an MGA double diffusion agar system seeded with 2×10^6 cfu/ml of an indicator, as previously described (Ishimaru *et al*., 1988) The indicators used included *E. amylovora* CIR556, which is sensitive to herbicolin O and I, and its spontaneous herbicolin-resistant derivatives, *E. amylovora* CIR555 (herbicolin O^RI^S) and *E. amylovora* CIR550 (herbicolin O^SI^R), as previously described (Kamber *et al*., 2012). Antibacterial activity was recorded as formation of clear zones of inhibition in bacterial lawns after incubation for 2 days at 26°C.

DNA isolation and manipulation. Plasmid DNA was isolated on a small scale by an alkaline mini-prep extraction procedure. Cosmid DNA was isolated according to the "very low copy" plasmid isolation procedure supplied by Qiagen (Santa Clarita, CA, USA). DNA T4 ligase was obtained from GIBCO BRL (Gaithersburg, MD, USA), and ligation of DNA was carried out as recommended by the manufacturer. Genomic DNA of *P. vagans* C9-1 was obtained by the CTAB method developed for Gram-negative bacterial genomic DNA extraction (Wilson, 2001). Restriction endonucleases were purchased from Promega Corp. (Madison, WI, USA) and digestion of DNA was carried out as recommended by the manufacturer.

Genomic library construction and screening. A cosmid library with inserts of about 25 kb was made in cloning vector pLAFR3 (22 kb) (Staskawicz *et al*., 1987). This vector included genes for mobilization in *E. coli*, tetracycline resistance, and the *lacZ* gene for selection of inserts by blue/white selection. Plasmid pLAFR3 was maintained in *E. coli* strain HB101. Approximately 5 µg of pLAFR3 DNA was digested with *Bam*HI and dephosphorylated with bacterial alkaline phosphatase. The dephosphorylated vector was ligated using T4 ligase to the sized fragments of *P.*

vagans C9-1 chromosomal DNA (1 µg) partially digested with *Sau*3A1. Ligated DNA was packaged using Gigapack II from Stratagene (now Agilent Technologies, Santa Clara, CA, USA) and amplified in *E. coli* DH5α. Individual clones of the cosmid library were assayed for antimicrobial activity, as described above. Clones were grown on MGA with tetracycline for 2 days at 26°C. Colonies were killed with chloroform vapor and overlaid with *E. amylovora* CIR576, a tetracycline resistant derivative of *E. amylovora* CIR556, to maintain tetracycline selection for pLAFR3 cosmids. *E. amylovora* CIR576 was created by a triparental mating between *E. coli* HB101 (pRK252), *E. coli* HB101 (pRK2013), and *E. amylovora* CIR556. *E. amylovora* transconjugants were selected on LB agar supplemented with tetracycline hydrochloride and rifampicin.

***In vitro* mutagenesis of herbicolin O biosynthesis genes.** EZ::TN™ (Epicenter) was used for *in vitro* mutagenesis of the herbicolin O-producing cosmid clone, pCIE85, according to the manufacturer's procedure. The transposition reaction was incubated at 37°C for 2 h and then stopped with SDS. Competent cells of *E. coli* DH5α were electroporated according to standard electroporation conditions for *E. coli. E. coli* (pCIE85::Tn<*Kan2*>) mutants were selected on the basis of resistance to kanamycin. Mutants were bioassayed for altered herbicolin O production, as described previously (Kamber *et al*., 2012). The mutants were tested for activity against *E. amylovora* CIR556, *E. amylovora* CIR550, and *E. amylovora* CIR555 (Kamber *et al*., 2012). In addition, antibiotics produced by the *E. coli* (pCIE85::Tn<*Kan2*>) mutants were screened for histidine reversal by adding histidine to the overlay medium seeded with the herbicolin O sensitive indicator, *E. amylovora* CIR550. Mutant phenotypes were verified by repeating the assays at least three times. All putative antibiotic deficient mutants were stored in glycerol at –80°C for future use.

Localization of EZ::TN™ insertions. Herbicolin O-deficient mutants were analyzed for the location and number of insertions by a combination of sequencing and Southern hybridization. Cosmid DNA preparations were digested with either *Eco*RI, which cuts outside of the borders of the EZ::TN insert, or *Hin*dIII, which cuts within the insert. For cosmid mutants, 1 µg of DNA was digested and separated by agarose gel electrophoresis, transferred to nylon membranes and probed with the EZ::TN insertion sequence labeled with digoxigenin-dUTP, according to protocols supplied with a DIG-High Prime Lab kit. DNA flanking the site of transposon inserts was sequenced using inverse primers supplied with the EZ::TN transposon. Big dye terminator cycle sequencing was performed on an ABI Prism 310 using standard manufacturer procedures (Applied Biosystems Inc., Foster City, CA, USA). Sequences for herbicolin O biosynthesis were located within the published genome sequence of *P. vagans* C9-1 (Smits *et al*., 2010) using the Lasergene Package (DNAStar, Madison, WI, USA). Comparisons to pantocin A biosynthesis genes and to the corresponding region in *P. agglomerans* ATCC 27155^T were completed as previously described (Kamber *et al*., 2012).

Isolation and structural analysis of histidine-sensitive antibiotic from CIE85. *E. coli* DH5α (pCIE85) was pregrown in MGA broth supplemented with 20 µg/ml tetracycline to an OD_{620} of 0.1 and used to create a 200 ml broth seed culture for large-scale antibiotic production in 10 l of MGA. Cells were fermented at 26°C for 15.5 h with maintenance of pH at 7.0 at the Colorado Bioprocessing Center (Fort Collins, CO). Cells were removed and the resultant supernatant was collected, concentrated by evaporation, and stored at –20°C. All herbicolin O-containing samples and fractions were collected and kept on ice during each procedure to retain bioactivity. For purification, samples were adjusted quickly to a pH 2.5-3.0 with sulfuric acid prior to cation exchange (Ag50 W-Y4, NH_4+ 2.5mM, pH 5). After elution with 20 mM ammonium acetate (pH 5) and desalting (ENVI™-Carb Packing 60 ml, 10 g cartridge; Supelco, Bellefonte, PA) as per the manufacturer's instructions, bioactive fractions were further separated by preparative and semi-preparative hydrophilic interaction (HILIC) (polyhydroxyethyl aspartimide™, PolyLC Inc., Columbia, MD, USA) in gradients containing acetonitrile, followed by desalting and concentration. Finally, antibiotic active fractions were separated by size exclusion chromatography with a polyhydroxyethyl aspartamide™ column (250 × 49.4 mm, 5 µm, 60 Å, PolyLC Inc.) equilibrated in 50 mM 1,1,1,3,3,3-hexafluoro-2-propanol (HF2P) (Aldrich, Milwaukee, WI, USA). Briefly, dry herbicolin O powder was resuspended completely in 1 ml cold HPLC grade H_2O and adjusted to 50 mM HF2P immediately prior to injection the column. Isocratic separation of bioactive peaks was accomplished in 50 mM HF2P at a flow rate of 0.4 ml/min. The absorbance was monitored with a photodiode array detector at 214-350 nm. Peaks with a retention time of approximately 42-46 min were collected, placed on ice, and immediately adjusted to pH 6-7 with 0.1 M ammonium hydroxide. The collected peaks were concentrated and desalted. Fractions with antimicrobial activity were lyophilized to dryness and stored at –20°C. Purified herbicolin O was analyzed by a combination of ^{1}H NMR and other NMR experiments, as previously described (Jin *et al*., 2003).

Temperature regulation of antibiotics from *P. vagans* C9-1. Herbicolin O producers *P. vagan*s C9-1 and *E. coli* (pCIE85) were grown on LB agar plates and incubated at 26°C and 37°C, respectively, for 24 h. Cell suspensions containing about 10^8 cfu/ml were made in potassium phosphate buffer (20 mM, pH 7.2). Ten µl of each producer was spotted onto MGA agar plates and incubated at either 26°C, 33°C or 37°C for 48 h. Viable bacteria were killed

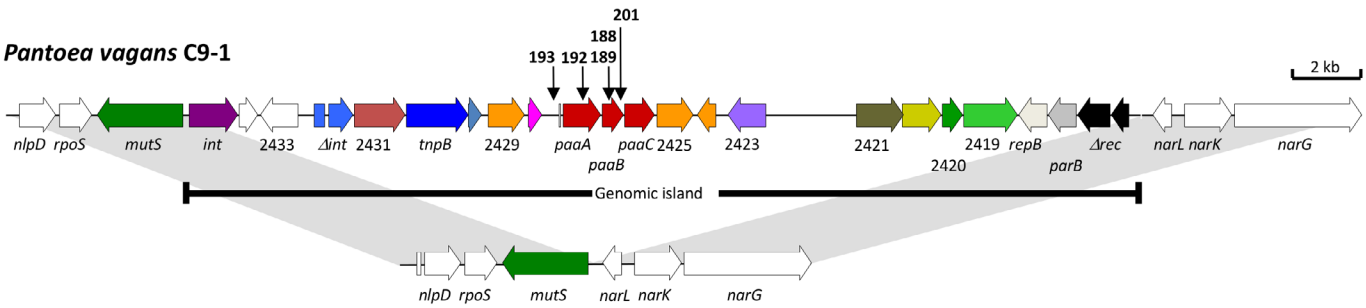

Fig. 1. Genomic island in *P. vagans* C9-1 containing the pantocin A gene cluster *paaABC*. Black vertical arrows indicate the loss of antibiotic production (188, 189, 192, 193 and 201) by transposon insertions located within the herbicolin O-producing cosmid clone, *E. coli* DH5α (pCIE85). The *P. vagans* C9-1 genomic island containing *paaABC* is absent from the corresponding genomic region of *P. agglomerans* ATCC 27155^T.

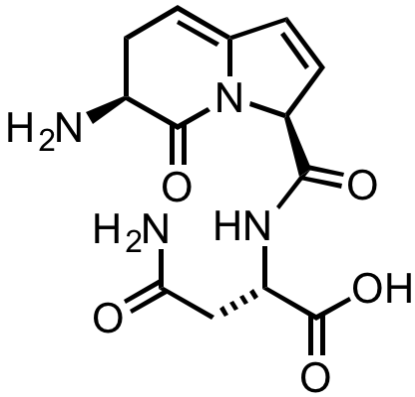

Fig. 2. Chemical structure of pantocin A and herbicolin O.

with chloroform vapor and overlaid with indicator strains varying in sensitivity to herbicolin I and herbicolin O, as described above. Antibacterial activity was recorded as formation of clear zones of inhibition after incubation for 2 days at 26°C.

RESULTS

Functional analysis of the *P. vagans* C9-1 herbicolin O biosynthetic cluster. One out of 2000 clones from a cosmid library of *P. vagans* C9-1 produced a zone of inhibition in lawn of the indicator *E. amylovora* CIR556. This activity was not present when histidine had been added to the overlay medium. The genes for antibiotic biosynthesis in this clone, *E. coli* DH5α (pCIE85), were delineated by directed mutagenesis with EZ::TN and sequencing. A total of 300 EZ::TN insertion mutants were screened for loss of herbicolin O production. Twenty-six mutations resulted in loss of herbicolin O on MGA medium. Southern hybridization experiments verified that all mutants contained single insertions of the transposon. Insertions associated with loss of function were localized within a 12.6 kb *Eco*RI fragment. The flanking DNA sequences of five transposon insertion sites (188, 189, 192, 193 and 201) associated with loss of herbicolin O production were obtained (Fig. 1). The overlapping sequences provided a complete sequence of the herbicolin O operon. Both the nucleotide sequences and the deduced amino acid sequences of the herbicolin O operon were highly similar to the promoter, pre-peptide, and *paaABC* gene sequences involved in the biosynthesis of pantocin A (Jin *et al.*, 2003).

Herbicolin O biosynthesis cluster in *P. vagans* C9-1. Comparisons between the EZ::TN insertions and the complete genome sequence of *P. vagans* C9-1 revealed that the genes required for herbicolin O biosynthesis are co-located on a 28-kb low G+C% genomic island containing the *paaABC* gene cluster. The genomic island present in *P. vagans* C9-1 containing *paaABC* genes was absent from the type strain *P. agglomerans* ATCC 27155, which does not produce the antibiotic (Fig. 1).

Isolation and structural identification of herbicolin O. The histidine-sensitive antibiotic produced by *P. vagans* C9-1 was isolated to homogeneity by a combination of chromatographic steps. The purity of the sample was confirmed by the presence of a single peak with characteristic dual absorption maxima at 214 and 275 nm in size exclusion HPLC chromatograms. The ^{1}H NMR spectra of herbicolin O closely resembled those of the bacterial metabolite pantocin A, and a more detailed analysis was undertaken to pinpoint any significant differences (Jin *et al.*, 2003). Detailed analysis of the proton spin systems *via* a dqf-COSY NMR experiment showed that the chemical shift values and coupling constants of the protons in herbicolin O corresponded exactly to those of pantocin A, suggesting that pantocin A and herbicolin O are identical. This dqf-COSY result was corroborated *via* an HMQC NMR experiment, which allowed for the assignment of all proton-bearing carbons in herbicolin O. Again, these data exactly matched those of pantocin A (data not shown). All measurements were within experimental error supporting the conclusion that herbicolin O is identical to pantocin A (Fig. 2).

Table 1. Temperature regulation of herbicolin O and I production in *Pantoea vagans* C9-1.

Producer[a]	*Erwinia amylovora* indicator strain[b]	Temperature (°C)	Herbicolin O inhibition zone (mm)	Herbicolin I[c] inhibition zone (mm)
P. vagans C9-1	CIR556 (O^SI^S)	26	40	20
		33	33	15
		37	0	0
	CIR555 (O^RI^S)	26	0	25
		33	0	12
		37	0	0
	CIR550 (O^SI^R)	26	39	0
		33	41	0
		37	0	0
E. coli (pCIE85)	CIR 556 (O^SI^S)	26	30	NA[d]
		37	35	NA

[a]Producers were grown on MGA agar medium and assayed for antimicrobial activity as described in methods.
[b]Indicators CIR555 and CIR550 were previously described derivatives of *Erwinia amylovora* CIR556 selected for sensitivity (S) or resistance (R) to herbicolin O or herbicolin I.
[c]Herbicolin I = 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine (APV)
[d]Not applicable; *E. coli* (pCIE85) produces only herbicolin O.

Temperature effects on antibiotic production and stability. The effect of temperature on the production of herbicolin peptide antibiotics was studied *in vitro*. The production of herbicolin O and I by *P. vagans* C9-1 was greatest at 26°C. Herbicolin O and I production was markedly reduced at 33°C and abolished at 37°C (Table 1). In contrast, production of herbicolin O by *E. coli* (pCIE85) was comparable at 26°C and 37°C (Table 1).

To determine the stability of herbicolin O, aliquots of purified herbicolin O were incubated at room temperature (20-23°C) for up to three days. The titer of herbicolin O was determined by bioassay with the herbicolin O and I sensitive indicator *E. amylovora* CIR556. No decrease in antibiotic activity was detected after 3-days of incubation (data not shown).

DISCUSSION

When first described, *Pantoea vagans* C9-1 was named *Erwinia herbicola*, in keeping with the taxonomy at the time (Ishimaru *et al.*, 1988). The antimicrobial activities produced *in vitro* by strain C9-1 were named "herbicolins" after the species epithet "herbicola". Three antimicrobial activities were characterized by a combination of *in vitro* assays using whole cells or partially purified preparations of the antibiotics. Herbicolin O inhibited the growth of *E. amylovora* and several members of the *Enterobacteriaceae*, while herbicolin I inhibited only *E. amylovora* and *Staphylococcus aureus*, under certain conditions. Another antibiotic produced, herbicolin 2C, seemed to have the same antimicrobial spectrum as herbicolin O. Initially, herbicolins O, I, and 2C were thought to be distinct structurally from other antibiotics produced by other *Erwinia herbicola/Pantoea agglomerans* strains with biocontrol activity against fire blight. One reason for this was an early report that partially purified herbicolins were sensitive to beta-lactamase and therefore belonged to the beta-lactam class of antibiotics (Ishimaru *et al.*, 1988). However, the presence of a beta-lactam ring claim was not supported by infrared spectroscopy (data not shown). Stockwell *et al.* found that commercially available β-lactamase preparations contain substantial protease activity (V. Stockwell, unpublished information). Repeated assays with highly purified preparations of herbicolin O and I against high quality enzyme preparations did not demonstrate beta-lactamase sensitivity (data not shown). Thus, the inactivation of herbicolin O and I reported in the earlier study was likely due to protease and not beta-lactamase activity.

Temperature is one factor affecting *in vitro* expression of herbicolin O in *P. vagans* C9-1 and other related antibiotics (Vanneste *et al.*, 2008; Jin *et al.*, 2003). Herbicolin O production by *P. vagans* C9-1 is inhibited at temperatures above the bacterium's optimal growth temperature of 26°C. Since elevated temperature did not affect herbicolin O production by *E. coli* pCIE85, the loss of herbicolin O activity observed with *P. vagans* C9-1 is not due to instability of herbicolin O at elevated temperatures. This conclusion is further supported by the observation that purified herbicolin O is stable at ambient temperatures for several days. Future studies on the effects of temperature on *in planta* regulation of pantocin A gene expression could aid in identifying environmental conditions that promote biological control of fire blight.

Knowledge of the diversity and novelty of antibiotics produced by common members of the microbial community in apples and pears has expanded greatly since the first reports of biocontrol of fire blight. It has become clear that while there are notable differences, similarities exist among the antibiotics produced by isolates independently selected for fire blight biocontrol activity. In the case of histidine-reversible antibiotics, obvious functional similarities occur among those produced by *P. agglomerans* Eh318, *P. agglomerans* Eh252, and *P. vagans* C9-1. In this report we have determined the structure of herbicolin O to be identical to that of pantocin A. This conclusion is

also supported by heterologous expression and directed mutagenesis of the *paaABC* orthologous gene cluster in *P. vagans* C9-1, where loss of herbicolin O production was associated with transposon insertions in *paaABC*. Thus, in all respects, pantocin A and herbicolin O are analogous. In the future, it is recommended that the histidine-reversible antibiotic produced by *P. vagans* C9-1 be referred to as pantocin A.

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