2-Amino-3-(Oxirane-2,3-Dicarboxamido)-Propanoyl-Valine, an Effective Peptide Antibiotic from the Epiphyte *Pantoea agglomerans* 48b/90[▽]

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The epiphyte *Pantoea agglomerans* 48b/90, which has been isolated from soybean leaves, belongs to the *Enterobacteriaceae*, as does the plant pathogen *Erwinia amylovora*, which causes fire blight on rosaceous plants such as apples and leads to severe economic losses. Since *P. agglomerans* efficiently antagonizes phytopathogenic bacteria, the *P. agglomerans* strain C9-1 is used as a biocontrol agent (BlightBan C9-1). Here we describe the bioassay-guided isolation of a peptide antibiotic that is highly active against the plant pathogen *E. amylovora* and pathovars of *Pseudomonas syringae*, and we elucidate its structure. Bioassay-guided fractionation using anion-exchange chromatography followed by hydrophobic interaction liquid chromatography yielded the bioactive, highly polar antibiotic. The compound was identified as 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine by using high-resolution electrospray ionization mass spectrometry and nuclear magnetic resonance techniques. This peptide was found to be produced by three of the nine *P. agglomerans* strains analyzed. Notably, the biocontrol strain *P. agglomerans* C9-1 also produces 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine. Previously, 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine has been characterized only from *Serratia plymuthica*. 2-Amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine has been shown to inhibit the growth of the human pathogen *Candida albicans* efficiently, but its involvement in the defense of epiphytes against phytopathogenic bacteria has not been investigated so far.

Microbial pathogens pose a major threat to many plants and can cause enormous losses in agriculture. Microorganisms that antagonize pathogens can offer a way to fight plant diseases that is more environmentally friendly than chemical treatment. Such diseases include fire blight, which is caused by *Erwinia amylovora* and affects many rosaceous plants, e.g., apple and pear (18, 25, 29, 38).

Suitable strains for biocontrol agents are often plant-associated microorganisms that are forced to defend their ecological niches under natural conditions and are thus adapted to competition with plant pathogens (2, 3). The species *Pantoea agglomerans* (formerly *Erwinia herbicola*) comprises many strains that are promising sources for biocontrol agents (8, 15, 30, 32, 43). *P. agglomerans* strains are ubiquitous in nature, inhabiting plant surfaces, water, soil, animals, and humans (9, 11). Several *Pantoea* isolates are known to inhibit *E. amylovora* efficiently in planta (39, 42). In vitro experiments have revealed some antibiotics from *P. agglomerans* and uncovered how they act

against *E. amylovora* (22, 43). The known antibiotics produced by *P. agglomerans* strains, which belong to diverse chemical classes and affect different molecular targets, exhibit both narrow- and broad-spectrum activities (21).

For example, *P. agglomerans* Eh318, isolated from apple leaves, produces two peptide antibiotics, pantocin A and pantocin B; both interfere with amino acid biosynthesis. Pantocin A blocks L-histidinol phosphate aminotransferase (20), and pantocin B acts as an N-acetylornithine transaminase inhibitor (5). Consequently, their inhibitory effects can be compensated for by supplementation with L-histidine and L-arginine, respectively (43). Giddens et al. (2002) described a phenazine antibiotic and its precursors, which were produced by *P. agglomerans* Eh1087 (10). Andrimid, a hybrid nonribosomal peptide polyketide antibiotic from *P. agglomerans* Eh335, selectively blocks the carboxyl transfer reaction of prokaryotic acetyl coenzyme A carboxylase; this reaction catalyzes the first committed step of fatty acid biosynthesis (19, 26). *P. agglomerans* E325 sold as Bloomtime Biological (Northwest Agricultural Products, Pasco, WA) acidifies flower stigmata, thus reducing the growth of *E. amylovora*. Simultaneously, it produces an antibiotic that has high specificity against *E. amylovora* and is effective under low-phosphate and low-pH conditions (34).

P. agglomerans C9-1, which is registered as the biocontrol agent BlightBan C9-1 (Nufarm Agricultural Inc.), produces two antibiotics, herbicolin O and herbicolin I (16). Like pantocin A, herbicolin O loses its activity in the presence of his-

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TABLE 1. *Pantoea agglomerans* strains used in this study

Strain designation(s)	Relevant characteristics	Source
48b/90, 39b/90, 33c/93, 18b/90, 15c/89, 29b/93, 2c/89, 23b/90	Wild type, from soybean; from fields around Dornburg, Germany	B. Völksch
C9-1	Wild type, from apple	K. Geider (V. O. Stockwell)

tidine. However, herbicolin I does not become ineffective in the presence of amino acids (17). Although C9-1 is registered as a biocontrol agent, the chemical nature of herbicolins has remained largely unknown (13, 14).

P. agglomerans 48b/90 (Pa48b), an epiphyte from soybean leaves (40), attracted our attention because it strongly inhibits the growth of *E. amylovora* and *Pseudomonas syringae* pv. *glycinea* (27), the pathogen that causes the bacterial blight of soybean. Since the mode of action of Pa48b against plant pathogens, in particular *E. amylovora*, is elusive, we looked for the molecular basis for the biocontrol potential of Pa48b. Here we describe the isolation, structure elucidation, and bioactivity of a potent antibiotic against plant pathogens that is produced by several *P. agglomerans* strains. The properties of this antibiotic perfectly match those of the chemically unidentified herbicolin I from *P. agglomerans* C9-1 (BlightBan C9-1).

MATERIALS AND METHODS

Strains and cultivation conditions. *P. agglomerans* strains and bacterial indicator strains were cultured and maintained on Standard I (St1) (Roth, Karlsruhe, Germany) agar plates. *P. syringae* strains were cultured and maintained on King's B agar plates (per liter, 20 g Bacto peptone, 10 g glycerol, 1.5 g K_2HPO_4 , and 15 g Bacto agar) (23). *P. agglomerans* strains were cultivated in liquid PIPES medium for antibiotic production [solution I contained, per 900 ml, 34 g piperazine- N,N' -bis(2-ethanesulfonic acid) (PIPES), 0.3 g KH_2PO_4 , 1 g NH_4Cl , and 1 g Na_2SO_4 ; the pH was adjusted to 6.8, solution II was added, containing, per 100 ml, 10 g glucose, 0.1 g $MgCl_2 \cdot 6H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 0.01 g $MnSO_4 \cdot 4H_2O$; the solutions were autoclaved separately] (36). The *P. agglomerans* strains used in this study are listed in Table 1, and the indicator strains are listed in Tables 2 and 3.

Agar diffusion assay. The antimicrobial activities of Pa48b against different indicator organisms (see Tables 2 and 3) were evaluated using spot assays. Portions (0.4 ml) of overnight St1 broth cultures of the bacterial indicator strains, adjusted to an optical density at 578 nm (OD_{578}) of 1, were added to 10 ml of melted 5b agar medium (12) and St1 agar medium at 50°C, and these cultures were poured into plates (diameter, 60 mm). Pa48b was spotted directly onto the agar surface, incubated at 28°C for 24 to 48 h, and analyzed. Alternatively, 100- μ l suspensions of fungi, with a McFarland standard of 0.5, were inoculated into 34 ml of sterile melted agar medium and poured into petri dishes. Holes (diameter, 9 mm) were cut out and filled with 50 μ l of the purified culture filtrates. As a reference compound, amphotericin B (10 μ g/ml) was used. Malt extract agar (per liter, 40 g malt extract [Becton Dickinson], 4 g yeast extract [Serva], and 5 g Bacto agar), Bacto yeast morphology agar (BYM; Difco Becton Dickinson & Company, Sparks, MD), and potato dextrose agar (Merck, Darmstadt, Germany) were used.

The success of the purification steps and the response to treatments were monitored using *E. amylovora* Ea7 as an indicator strain in the agar plate diffusion assay. Two milliliters of the bacterial suspension (OD_{578} of 1) was added to 48 ml of 5b agar medium and poured into plates (diameter, 120 mm). Holes (diameter, 9 mm) were cut out and filled with 50 μ l of the test samples. As a reference compound, spectinomycin (5 mg/ml) was used. Agar diffusion assays with the bacterial indicator organisms listed in Table 3 were carried out in the same way.

In order to investigate the effects of *N*-acetylglucosamine and casein hydrolysate on antibiotic activity, 50 μ l of either an *N*-acetylglucosamine solution (100

μ g/ μ l), a casein hydrolysate solution (0.3%, wt/vol), or water (control) was added to 50 μ l of a sterile culture supernatant. These samples were tested in agar diffusion assays with *Erwinia amylovora* Ea7 as the indicator strain as described above.

Growth curve and physical and biochemical characteristics. The growth curve was determined in triplicate from Pa48b cultures grown in 500-ml Erlenmeyer flasks containing 100 ml PIPES medium (36) and shaken at 200 rpm for 100 h at 10°C (Innova 4230 incubator shaker; New Brunswick Scientific, Edison, NJ), starting at an OD_{578} of 0.15. The cultures were inoculated with an overnight liquid culture of Pa48b (PIPES medium, 28°C, 200 rpm). The temperature dependence of antibiotic production by Pa48b was studied at 8, 10, 12, 18, and 28°C. The cells were grown to an OD_{578} of 5.5 and were then analyzed for their antibiotic activity. Statistical analysis was performed using Sigma Plot, version 9.01 (Systat Software, Inc., 2004).

In order to determine the influence of proteases on antibiotic activity, aliquots of the bioactive supernatant were incubated with proteinase K (final concentration, 20 mg/ml; Fermentas, St. Leon-Rot, Germany) for 12 h at room temperature or with type I *Bacillus cereus* β -lactamase (final concentrations, 2.5, 5, and 50 mg/ml; Sigma-Aldrich, Steinheim, Germany) for 1 h at 37°C. The heat stability of the antibiotic activity of Pa48b was analyzed by incubating the culture supernatant for 10 min at 60, 80, and 100°C and for 30 and 60 min at 100°C. The pH stability of the antibiotic activity of Pa48b was investigated by adjusting the culture supernatant to pH 3 or pH 10 for 20 min at room temperature. After the treatment, the pH was readjusted to 6.8, and the antibiotic activity against *E. amylovora* was tested in the agar diffusion assay.

Purification of the *E. amylovora* growth-inhibiting compound. Pa48b (a 1-liter culture) was grown in PIPES medium for 72 h at 10°C and was subsequently centrifuged, after which the supernatant was collected. The supernatant was extracted with ethyl acetate at a ratio of 1:1. The water phase was adjusted to pH 2 with HCl (36%) to precipitate PIPES. After filtration, the aqueous solution was concentrated in vacuo and the residue resuspended in water. The samples were directly applied to an anion-exchange column (Sephadex QAE, A-25; Pharmacia Fine Chemicals, Uppsala, Sweden), followed by washing with 150 ml water and 100 ml 0.2 M ammonium hydrogen carbonate. The bioactive fractions were obtained after elution with 100 ml of 30% acetic acid (AcOH) and testing of the fractions against *E. amylovora* Ea7 in the agar diffusion assay (5b agar medium). Active fractions were pooled and concentrated in vacuo and were subjected to high-pressure liquid chromatography (HPLC) using hydrophobic interaction liquid chromatography (HILIC) conditions (6). A Phenomenex Luna NH_2 HPLC column (250 mm by 2 mm; inner diameter, 5 μ m; Phenomenex, Aschaffenburg, Germany) was used with programmed gradient elution: 3 min at 0% B, with a gradient to 100% B in 27 min at a flow rate of 0.2 ml/min (where A is acetonitrile–0.1% AcOH and B is H_2O –0.1% AcOH).

HPLC was performed using an HP1100 HPLC system (Agilent, Waldbronn, Germany) connected either to an LTQ electrospray ionization mass spectrometer (ESI-MS) (Thermo Fisher, Egelsbach, Germany) or to a Gilson 207 fraction collector. All fractions (fraction size, 0.5 min) were subjected to the agar diffusion assay in order to detect compounds active against *E. amylovora*. In order to collect enough sample for nuclear magnetic resonance (NMR) spectroscopy and biological testing, separation was performed repeatedly using a semipreparative HPLC column (Nucleodur NH_2 ; 250 mm by 10 mm; inner diameter, 5 μ m; Macherey Nagel, Düren, Germany) with programmed gradient elution [3 min at 0% B, with a gradient to 100% B in 27 min at a flow rate of 3 ml/min (where A is methyl cyanide–0.1% AcOH and B is H_2O –0.1% AcOH)], a retention time of 16 min, and a yield of 1 to 2 mg from 1 liter of culture. High-resolution mass spectrometry (HR-MS) was performed by directly injecting the purified sample into an LTQ Orbitrap MS (Thermo Fisher, Egelsbach, Germany) by using a syringe pump.

Elucidation of the structure of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine. The retention time (with a Phenomenex Luna NH_2 column) was 21.7 min. HR-ESI-MS: $[M+H]^+$ measured: 317.14514, calculated: 317.14556 ($C_{12}H_{21}N_4O_6$); ESI-MS¹ of 317 ($[M+H]^+$): measured: 299.13580 (100), calculated: 299.13500 ($C_{12}H_{19}N_4O_5$); measured: 271.13926 (2), calculated: 271.13929 ($C_{12}H_{17}N_4O_4$); ESI-MS² of 299 ($[M+H-H_2O]^+$): 281 (4), 270 (3), 253 (100), 236 (7), 227 (2), 210 (2), 200 (2), 168 (1); ESI-MS³ of 253 ($[M+H-HCOOH]^+$): 236 (100), 224 (15), 210 (22), 208 (18), 196 (12), 181 (13), 168 (17), 154 (24), 137 (14), 125 (5), 111 (26), 97 (3).

NMR spectra were recorded using a Bruker DMX 600 spectrometer (Bruker, Rheinstetten, Germany) fitted with a TXI cryoprobe. For calibration of the ¹H NMR spectra, the solvent signal (D_2O δ = 4.65) was used. ¹H NMR (500 MHz, D_2O , 300K): δ 0.90 (d, J = 6.90, 3H, H_3C -4/5), 0.93 (d, J = 6.90, 3H, H_3C -4/5), 1.8 to 1.9 (m, J = 1.8, 1H, CH-3), 3.68 (d, J = 1.8, 1H, CH-2''), 3.69 (d, J = 1.8, 1H, CH-3'), 3.74 (dd, J = 5.95 J = 14.70, 1H, H_2C -3'), 3.82 (dd, J = 5.18 J =

TABLE 2. Indicator strains used in this study and inhibition of their growth by *P. agglomerans* 48b/90

Species	Strain designation(s)	Origin	Source ^a	Inhibition in the agar diffusion assay using ^b :	
				SM	CM
<i>Agrobacterium tumefaciens</i>	10621		IMET	+	– (St1)
<i>Alcaligenes faecalis</i> subsp. <i>faecalis</i>	DSM 30030		DSMZ	+	– (St1)
<i>Chromobacterium violaceum</i>	CVO26		K. Geider	+	– (St1)
<i>Erwinia amylovora</i>	Ea7	Pear; Germany	K. Naumann ^c	+	– (St1)
	Ea1/79	Cotoneaster; Germany	K. Geider	+	– (St1)
	S59/5	Pear; United Kingdom	K. Geider	+	– (St1)
	Ea273	Apple; United States	K. Geider	+	– (St1)
	GSPB1189		GSPB	+	– (St1)
	Ea266	Pear; Canada	M. Ullrich	+	– (St1)
	IL-6	Raspberry; United States	M. Ullrich	+	– (St1)
	PD576, PD579	Hawthorn; The Netherlands	M. Ullrich	+	– (St1)
<i>Escherichia coli</i>	N100		K. Rudolph	+	– (St1)
	K12		DSMZ	+	– (St1)
<i>Pectobacterium atrosepticum</i>		Potato	IMET	+	– (St1)
<i>Providencia rettgeri</i>	10209		IMET	+	– (St1)
<i>Pseudomonas fluorescens</i>	DSM 50090		DSMZ	+	– (St1)
<i>Pseudomonas putida</i>	6235		B. Völksch	+	– (St1)
<i>Pseudomonas syringae</i> pv. <i>atropurpurea</i>	MAFF301309	Ryegrass; Italy	K. Nishiyama	+	– (St1)
<i>Pseudomonas syringae</i> pv. <i>glycinea</i>	8/83, 1a/96, 13a/94	Soybean	B. Völksch	+	– (St1)
<i>Pseudomonas syringae</i> pv. <i>morsprunorum</i>	886	Cherry	K. Rudolph	+	– (St1)
<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i>	KZ667	Kudzu vine	Y. Takikawa	+	– (St1)
	Hb-1b, 1321	Bush bean	K. Naumann	+	– (St1)
<i>Pseudomonas syringae</i> pv. <i>sesami</i>	SUPP 1473	Sesame	Y. Takikawa	+	– (St1)
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	B301D	Pear	M. Ullrich	+	– (St1)
<i>Pseudomonas syringae</i> pv. <i>tabaci</i>	pp112	Tobacco	K. Naumann	+	– (St1)
<i>Pseudomonas syringae</i> pv. <i>tomato</i>	479	Tomato	K. Rudolph	+	– (St1)
		Tomato	K. Naumann	+	– (St1)
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	M1/3/98	Cauliflower	K. Naumann	+	– (St1)
<i>Xanthomonas campestris</i> pv. <i>holcicola</i>	3851	<i>Holcus</i> sp.	DSMZ	+	– (St1)
<i>Bacillus subtilis</i>			M. Ramm	+	– (St1)
<i>Micrococcus luteus</i>	DSM 20030		DSMZ	+	– (St1)
<i>Staphylococcus epidermidis</i>	SE1		B. Völksch	NG	+
<i>Candida albicans</i>	BMSY212		Bayer AG	+	(BYM) – (MEX)

^a IMET, strain collection of the former Zentralinstitut für Mikrobiologie und Experimentelle Therapie, Jena, Germany; DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; GSPB, Göttinger Sammlung Phytopathogener Bakterien, Germany.

^b +, inhibition; –, no inhibition; NG, not grown. SM, synthetic medium; CM, complex medium; St1, St1 agar; MEX, malt extract agar; 5b, 5b agar.

^c Strain collection of the former Bundesanstalt für Züchtungsforschung an Kulturpflanzen, Aschersleben, Germany.

14.73, 1H, H₂C-3'), 4.06 (d, *J* = 5.70, 1H, CH-2), 4.23 (t, *J* = 5.53, 1H, HC-2').
¹³C NMR (150 MHz, D₂O, 300K): δ 18.7 (C-4/5), 20.1 (C-4/5), 31.5 (C-3), 41.1 (C-3'), 54.1 (C-2'), 54.8 (C-2'), 55.0 (C-3'), 62.9 (C-2), 168.5, 171.1, 172.3, 179.4.

Screening of *Pantoea* strains for 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine. In order to test for the production of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine, nine *P. agglomerans* strains (Table 1) were grown in 50 ml PIPES medium. One milliliter of each culture was withdrawn and centrifuged to pellet the cells, and 1 to 5 µl was analyzed by LC-ESI-MS as described above. In addition, 40 ml of the supernatants was concentrated and used for the agar diffusion assay against *E. amylovora*.

RESULTS

Primary screening of the bioactivity of Pa48b. In the agar diffusion assay with 5b agar, Pa48b inhibited the growth of a large variety of gram-negative bacteria and several gram-positive bacteria (Table 2).

Changing from the synthetic medium (5b agar) to a complex medium (St1 agar) resulted in a loss of the inhibitory effect against most of the microorganisms tested (Table 2), suggesting that one or more medium constituents compensated for the antibacterial activity of Pa48b. Only the growth of *Staphylococcus epidermidis* was inhibited when the complex St1 agar medium was used. The inhibitory effects were not abolished by

supplementing the medium with any of the 20 proteinogenic amino acids; with citrulline, homoserine, isoleucine, or ornithine (40); or with casein hydrolysate (0.15%) (data not shown). None of the fungi tested (*Aspergillus nidulans*, *Alternaria* sp., *Botrytis cinerea*, *Geotrichum candidum*, *Fusarium solani*, *Mucor mucedo*, *Penicillium claviforme*) except *Candida albicans*, which was very sensitive to Pa48b, were affected by Pa48b in agar diffusion assays with potato dextrose agar (data not shown). Cytopathic effect inhibition assays (35) showed that Pa48b had no antiviral effect on influenza A virus/Hongkong/1/68 and also failed to exhibit any cytotoxicity against Madin-Darby canine kidney cells (data not shown).

Antibiotic production and its biochemical characteristics. The antibiotic production of Pa48b depends greatly on the cultivation temperature. Surprisingly, the highest rate of production was reached at 8°C to 12°C and was about 34-fold the rate of production at 28°C (Fig. 1). Interestingly, in contrast to antibiotic production, the population size of Pa48b was not significantly influenced by the different temperatures (10°C, 18°C, 28°C) (data not shown). The growth curve and the antibiotic production of Pa48b in a shaken culture at 10°C are shown in Fig. 2. The production of the antibiotic is strictly

FIG. 1. Temperature dependence of antibiotic production by Pa48b in liquid PIPES medium. Cultures were analyzed at an OD_{578} of 5.5. Bars represent means from three independent experiments; error bars, standard deviations. Different letters indicate significant differences ($P \leq 0.05$).

growth associated, starting at the early-exponential growth phase. The highest antibiotic concentration was detected when the strain reached the stationary-growth phase; thereafter, the antibiotic concentration remained stable.

The antibiotic activity of Pa48b was not inactivated by proteinase K or β -lactamase. The antibiotic isolated from Pa48b was stable when treated with heat for 10 min at 80°C. Even after 30 min of exposure to 100°C, some antibiotic activity remained (data not shown). The antibiotic activity was also stable at extreme pH values (pH 3 and 10) (data not shown).

Bioassay-guided isolation and structure elucidation. In order to characterize the antibiotic compound that is responsible for the observed inhibition of the growth of *E. amylovora*, the culture supernatant was subjected to bioassay-guided fractionation. Since the antibiotic turned out to be highly polar, it could not be extracted with organic solvents. Therefore, the supernatant was freeze-dried and then subjected to anion-exchange chromatography. The combined active fractions of the highly polar antibiotic were further purified by HILIC using an aminopropyl column (6). In LC-MS, the bioactive fraction exhibited a $[M+H]^+$ ion of 317 (Fig. 3A). HR-MS of the purified compound revealed its molecular composition to be $C_{12}H_{20}N_4O_6$, a compound with 5 double-bond units. The high oxygen and nitrogen contents of the compound and its chromatographic behavior pointed to a peptide-like structure. Tandem MS (MS-MS) analysis showed the loss of H_2O and of $HCOOH$. The MS^3 of the $[M+H-H_2O-HCOOH]^+$ ion gave a highly characteristic fragmentation pattern (Fig. 3A). In order to elucidate the structure of the suspected peptide, the compound was purified from 1 liter of culture for detailed NMR analysis. In the 1H NMR (Fig. 3B) at 0.90 and 0.93 ppm, two doublets with an integral of three were observed; these corresponded to two methyl groups [C-4 and C-5 of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine], which had a methine group at 1.8 to 1.9 ppm (C-3) as their neighbor. The doublet signal at 4.09 ppm corresponded to an amino methine moiety (C-2) that showed a cross-signal in the correlation spectroscopy (COSY) to C-3. From these data, valine was identified as a structural element of the antibiotic. A 2,3-diamino-

FIG. 2. Growth curve and antibiotic production of Pa48b in liquid PIPES medium at 10°C. Bars represent means from three independent experiments; error bars, standard deviations.

propionic acid moiety was deduced for the signals at 3.74 (C-3'), 3.82 (C-3'), and 4.23 ppm (C-2'), each corresponding to 1 proton, which showed correlations in the COSY. The two signals at 3.68 and 3.69 exhibited a small coupling constant of $J = 1.8$ Hz, which is characteristic for an epoxide moiety (C-2'' and C-3''). The ^{13}C NMR and attached proton test NMR spectra (Fig. 3C) showed four carbonyl signals at 168.5, 171.1, 172.3, and 179.4 ppm, which account for one carboxylic acid moiety and three amide groups, respectively. At 18.7 (C-4/5) and 20.1 (C-4/5) ppm, both methyl groups were observed, and at 31.5 ppm, the β -CH moiety (C-3) of the valine was observed. The signals at 54.1 and 62.9 ppm correspond to two amino methine moieties: C-2' of the 2,3-diaminopropionic acid and C-2 of valine. At 41.1 ppm, a signal for an amino methylene carbon, C-3' of the 2,3-diamino propionic acid, appeared. The remaining two signals at 54.8 and 55.0 are characteristic for CH carbons of the epoxide moiety (C-2'' and C-3'') of the epoxy-fumaric acid amide. By following the connectivity using H,H-COSY, heteronuclear multiple-bond correlation, and heteronuclear single-quantum coherence spectra, the structure of the antibiotic was identified as 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine (Fig. 3D). Since 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine has been characterized from *Serratia plymuthica* (37), it was possible to compare our proposed structure with the data in the literature, and they matched perfectly.

After identifying the bioactive compound from Pa48b, we analyzed seven additional *P. agglomerans* isolates and *P. agglomerans* C9-1 (biocontrol agent BlightBan C9-1) for their potential abilities to produce 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine. Like Pa48b, C9-1 and 39b/90 also produced 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine (data not shown).

Biological activity of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine. A secondary bioactivity screening (Table 3) with pure 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine led to results similar to those of the primary screening with the extract of Pa48b (Table 2). 2-Amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine exhibits broad antimicrobial potential and inhibited *Erwinia amylovora*, *Agrobacterium tumefaciens*, and *Pseudomonas syringae* (data not shown).

FIG. 3. Identification of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine from Pa48b as an antibiotic active against *E. amylovora*. (A) ESI-MS and ESI-MS-MS spectra of purified 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine. TIC, total ion current. (B and C) ¹H NMR and ¹³C NMR spectra, respectively, of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine. (D) Structure of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine. (E) Results of agar diffusion assays (on 5b agar) against *E. amylovora* Ea7 with 3.5 μg/μl amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine (left) and 5 μg/μl spectinomycin (right).

faciens, *Escherichia coli*, several *Pseudomonas syringae* pathovars, *Serratia marcescens*, *Bacillus subtilis*, different *Candida albicans* strains, and the yeast *Yarrowia lipolytica* (Table 3).

Because of the epiphytic origin of *P. agglomerans* and its

use as a biocontrol organism against plant diseases, we concentrated mainly on testing the potential of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine against bacterial phytopathogens. 2-Amino-3-(oxirane-2,3-dicarboxamido)-

TABLE 3. Inhibition of selected strains by different concentrations of the Pa48b antibiotic 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine in synthetic medium (5b agar [bacteria] or BYM [yeasts])

Species	Strain designation	Origin	Source ^a	Inhibition zone (mm) with antibiotic at:	
				20 µg/ml	200 µg/ml
<i>Agrobacterium tumefaciens</i>	10621	Pear; Germany	IMET	0	17.5
<i>Erwinia amylovora</i>	Ea7		K. Naumann ^b	19.5	35.5
<i>Escherichia coli</i>	K12		DSMZ	0	23.0
<i>Pseudomonas syringae</i>					
<i>Pseudomonas syringae</i> pv. <i>glycinea</i>	1a/96	Soybean	B. Völksch	0	13.0
<i>Pseudomonas syringae</i> pv. <i>morsprunorum</i>	886	Cherry	K. Rudolph	16.5	25.5
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	B301D	Pear	M. Ullrich	0	19.0
<i>Pseudomonas syringae</i> pv. <i>tabaci</i>	pp112	Tobacco	K. Naumann	0	12.0
<i>Pseudomonas syringae</i> pv. <i>tomato</i>	479	Tomato	K. Rudolph	0	28.5
<i>Serratia marcescens</i>	SM1		B. Völksch	11.0	16.0
<i>Bacillus subtilis</i> ^c	10		M. Ramm	0	0
<i>Candida albicans</i>	25000		IMET	20.0	30.0
<i>Candida albicans</i>	BMSY212		Bayer AG	28.0	NT ^d
<i>Yarrowia lipolytica</i>	25130		IMET	0	21.0

^a IMET, strain collection of the former Zentralinstitut für Mikrobiologie und Experimentelle Therapie, Jena, Germany; DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.

^b Strain collection of the former Bundesanstalt für Züchtungsforschung an Kulturpflanzen, Aschersleben, Germany.

^c With the antibiotic at 1 mg/ml, the inhibition zone was 115 mm.

^d NT, not tested.

propanoyl-valine strongly inhibited all tested *E. amylovora* strains that cause the economically important fire blight disease (Fig. 3E; Table 2). The minimal concentration of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine necessary to inhibit *E. amylovora* Ea7 and *P. syringae* pv. *morsprunorum* in 5b agar was less than 20 µg/ml. A minimum of 200 µg/ml of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine inhibited several plant-pathogenic *P. syringae* pathovars (Table 3).

However, 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine did not prevent the growth of either of these plant pathogens if the agar diffusion assays were performed in a complex medium such as St1 agar. Because the inhibitory effect of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine can be compensated for by adding *N*-acetylglucosamine to 5b medium (Fig. 4), it is likely that a sufficient supply of *N*-acetylglucosamine in the complex medium (St1) prevents Pa48b from inhibiting the growth of the microorganisms tested (Table 2).

In order to inhibit the growth of the human-pathogenic fungus *C. albicans* by 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine, concentrations as low as 1.5 µg/ml were sufficient. Thus, 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine was active against *C. albicans* at a level comparable to that of the established antifungal amphotericin B. However, after 4 days of incubation, a few presumably resistant or adapted colonies of *C. albicans* arose within the inhibition zones of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine but not within the inhibition zones of amphotericin B (data not shown).

DISCUSSION

The species *P. agglomerans* is known to colonize a variety of habitats, for example, plant surfaces (33). The epiphyte Pa48b has been isolated from soybean leaves and found to be well adapted to its niche (40). Besides coping with low nutrient availability, Pa48b efficiently suppresses competitors, as our in

vitro screening of bacterial phytopathogens for growth inhibition revealed (Table 2). Pa48b produces an antibiotic with broad activity against the gram-negative bacteria tested and the gram-positive bacterium *Bacillus subtilis* (Table 3). Thus, Pa48b is a promising biocontrol agent against various microbial plant diseases.

In order to characterize the compound(s) with high activity against plant pathogens, in particular against *E. amylovora*, a bioassay-guided isolation approach was used. A highly polar antibiotic was obtained after anion-exchange chromatography and HILIC-HPLC purification (6). The purified antibiotic turned out to be stable at extreme pHs; in addition, it was

FIG. 4. (A) Growth of *Candida albicans* in the presence of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine. (Left) BYM agar; (right) BYM agar supplemented with *N*-acetylglucosamine (4 mg/ml). (B) Growth of *Erwinia amylovora* in the presence of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine. (Left) 5b agar; (right) 5b agar supplemented with *N*-acetylglucosamine (4 mg/ml).

resistant to heat and treatment with proteinase K and β -lactamase.

Using HR-ESI-MS and NMR experiments, the structure of the compound was identified as 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine; this compound has already been isolated by Shoji et al. from *Serratia plymuthica* CB-25 (37). In addition, there are two closely related peptides, A19009 and Sch37137, from *Streptomyces collinus* and *Micromonospora* sp., respectively (7, 28). However, neither 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine nor the related peptides A19009 and Sch37137 had been isolated from *P. agglomerans* or characterized as highly active against plant pathogens such as *E. amylovora* and *P. syringae* pathovars. But Shoji et al. characterized 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine as an excellent inhibitor of the human pathogen *C. albicans* (37), which we also observed in our biological screening.

2-Amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine formation is associated with growth. The production of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine is temperature dependent: its rate of production is optimal between 8°C and 12°C. Interestingly, not only is the rate of antibiotic production highest at low temperatures, but after a short adaptation phase, Pa48b grew as well at 10°C as at 28°C. Secondary metabolite biosynthesis at low temperatures has been observed for phytotoxins such as persicomyacin (1), phaseolotoxin, or coronatin (4), which play important roles in the progression of plant diseases in different *P. syringae* pathovars. In the case of Pa48b, the epiphyte might benefit from its substantial growth and the high rate of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine production at low temperatures in springtime to outcompete other epiphytic and phytopathogenic microorganisms.

In contrast to the impact of many antibiotics from *P. agglomerans*, such as pantocins A and B (43) or herbicolin O (17), the impact of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine cannot be compensated for by supplementing the medium with amino acids or casein hydrolysate. Therefore, this compound is different from most other antibiotics from *P. agglomerans* strains. More than 90% of the 90 strains tested did not inhibit *E. amylovora* in the presence of different amino acids (41). Still, the growth-inhibiting effect of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine is compensated for if *E. amylovora* is grown on agar plates containing complex medium. In this respect, it was interesting to see how 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine acted. Since the related peptides Sch37137 and A19009 from *Micromonospora* sp. and *Streptomyces collinus*, respectively, inhibit the glucosamine-6-phosphate synthase (GlmS; EC 2.6.1.16) (7, 28), we suspected a similar mode of action for 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine. GlmS catalyzes the formation of glucosamine-6-phosphate (31), which is a key substrate of bacterial peptidoglycan and fungal chitin (24).

Supplementation of our synthetic medium with *N*-acetylglucosamine caused 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine to become inactive against *E. amylovora*, suggesting that 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine acts as a GlmS inhibitor, as do the peptides Sch37137 and A19009 (Fig. 4). Thus, as for other antibiotics from *P. agglomerans* strains, the ability of 2-amino-3-(oxirane-2,3-dicarbox-

amido)-propanoyl-valine to antagonize phytopathogens such as *E. amylovora* depends strongly on nutrient availability.

In order to study the distribution of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine among *P. agglomerans* strains, we analyzed seven additional selected *Pantoea* isolates (all from Dornburg, Germany [Table 1]) and the *P. agglomerans* strain C9-1. The active compound was found in the culture supernatants of Pa48b, Pa39b, and the biocontrol strain *P. agglomerans* C9-1 (BlightBan). Comparing the properties of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine with the information available (molecular formula, chemical and biological properties) for the antimicrobial herbicolin I from *P. agglomerans* C9-1, we conclude that the two compounds are identical (16, 17). In order to characterize the role of each herbicolin, Ishimaru et al. (1988) used *E. amylovora* strains resistant to herbicolin O and herbicolin I in immature pear fruit assays with *P. agglomerans* C9-1 (17). Herbicolin O-resistant strains were sensitive only to herbicolin I. Disease symptoms were suppressed half as efficiently as those due to wild-type strains when herbicolin I-resistant strains were used in these assays. Therefore, herbicolin I was found to have a strong impact on the biological control of fire blight disease. A comparison of our agar diffusion assays with the assays used for *E. amylovora* and *Pseudomonas syringae* pv. *glycinea* suggests that 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine is more important in suppressing *E. amylovora* than in suppressing *P. syringae* pathovars (Table 3).

In summary, the epiphyte Pa48b produces 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine, which is highly active against bacterial plant pathogens, in particular *E. amylovora*, in vitro. To date, 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine has been known only from *S. plymuthica* CB-25 and not from *Pantoea agglomerans* strains. The antibiotic herbicolin I from *P. agglomerans* C9-1, the structure of which has not been elucidated beyond its molecular formula (16), appears to be identical to 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine. Future in planta experiments will reveal the potential of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine as an agent against plant diseases and will allow us to understand its role in the antagonism of Pa48b against plant pathogens.

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