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The *Pseudomonas aeruginosa* antimetabolite L-2-amino-4-methoxy-*trans*-3-butenoic acid inhibits growth of *Erwinia amylovora* and acts as a seed germination-arrest factor

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Summary

The *Pseudomonas aeruginosa* antimetabolite L-2-amino-4-methoxy-*trans*-3-butenoic acid (AMB) shares biological activities with 4-formylaminooxyvinylglycine, a related molecule produced by *Pseudomonas fluorescens* WH6. We found that culture filtrates of a *P. aeruginosa* strain overproducing AMB weakly interfered with seed germination of the grassy weed *Poa annua* and strongly inhibited growth of *Erwinia amylovora*, the causal agent of the devastating orchard crop disease known as fire blight. AMB was active against a 4-formylaminooxyvinylglycine-resistant isolate of *E. amylovora*, suggesting that the molecular targets of the two oxyvinylglycines in *Erwinia* do not, or not entirely, overlap. The AMB biosynthesis and transport genes were shown to be organized in two separate transcriptional units, *ambA* and *ambBCDE*, which were successfully expressed from IPTG-inducible *tac* promoters in the heterologous host *P. fluorescens* CHA0. Engineered AMB production enabled this model biocontrol strain to become inhibitory against *E. amylovora* and to weakly interfere with the germination of several graminaceous seeds. We conclude that AMB production requires no additional genes besides *ambABCDE* and we speculate that their expression in marketed fire blight biocontrol strains could potentially contribute to disease control.

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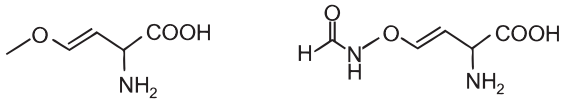
Introduction

L-2-amino-4-methoxy-*trans*-3-butenoic acid (AMB; Fig. 1) belongs to a small group of natural compounds known as oxyvinylglycines which have been isolated from *Pseudomonas aeruginosa* (Scannell *et al*., 1972), *Streptomyces* sp. (Pruess *et al*., 1974; Hirata *et al*., 1993), *Pseudomonas fluorescens* (McPhail *et al*., 2010), *Rhizobium japonicum* and *Pseudomonas andropogonis* (Owens *et al*., 1972). Oxyvinylglycines act as inhibitors of pyridoxal phosphate-dependent enzymes and as such have multiple targets in bacteria, animals and plants (Berkowitz *et al*., 2006). A well-known plant target is the ethylene biosynthesis enzyme ACC synthase, which is inhibited by 4-aminoethoxyvinylglycine (AVG), an oxyvinylglycine produced by a *Streptomyces* species. AVG is the active ingredient of ReTain®, a commercially available product marketed as a plant growth regulator for regulation of fruit set in orchard crops. AVG was recently shown to be active as a germination-arrest factor (GAF) that rather specifically inhibits germination of the seeds of graminaceous species, including a number of invasive grassy weeds that reduce the forage and turf quality of grasslands worldwide. In this activity, AVG resembles the *P. fluorescens* compound 4-formylaminooxyvinylglycine (FVG; Fig. 1) with which GAF activity was observed initially (McPhail *et al*., 2010). Interestingly, FVG also selectively inhibits the growth of several plant pathogens, including the fire blight bacterium *Erwinia amylovora*, illustrating again the broad target specificity of these compounds and the application potential they may have (Halgren *et al*., 2011). Different targets have also been reported for AMB (Berkowitz *et al*., 2006), and we recently evaluated its importance as a *P. aeruginosa* virulence factor using an *Acanthamoeba castellanii* cell model. Although AMB inhibited growth and induced encystment in *A. castellanii* (Lee *et al*., 2012), the required concentrations were rather high, making a strong contribution of AMB to the virulence of *P. aeruginosa* unlikely.

The biosynthesis of AMB requires a five-gene cluster, which specifies an export protein (AmbA), two non-ribosomal peptide synthetases (AmbB and AmbE), and two α-ketoglutarate-dependent non-haem iron oxygenases of the TauD family (AmbC and AmbD), suggesting



L-2-amino-4-methoxy-*trans*-3-butenoic acid (AMB) (*P. aeruginosa*)

4-formylaminooxyvinylglycine (FVG) (*P. fluorescens* WH6)

Fig. 1. Chemical structures of AMB and FVG.

that AMB is assembled by a thiotemplate mechanism (Lee *et al*., 2010). Although the biosynthetic pathway for AMB is not yet fully understood, the identification of the *amb* gene cluster has allowed for the construction of AMB-negative mutants and overexpression strains, which are instrumental for investigating the role of AMB in different biological interactions. Here we demonstrate that culture filtrates of a *P. aeruginosa* strain overproducing AMB displayed GAF activity and strongly inhibited the growth of FVG-sensitive and FVG-resistant *E. amylovora* isolates. The same inhibitory activity was observed when the *amb* genes were heterologously expressed in *P. fluorescens* CHA0, illustrating that the activity spectrum of a given biocontrol strain can be widened by engineered AMB production.

Results and discussion

AMB overproduction in P. aeruginosa *correlates with GAF activity and growth inhibition of* E. amylovora

The structural identification of the herbicidal GAF from *P. fluorescens* WH6 as FVG (McPhail *et al*., 2010), a novel member of the oxyvinylglycine family, suggested that AMB could have similar biological activities. We first evaluated its potential to inhibit germination of the grassy weed *Poa annua* (annual bluegrass) following a previously established procedure (Banowetz *et al*., 2008). Cell-free culture filtrates of PAO1 (wild type), PAO6665 (AMB-negative mutant) and PAO1/pME9618 (AMB overexpression strain) were tested for activity at several dilutions and compared with the activity present in culture filtrates of *P. fluorescens* WH6. As shown in Table 1, GAF activity was neither detected in culture filtrates of the AMB-negative mutant nor in those of the wild type, and culture filtrates of strain PAO1 carrying the IPTG-inducible P_{tac}-*ambABCDE* construct (pME9618) had only a modest GAF activity compared with that observed with filtrates of *P. fluorescens* WH6. However, AMB overproduction correlated with a very strong growth inhibition of *E. amylovora.* Culture filtrates from PAO1/pME9618 were at least as inhibitory for *E. amylovora* 153 as those from *P. fluorescens* WH6 and were also active towards a FVG-resistant sub-isolate (Fig. 2).

Expression of the ambABCDE *genes in the heterologous host* P. fluorescens *CHA0*

Being a pathogenic bacterium, *P. aeruginosa* is not suitable as a biocontrol agent. Plasmid pME9618 was therefore transferred to *P. fluorescens* CHA0 (Table S1) to engineer AMB production in this well-established biocontrol strain (Haas and Défago, 2005). Surprisingly, CHA0/pME9618 did not inhibit growth of *Escherichia coli* K12 (data not shown) in our routine plate overlay bioassay (Lee *et al*., 2010), indicating either that the production of AMB in strain CHA0 would require additional genes or that the *ambABCDE* genes were not correctly expressed

Table 1. AMB production correlates with GAF activity on *Poa annua*.

Filtrate source	AMB production	Germination score at relative filtrate concentration			
		0	0.03×	0.1×	0.3×
(PMS growth medium)	–		4.0 ± 0.00	4.0 ± 0.00	4.0 ± 0.00
P. aeruginosa PAO1	+		4.0 ± 0.00	4.0 ± 0.00	4.0 ± 0.00
P. aeruginosa PAO6665	–		4.0 ± 0.00	4.0 ± 0.00	4.0 ± 0.00
P. aeruginosa PAO1/pME9618	+++[a]	4.0 ± 0.00	4.0 ± 0.00	4.0 ± 0.00	2.2 ± 0.12
P. fluorescens CHA0	–		4.0 ± 0.00	4.0 ± 0.00	4.0 ± 0.00
P. fluorescens CHA1351	–		4.0 ± 0.00	4.0 ± 0.00	4.0 ± 0.00
P. fluorescens CHA1351/pME6032	–		4.0 ± 0.00	4.0 ± 0.00	4.0 ± 0.00
P. fluorescens CHA1351/pME9725	+++[a]		4.0 ± 0.00	3.4 ± 0.18	2.6 ± 0.16
P. fluorescens WH6	–[b]		1.9 ± 0.07	1.3 ± 0.09	1.0 ± 0.00

a. The identity of AMB concentration in culture supernatants with GAF activity was confirmed by tandem MS (Lee *et al*., 2010) and its concentration determined as 99 μM for *P. aeruginosa* PAO1/pME9618 and 168 μM for *P. fluorescens* CHA1351/pME9725.
b. Note that GAF activity of *P. fluorescens* WH6 is caused by the AMB-related compound FVG for which there is currently no quantification method available.
Bacterial strains (Table S1) were grown for 7 days in PMS medium (Banowetz *et al*., 2008) ± 1 mM IPTG at 30°C (*P. fluorescens*) or 37°C (*P. aeruginosa*). Cell-free culture supernatants were diluted in the same medium as indicated and tested for GAF activity as described by Banowetz and colleagues (2008). The relative abundance of AMB in cell-free culture supernatants was tested with an *E. coli* K12-based bioassay (Lee *et al*., 2010). A germination score of 1.0 corresponds to high GAF activity (complete germination arrest immediately after coleorhiza and plumule emergence); a score of 4.0 indicates no GAF activity (germination and seedling development same as in control). Values represent the mean ± standard error of three replicates with three *P. annua* seeds per treatment.

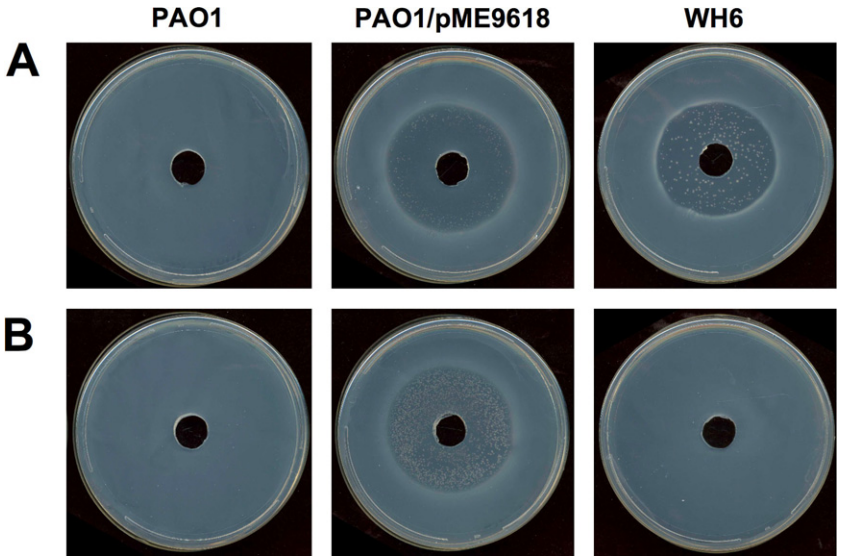


Fig. 2. Overproduction of AMB in *P. aeruginosa* results in growth inhibition of *E. amylovora*. Three hundred microlitres of undiluted cell-free culture filtrates of *P. aeruginosa* PAO1, *P. aeruginosa* PAO1/pME9618 and *P. fluorescens* WH6 (see Table S1 for strains and plasmids and legend of Table 1 for culture conditions) were deposited, as described previously (Halgren *et al*., 2011), into the central well of bacterial lawns of *E. amylovora* 153 (A) and of a FVG-resistant sub-isolate of *E. amylovora* 153 (B). Plates were incubated at 28°C for 2 days.

from the vector's *tac* promoter in the heterologous host. Indeed, operon predictions at http://www.pseudomonas.com suggested separate transcriptional units for *ambA* and *ambBCDE* in *P. aeruginosa* (Winsor *et al*., 2011). This prediction was tested and confirmed experimentally by RT-PCR experiments, which revealed co-transcription of *ambB* with *ambC* and co-transcription of *ambD* with *ambE*, but gave no evidence for an *ambA*/*ambB* co-transcription (Fig. 3). To achieve IPTG-inducible AMB production in a heterologous host such as *P. fluorescens* CHA0, the *ambA* and *ambBCDE* genes were therefore expressed separately as follows. P_{tac}-*ambA* was cloned into the mini-Tn*7* delivery vector pME3280a (to give pME9722) and chromosomally integrated in strain CHA0 using the Tn*7* transposition genes present on pUX-BF13 (Zuber *et al*., 2003). The resulting strain CHA1351 was then transformed with pME9725, carrying *ambBCDE* under P_{tac} control. (For details of strains and plasmids see Table S1 and Fig. 4). When grown with IPTG, CHA1351/pME9725 inhibited growth of *E. coli* K12 in our plate

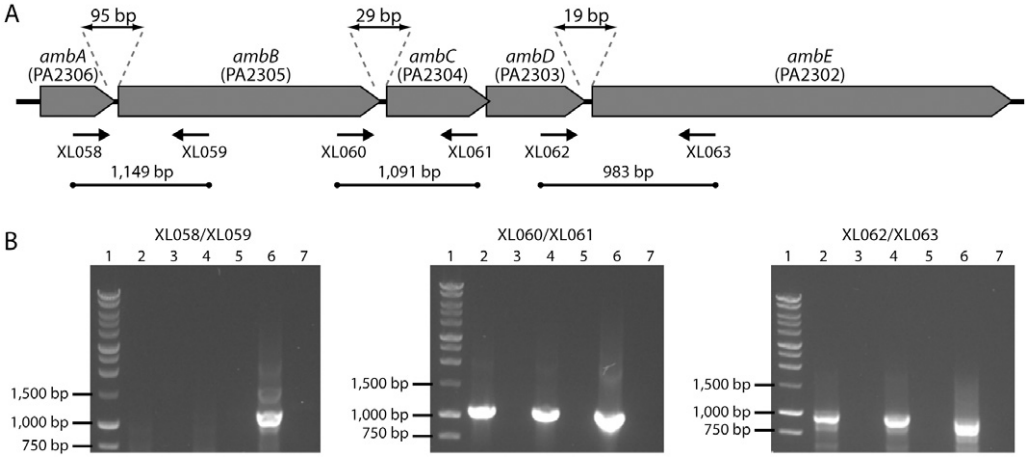


Fig. 3. RT-PCR analysis of the *amb* gene cluster reveals separate transcriptional units for *ambA* and *ambBCDE*.
A. Schematic representation of the *ambABCDE* gene cluster with sizes of PCR fragments generated with the primer pairs indicated.
B. RT-PCR analysis of the *ambABCDE* genes. Cultures of *P. aeruginosa* PAO1 were grown in triplicate to $OD_{600} = 1$ and $OD_{600} = 2$ and total RNA was extracted using the hot phenol method (Leoni *et al*., 1996). Following DNase I treatment (Roche) and confirmation of RNA integrity by agarose gel electrophoresis, cDNA synthesis was performed using Superscript III (Roche) and random primers, according to the manufacturer's instructions. RT-PCR reactions were then performed using primer pairs XL058 and XL059, XL060 and XL061, and XL062 and XL063 (Table S1), and GoTaq® DNA polymerase (Promega) according to the manufacturer's instructions. Lane 1, 1 kb DNA ladder (Promega); lane 2, cDNA from $OD_{600} = 1$ culture; lane 3, RNA from $OD_{600} = 1$ culture; lane 4, cDNA from $OD_{600} = 2$ culture; lane 5, RNA from $OD_{600} = 2$ culture; lane 6, *P. aeruginosa* PAO1 genomic DNA; lane 7, PCR reaction without DNA or RNA template. Results shown for each OD are from a typical experiment performed in triplicate. PCR reactions were analysed on a 0.9% agarose gel.

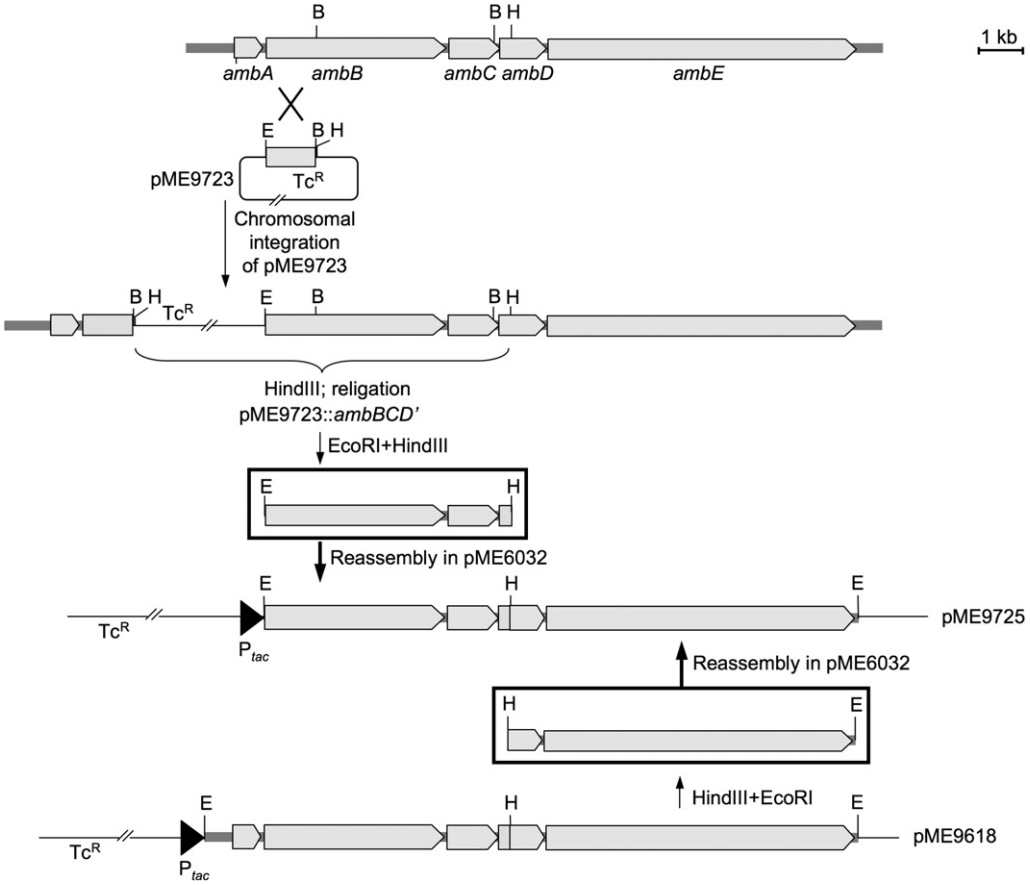


Fig. 4. Reassembly of the *ambBCDE* genes in the pME6032 expression plasmid. (For details of construction see also Table S1.)

overlay bioassay (not shown), indicating that (i) *ambA* and *ambBCDE* are sufficient to confer the ability of AMB production to a heterologous host and that (ii) both transcriptional units were expressed. The identity of AMB in culture supernatants of CHA1351/pME9725 was confirmed by tandem MS (Lee *et al*., 2010).

AMB production in P. fluorescens *results in GAF activity and growth inhibition of* E. amylovora *and* Bacillus megaterium

We next tested whether AMB production in *P. fluorescens* would correlate with GAF activity. As expected, GAF activity was neither detected in the growth medium nor in culture filtrates of strain CHA0 and the expression of *ambA* in strain CHA1351 was not sufficient for GAF activity either (Table 1). In contrast, culture filtrates of strain CHA1351/pME9725, in which *ambA* was coexpressed with *ambBCDE*, significantly reduced germination of *P. annua* seeds, although the activity was still lower than with culture filtrates of the reference strain WH6 (Table 1 and Fig. S1). As a further control, we also verified that culture filtrates of CHA1351 carrying the empty vector pME6032 lacked GAF activity.

Because GAF activity of the AMB-related compound FVG is not restricted to *P. annua* but has been observed with many other graminaceous seeds (Banowetz *et al*., 2008), we tested the effect of AMB on the germination of *Lolium temulentum* (darnel ryegrass) and *Triticum aestivum* (wheat cv. Madsen). As shown in Table 2, germination of seeds from both species was weakly arrested by culture filtrates from the AMB-overexpressing *P. fluorescens* strain CHA1351/pME9725, while no GAF activity was observed with filtrates from the empty vector control strain CHA1351/pME6032. The activity of culture filtrates with AMB was much lower than that of filtrates containing FVG which is in agreement with data from the *P. annua* experiments. We thus conclude that AMB, like FVG, is able to arrest seed germination of a number of graminaceous species.

Bacterial culture filtrates were next tested for growth inhibition of *E. amylovora*. In agreement with the GAF assays, only CHA1351/pME9725 was found to be active against *Erwinia*. As shown in Table 3, the inhibition zones

Table 2. GAF activity of AMB and FVG from *P. fluorescens* culture filtrates on *Lolium temulentum* and *Triticum aestivum*.

Species (common name)	Filtrate source	Germination score at relative filtrate concentration		
		0× (control)	0.1×	0.3×
L. temulentum (darnel ryegrass)	(PMS medium)	3.8 ± 0.12	3.7 ± 0.24	4.0 ± 0.00
	WH6		4.0 ± 0.00	2.9 ± 0.35
	CHA1351/pME6032		4.0 ± 0.00	3.7 ± 0.17
	CHA1351/pME9725		3.8 ± 0.12	3.4 ± 0.26
T. aestivum (wheat cv. Madsen)	(PMS medium)	3.2 ± 0.28	3.4 ± 0.29	3.1 ± 0.39
	WH6		2.0 ± 0.12	1.7 ± 0.08
	CHA1351/pME6032		4.0 ± 0.00	3.4 ± 0.30
	CHA1351/pME9725		3.5 ± 0.30	2.7 ± 0.28

Culture filtrates of *P. fluorescens* strains were prepared as described for Table 1 and tested for GAF activity on *L. temulentum* and *T. aestivum* according to Banowetz and colleagues (2008). The scoring system for evaluating seed germination was identical to that of *P. annua* and has been described (Banowetz *et al*., 2008). Values represent the mean ± standard error of six replicates with two seeds per treatment for *L. temulentum*, and of 12 replicates with one seed per treatment for *T. aestivum*.

caused by culture filtrates of CHA1351/pME9725 were almost twice as large as those caused by the WH6 filtrate. Moreover, the AMB-containing filtrate was active against a FVG-resistant sub-isolate of strain 153, suggesting that different *E. amylovora* enzymes were targeted by AMB and FVG.

Finally, we evaluated whether AMB-containing culture filtrates would be active against additional plant-associated bacterial species such as *B. megaterium* Km, *Pantoea agglomerans* EH252, *Pectobacterium carotovora* cc101, *P. fluorescens* WH6, *Pseudomonas syringae* pv. syringae PSS61 and *Rhodococcus fascians* 02-815. Of these, *B. megaterium* Km was strongly inhibited by culture filtrates of CHA1351/pME9725 (but not by filtrates of CHA0), which caused an inhibition zone of 38.85 ± 0.57 cm^2 around the central well.

Anti-Erwinia *activity in culture filtrates of CHA1351/pME9725 is due to AMB*

Pseudomonas fluorescens CHA0 makes a large variety of secondary metabolites which are instrumental to its activity as a biocontrol agent (Haas and Défago, 2005). To confirm that the anti-*Erwinia* effect of CHA1351/pME9725 is caused by AMB itself, and not by an AMB-induced increase of a pre-existing metabolite, we fractionated the solids from dried culture filtrates of CHA0, CHA1351, CHA1351/pME6032 and CHA1351/pME9725 by successive extractions with 90% and 76% ethanol, as described by Armstrong and colleagues (2009), followed by thin layer chromatography (TLC) analysis of the extracts. The distribution of anti-*Erwinia* activity in the extracts and residue is shown in Table 4. The largest proportion of the activity was found in the 90% ethanol extract, but substantial activity was also found in the subsequent 76% extract and a trace of activity remained in the residue. Following TLC fractionation of the 90% ethanol extract, ninhydrin staining revealed a very prominent band in the CHA1351/pME9725 TLC plate. This band was absent from the TLC plates in fractionations of extracts from all other strains analysed (Fig. S2). Different fractions of an unstained CHA1351/pME9725 chromatogram were then extracted and tested for anti-*Erwinia* activity as described previously (Halgren *et al*., 2011). As shown in Fig. 5, the

Table 3. Heterologous expression of *amb* genes in *P. fluorescens* results in growth inhibition of *E. amylovora* 153.

Filtrate source	AMB production	Inhibition zone (cm^2) in bacterial lawn	
		E. amylovora 153	FVG-resistant sub-isolate of *E. amylovora* 153
P. fluorescens WH6	–[a]	20.3 ± 0.22	0.0 ± 0.00
P. fluorescens CHA0	–	0.0 ± 0.00	0.0 ± 0.00
P. fluorescens CHA1351	–	0.0 ± 0.00	0.0 ± 0.00
P. fluorescens CHA1351/pME6032	–	0.0 ± 0.00	0.0 ± 0.00
P. fluorescens CHA1351/pME9725	+++[b]	39.4 ± 1.12	39.2 ± 0.14

a. Note that the anti-*Erwinia* activity of *P. fluorescens* WH6 is caused by the AMB-related compound FVG.
b. The concentration of AMB in culture supernatants of *P. fluorescens* CHA1351/pME9725 was 168 μM (Table 1).
Three hundred microlitres of undiluted cell-free culture filtrates of the indicated *P. fluorescens* strains (Table S1) were deposited, as described previously (Halgren *et al*., 2011), into the central well of bacterial lawns of *E. amylovora* 153 and a FVG-resistant sub-isolate of *E. amylovora* 153. Plates were incubated at 28°C and inhibition zones were measured after 48 h. Data represent the average of three replicate plates ± standard errors of the mean.

Table 4. Anti-*Erwinia* activity of extracts of culture filtrate from *P. fluorescens* CHA1351/pME9725.

Filtrate extracts (1× concentration[a])	Inhibition zone (cm^2) in bacterial lawn	
	E. amylovora 153	FVG-resistant sub-isolate of *E. amylovora* 153
Original culture filtrate	39.4 ± 1.12	39.2 ± 0.14
90% ethanol extract of dried filtrate solids	32.8 ± 0.50	32.9 ± 0.29
76% ethanol extract of 90% extraction residue	26.0 ± 1.07	24.0 ± 0.34
Residue from all extractions	10.8 ± 0.51	10.4 ± 0.21

a. Prior to testing in the *Erwinia* assay, extracts and residue were taken to dryness and the recovered solids dissolved in a volume of water equivalent to the volume of the original culture filtrate (= 1× concentration).

anti-*Erwinia* activity was present in two TLC fractions, with the major part of the activity coinciding with the position of the large, brown, ninhydrin-stained band specific to the CHA1351/pME9725 extracts and presumed to be characteristic for the non-proteinogenic amino acid AMB, and with some activity trailing in the adjacent lower fraction. TLC analysis of a 76% ethanol extract of the residue from the 90% extraction revealed that the anti-*Erwinia* activity left behind by the 90% extraction also chromatographed at the same position as a brown band visible after ninhydrin staining and presumed to correspond to AMB (data not shown). Together with the AMB quantification data (Table 1), it appears that all, or at least most, of the activity in the CHA1351/pME9725 culture filtrate is attributable to AMB.

In conclusion, we have shown here that AMB strongly inhibits the fire blight pathogen *E. amylovora* and possesses minor activity as an inhibitor of seed germination in tests with three graminaceous plants. The weak GAF activity of AMB compared with the previously demonstrated high activity of AVG and FVG (although the absolute concentration of the latter in culture supernatants is not known) raises interest with regard to the structure/activity requirements of oxyvinylglycines for GAF activity. Similarly, the anti-*Erwinia* activity of AMB is of particular interest as AMB remained active even towards a FVG-resistant *E. amylovora* isolate. Thus, changes in the structure of the side-chain of these oxyvinylglycine compounds can result in rather subtle differences in their antimicrobial properties. It would be exciting to express the *amb* genes in a marketed fire blight biocontrol agent and evaluate if AMB could contribute to disease control.

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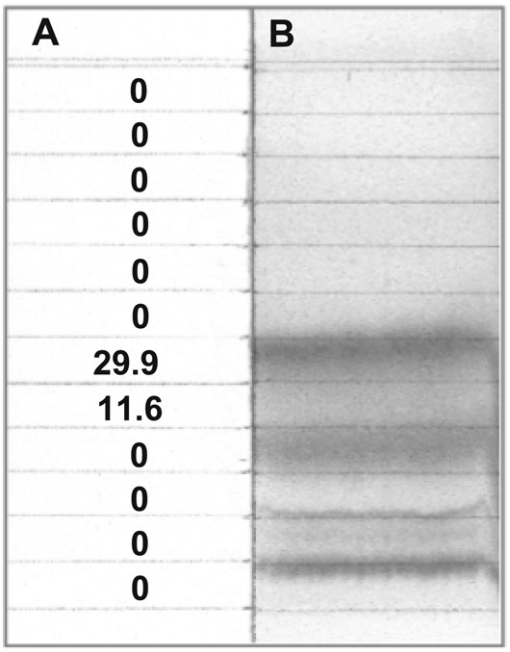

Fig. 5. Anti-*Erwinia* activity co-migrates with a ninhydrin-reacting band on TLC chromatograms. A 90% ethanol extract of the solids recovered from evaporation of the CHA1351/pME9725 culture filtrate was chromatographed on two cellulose TLC plates. Plates were developed over a distance of 12 cm in a mixture of ethylacetate/isopropanol/water (7.5/15/10). Twelve zones were recovered individually from plate A by aqueous extraction and tested for inhibitory activity against the FVG-resistant sub-isolate of *E. amylovora* 153. (For experimental details see Halgren *et al.*, 2011.) Plate B was stained with ninhydrin. Values are in cm^2 and indicate the size of inhibition zones.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Growth differences of *P. annua* seeds as a result of AMB overproduction in *P. fluorescens*. Strains (Table S1) were grown for 7 days in PMS medium $\pm$ 1 mM IPTG at 30°C. Cell-free culture supernatants were diluted in the same medium to 0.3× concentration and tested for GAF activity as described by Banowetz and colleagues (2008). Three surface-sterilized annual bluegrass seeds and 200 µl of test solution were placed in each well. Three replicate wells were set up for each treatment.

Fig. S2. TLC analysis of *P. fluorescens* culture filtrates. Samples were treated and analysed as described in detail by Halgren and colleagues (2011). Briefly, culture filtrates were dried under vacuum, extracted with 90% ethanol and applied to Avicel Microcrystalline Cellulose TLC plates (Analtech, Inc.). Plates were developed over a distance of 12 cm in a mixture of ethylacetate/isopropanol/water (7.5/15/10), air-dried, and sprayed with a ninhydrin solution to visualize AMB (indicated by an arrow).

Table S1. Bacterial strains, plasmids and oligonucleotides.