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# Biosynthetic Origin of Formylaminooxyvinylglycine and Characterization of the Formyltransferase GvgI

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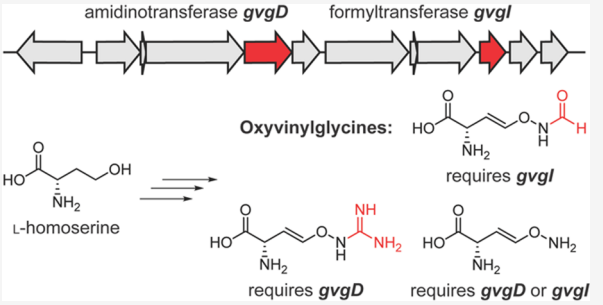
**ABSTRACT:** 4-Formylaminooxyvinylglycine (FVG) is an herbicidal and antibacterial nonproteinogenic amino acid produced by several strains of the *Pseudomonas fluorescens* species complex. It contains a unique vinyl alkoxyamine moiety with an O−N bond, and its biosynthetic origin remains unknown. Here, we show that the *gvg* cluster from *P. fluorescens* WH6 is responsible for the biosynthesis of FVG and two additional O−N bond-containing oxyvinylglycines, guanidinooxyvinylglycine and aminooxyvinylglycine. Feeding studies in the producing bacteria indicate that these compounds originate from homoserine. We identify a formyltransferase *gvgI* that is required for the production of FVG and characterize the activity of this enzyme *in vitro* toward amino acids with a side chain amine. Sequence similarity network analysis reveals that GvgI and homologues make up a distinct group from the main classes of formyltransferases.






## 1. INTRODUCTION

Rhizosphere bacteria live in close association with plant roots and produce secondary metabolites that affect the growth of plants and other microbes.[1] The rhizosphere bacterial group *Pseudomonas fluorescens* includes both biocontrol strains, which protect the plant from harmful microbes by secreting antimicrobial compounds like 2,4-diacetylphloroglucinol and phenazines,[2] and strains that are deleterious to plant growth. Five *P. fluorescens* strains were found to arrest seed germination of certain monocot weeds.[3] One strain, *P. fluorescens* WH6 (WH6 for short), was further investigated for this bioactivity. The culture filtrate of WH6 also inhibits growth of the plant pathogen *Erwinia amylovora*, which causes the destructive fireblight in orchard crops.[4]

The compound responsible for the dual herbicidal and antibacterial activity was identified and structurally characterized as 4-formylaminooxyvinylglycine (FVG).[5] Oxyvinylglycines are a small group of nonproteinogenic amino acids characterized by a side chain vinyl oxygen moiety, including L-2-amino-4-methoxy-*trans*-3-butenoic acid (AMB),[6] 4-(2′-aminoethoxy)vinylglycine (AVG),[7] and rhizobitoxine[8] (Figure 1A). Notably, AVG inhibits the biosynthesis of the plant hormone ethylene[9] and has been marketed as the plant growth modulator ReTain. The biosyntheses of these compounds have been studied to varying degrees.[10−13] The germination-arrest activity of FVG was linked to a 12-kb gene cluster in WH6 named *gvg*, and the genes essential for bioactivity were identified: *gvgR*, *gvgA*, *gvgB*, *gvgC*, *gvgF*, *gvgH*, *gvgI*, and *gvgJ* (Figure 1B).[14] Among these genes, *gvgA*, *gvgC*, *gvgF*, *gvgH*, and *gvgI* encode putative enzymes, which we predict play a role in FVG biosynthesis (Figure 1B). The *gvg* biosynthetic gene cluster differs from those of AMB, AVG, and rhizobitoxine, which suggests a different biosynthetic route for installing the vinyl alkoxyamine moiety in FVG. In particular, the genes *gvgA*, *gvgC*, *gvgD*, *gvgF*, and *gvgI* are not present in other oxyvinylglycine gene clusters. In this study, we identify two additional products of *gvg*, investigate the functions of the genes *gvgD* and *gvgI* in a heterologous host, characterize activity of the formyltransferase GvgI toward two substrates *in vitro*, and identify homoserine as the biosynthetic precursor of *gvg*-derived compounds.

## 2. RESULTS AND DISCUSSION

While we previously used bioactivity and thin-layer chromatography analysis to evaluate the production of FVG and other unidentified *gvg*-derived compounds,[14] here, we used liquid chromatography−high-resolution mass spectrometry (LC-HRMS) to directly assess compound production and identify genes essential for FVG biosynthesis. We cultured the wildtype WH6 and five single-gene knockout strains in the putative biosynthetic genes, Δ*gvgA*, Δ*gvgC*, Δ*gvgF*, Δ*gvgH*, or Δ*gvgI*. To better separate and detect FVG and related compounds from the metabolome, we derivatized compounds in culture extracts that contain a primary or secondary amine using

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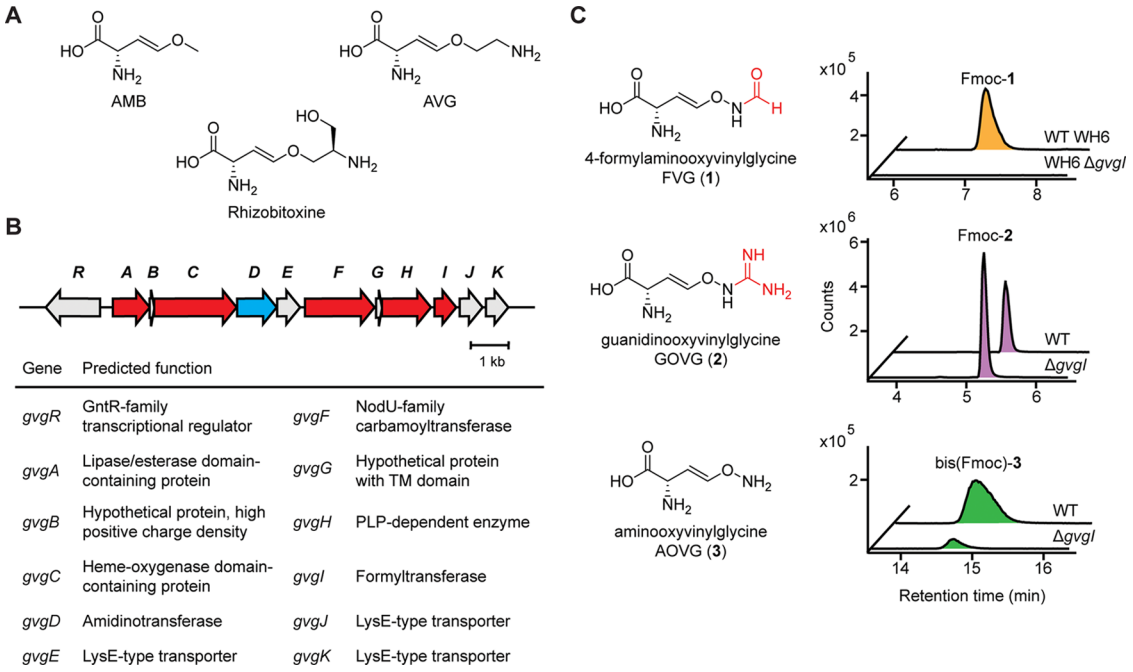


**Figure 1.** (A) Structures of oxyvinylglycines AMB, AVG, and rhizobitoxine. (B) *gvg* cluster in *P. fluorescens* WH6 with predicted functions below. Colored arrows indicate putative biosynthetic genes, with red arrows likely involved in FVG biosynthesis. (C) Structures of three products of the *gvg* cluster and production in wildtype WH6 and the Δ*gvgI* strain. Extracted ion chromatograms (EICs) are shown (Fmoc-FVG, *m/z* $[M + Na]^+$ 405.1057; Fmoc-GOVG, *m/z* $[M + H]^+$ 397.1506; bis(Fmoc)-AOVG, *m/z* $[M + H]^+$ 577.1969). The sodium adduct of Fmoc-FVG is used because it is more abundant than the proton adduct. Structures and mass spectra of the derivatized products are shown in Figure S1. Data are representative of three independent experiments with biological triplicates. PLP, pyridoxal 5'-phosphate. TM, transmembrane.

fluorenylmethyloxycarbonyl (Fmoc) chloride and then analyzed the metabolomes using LC-HRMS. We detected the Fmoc-derivatives of FVG and two additional oxyvinylglycines, aminooxyvinylglycine (AOVG) and guanidinooxyvinylglycine (GOVG) (Figures 1C and S1). AOVG contains a free vinyl alkoxyamine and GOVG has an amidino substituent at the amine (Figure 1C). AOVG and GOVG have been identified as antifungals extracted from cultures of *Streptomyces*,[15] but their production or biosynthesis in *P. fluorescens* have not been reported. All three compounds were detected in extract of the wildtype WH6 strain, but only GOVG and AOVG were detected in the Δ*gvgI* strain, which lacks the encoded formyltransferase (Figure 1C). None of the three compounds were detected in the other four knockout strains (Figure S2), suggesting that *gvgA*, *gvgC*, *gvgF*, and *gvgH* are required for production of all three compounds, whereas *gvgI* is only required for FVG production.

We next examined which *gvg* genes are sufficient for biosynthesis by constructing plasmids containing combinations of *gvg* genes for heterologous expression. We used *P. fluorescens* SBW25 (SBW25 for short) as a heterologous host because it is sequenced and closely related to WH6[16] but does not contain *gvg* in its genome. To verify the compounds are produced as a direct result of *gvg* expression, we constructed the plasmid pPSV35-*gvg*(*A*−*K*) which contains the entire cluster minus the regulator *gvgR* under the control of the *lacUV5* promoter. Expression of the cluster in SBW25 resulted in the production of all three oxyvinylglycines, but expression of the empty vector did not (Figure 2A). We then examined which genes are necessary for the production of each oxyvinylglycine by omitting genes. Because FVG and GOVG contain substituents at the amine of AOVG, we hypothesized that AOVG is a branching point for the biosynthesis of FVG and GOVG. Therefore, we constructed and expressed three plasmids: pPSV35-*gvg*(*A*−*H*), which omits *gvgI* and two of the three putative transporter genes *gvgJ* and *gvgK*; pPSV35-*gvg*(−*DE*), which omits the putative amidinotransferase-encoding gene *gvgD* and transporter gene *gvgE*; and pPSV35-*gvg*(−*DEI*), which omits *gvgD*, *gvgE*, and *gvgI* (Figure S3). As expected, FVG was only produced by the SBW25 + *gvg*(−*DE*) strain, and GOVG was only produced by the SBW25 + *gvg*(*A*−*H*) strain (Figure 2B). AOVG is produced by both the SBW25 + *gvg*(−*DE*) and SBW25 + *gvg*(*A*−*H*) strains but not by the SBW25 + *gvg*(−*DEI*) strain. These data suggest that the formyltransferase gene *gvgI* is necessary for the production of the formyl group-containing FVG, and the amidinotransferase gene *gvgD* is necessary for the production of the amidino group-containing GOVG, but at least one of the two genes, *gvgD* or *gvgI*, is required for AOVG production in the heterologous expression system.

Upon searching for biosynthetic intermediates in the lysates of the native FVG producer WH6 and the knockout strains of *gvg* genes, we detected a compound in the wildtype that was in very low abundance in the knockout strains (Figure S4). After derivatization using Fmoc-Cl and isolation, the compound was characterized by NMR as Fmoc-$N^5$-formyl-L-ornithine (Figures S5−S8). We postulated that the formyltransferase GvgI catalyzes formylation of L-ornithine as well as AOVG and that formylation of AOVG is on route to FVG biosynthesis. To test this hypothesis, we expressed GvgI (PFWH6_5257, UniProtKB E2XYY4) in *E. coli*, purified recombinant protein, and reconstituted its activity *in vitro*. Since we could not isolate AOVG from bacterial cultures because of its low abundance and stability, we tested L-canaline, a plant antimetabolite that is the 3,4-dihydro form of AOVG, as a GvgI substrate. We examined the formylation activity of GvgI toward ornithine


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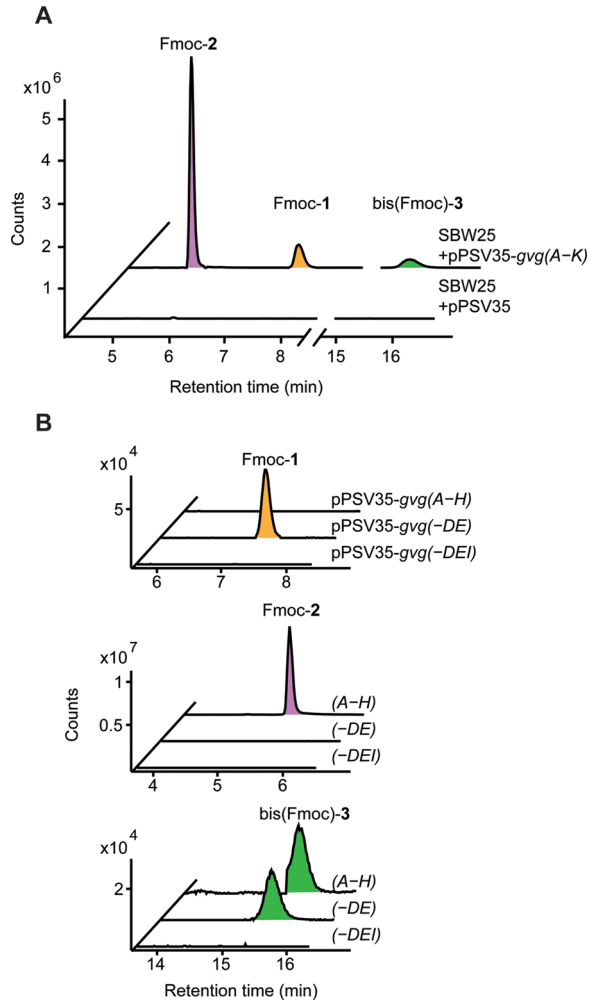


**Figure 2.** Detection of FVG (**1**), GOVG (**2**), and AOVG (**3**) in the SBW25 heterologous expression strain. Data are representative of three independent experiments with biological triplicates. (A) EICs of Fmoc-derivatized products from culture extract of SBW25 that expresses *gvgA*−*gvgK*. (B) EICs of Fmoc-derivatized products from culture extract of SBW25 that expresses partial clusters.

and canaline using $N^{10}$-formyl-tetrahydrofolate ($N^{10}$-fTHF) as the formyl donor. GvgI converted ornithine to $N^5$-formylornithine (Figure 3A), and after derivatization using Fmoc-Cl, this product exhibits identical tandem MS fragmentation as the Fmoc-$N^5$-formyl-L-ornithine that we obtained from bacterial culture (Figure S9A). When GvgI was incubated with canaline as a substrate followed by Fmoc-Cl derivatization at pH 10.5, two products with masses corresponding to Fmoc-formylcanaline were observed (Figures 3A and S9C). Tandem MS analysis identified fragments that suggest the major product is formylated at the side chain alkoxyamine and derivatized at the $\alpha$-amine (Fmoc-$N^5$-formylcanaline), and the minor product is formylated at the $\alpha$-amine and derivatized at the alkoxyamine (Fmoc-*N*-$\alpha$-formylcanaline) (Figure S9B). Derivatizing the GvgI and canaline reaction with Fmoc-Cl at pH 7.0 led to reduced Fmoc-derivatization of the major product (Figure S9C), but Fmoc-derivatization of the minor product remained unchanged. Because primary ammonium is less acidic than a protonated alkoxyamine, reduced Fmoc-derivatization at pH 7 further supports that the major product of GvgI is formylated at the side-chain alkoxyamine and derivatized by Fmoc at the $\alpha$-amine.

To examine the possibility of ornithine as a precursor to FVG and GOVG, we performed a direct feeding experiment using U-[$^{13}$C,$^{15}$N]-L-ornithine in wildtype WH6 cultures. Only nonspecific labeling in FVG and GOVG (+1 or +2 increase in *m*/*z*) was observed (Figure S10B), which suggests that ornithine is not a direct biosynthetic precursor, and therefore, formylation of ornithine by GvgI is not on pathway to FVG or GOVG biosynthesis. Canaline inhibits the growth of WH6 and cannot be tested in a feeding study.

To determine the substrate preference of GvgI, we performed a competition assay with both canaline and ornithine *in vitro*. We compared product formation using extracted ion chromatogram peak areas of Fmoc-formylcanaline and Fmoc-formylornithine in single-substrate assays and a competition assay with both substrates present. The production curve of formylcanaline was unchanged between the single-substrate and competition assays (Figure 3B, top); however, formation of formylornithine was substantially slower in the competition assay relative to the single-substrate assay (Figure 3B, bottom). The higher formylation rate of canaline in the competition assay suggests that GvgI prefers canaline over ornithine. To quantify the degree of substrate preference, we used an HPLC-based assay to measure substrate consumption over time for each substrate. Using 1 mM canaline or ornithine, 1 mM $N^{10}$-formyl-tetrahydrofolate, and 2.5 or 10 $\mu$M GvgI, respectively, GvgI consumed 1.3 ± 0.1 $\mu$M canaline per second over 90 s, for a turnover of 31 ± 2 per minute, whereas GvgI consumed 7 ± 2 $\mu$M ornithine per minute over 15 min, for a turnover of 0.7 ± 0.2 per minute (Figure S11). Thus, consumption of canaline under these conditions is an order of magnitude higher than that for ornithine, or 32−66 times faster.

We sought to identify precursors of FVG, GOVG, and AOVG. The oxyvinylglycines rhizobitoxine and AVG have been proposed to come from L-homoserine based on feeding and gene disruption studies.[11,12] To test whether FVG, AOVG, and GOVG also originate from homoserine, we examined the incorporation of homoserine into each compound using both direct and inverse feeding. We also tested whether the amidino nitrogen atoms of GOVG originate from arginine using direct feeding. For direct feeding, cultures of wildtype WH6 were supplemented with $^{15}$N-L-homoserine or [$^{15}$N$_2$-amidino]-L-arginine, and the metabolomes were analyzed using LC-HRMS after derivatization with Fmoc-Cl. The major ion for all three compounds in the cultures supplemented with $^{15}$N-L-homoserine was 1 *m*/*z* unit higher than that in control cultures (Figure S12), which suggests that one $^{15}$N atom was incorporated. In the cultures supplemented with [$^{15}$N$_2$-amidino]-L-arginine, the major ion for Fmoc-GOVG was 2 *m*/*z* units higher, but the major ion for FVG and AOVG was the same as that in control cultures (Figure S12B). The +1 *m*/*z* ions also exhibited increased abundance, which is likely due to nonspecific labeling (Figure S13).

Because fully $^{13}$C, $^{15}$N-labeled homoserine is not commercially available, we used an inverse feeding experiment[17] to investigate whether the carbon backbones of FVG and GOVG come from homoserine. Cultures were grown in $^{13}$C, $^{15}$N medium that was supplemented with unlabeled homoserine, and the isotope distributions of each compound were analyzed. The major ions of Fmoc-FVG were 5 and 6 *m*/*z* less than the fully $^{13}$C, $^{15}$N-labeled ion, corresponding to the incorporation of 5 or 6 $^{12}$C and $^{14}$N atoms from homoserine (Figure 4A). Because homoserine only contains five total C and N atoms,


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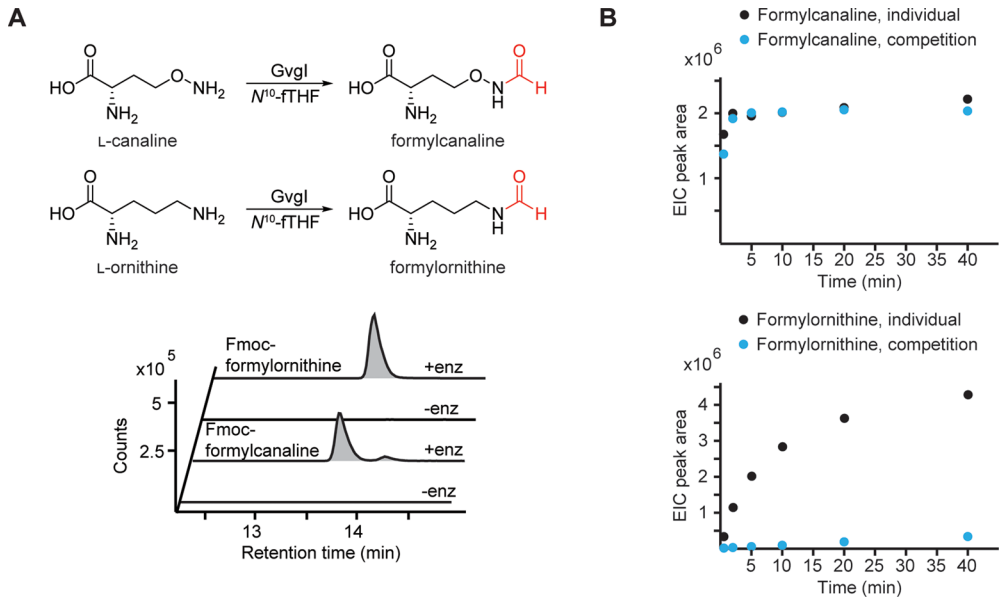


**Figure 3.** Biochemical characterization of the formyltransferase GvgI. (A) (Top) GvgI reaction schemes for the formylation of canaline and ornithine. (Bottom) EICs of Fmoc-formylcanaline ($m/z$ $[M + H]^+$ 385.1394) and Fmoc-formylornithine ($m/z$ $[M + H]^+$ 383.1601) in GvgI reactions and the minus enzyme control reactions. (B) Formation of Fmoc-formylcanaline (top) and Fmoc-formylornithine (bottom) in the individual or competition assays with GvgI. EIC peak areas of products were plotted over time. Data represent three separate experiments.

the incorporation of the additional atom may be due to the catabolism of homoserine and recycling of $^{12}C$ atoms into the one-carbon pathway, where they could be incorporated into FVG as a formyl group. Recycling and incorporation of the additional $^{12}C$ might also explain the high abundance of the −1 $m/z$ peak. For Fmoc-GOVG and bis(Fmoc)-AOVG, the major ions were 5 $m/z$ less than the fully $^{13}C$, $^{15}N$-labeled ion, which indicates that 5 atoms from homoserine were incorporated (Figures 4B and S14A). To determine which atoms come from the unlabeled homoserine, we performed tandem MS analysis on the major ions of interest: 385.1228 for Fmoc-FVG (5 $m/z$ less than the fully $^{13}C$, $^{15}N$-labeled ion), 401.1464 for Fmoc-GOVG, and 578.1916 for bis(Fmoc)-AOVG. The fragmentation patterns support the proposal that the carbon backbones, but not the aminooxy substituents, of each compound are derived from homoserine (Figures S14B and S15). Using 5 mM L-phenylalanine as an inverse feeding control resulted in a nonspecific incorporation pattern for both Fmoc-FVG and Fmoc-GOVG (Figure S10A), consistent with our conclusion.

Requirement of *gvgI* for FVG but not GOVG and AOVG production suggests that GvgI may convert AOVG to FVG as the last biosynthetic step. Similarly, AOVG may be transformed into GOVG by the putative amidinotransferase GvgD (Figure 4C, top). Because of its instability, we could not isolate AOVG to test as a substrate for GvgI. Using canaline as a proxy for AOVG suggests that GvgI prefers canaline as a substrate over ornithine. Although canaline and ornithine share the same side chain length, the alkoxyamine of canaline is more nucleophilic than the amine in ornithine. This difference likely contributes to the preference of GvgI for canaline. However, toward canaline as a substrate, GvgI appears not to be completely regioselective for the side chain alkoxyamine because the $\alpha$-amine of canaline is also formylated to an *N*-formamide product, which consistently constitutes ∼11 to 14% of the total product regardless of reaction time (Figure S16). Nonetheless, GvgI is specific for the side chain length of canaline and ornithine because it cannot formylate L-lysine under the same conditions (Figure S17).

Another possibility is that FVG and GOVG are formed first, then hydrolyzed to AOVG either spontaneously or enzymatically (Figure 4C, bottom), because AOVG was only observed alongside FVG or GOVG in the partial cluster expressions. Furthermore, treating GOVG with arginase has been shown to produce AOVG.[15] In this scenario, formation of FVG and GOVG would still require GvgI and GvgD, respectively. When neither *gvgI* nor *gvgD* is present, no AOVG is produced.

To contextualize the GvgI sequence and function with other known formyltransferases, we built a sequence similarity network[18] that includes GvgI-like proteins and enzymes from each major formyltransferase class: methionyl-tRNA formyltransferases,[19] sugar formyltransferases,[20,21] and formyltransferases in purine biosynthesis[22] (Figure S18). Each class of formyltransferase forms a distinct group, and GvgI has low (<25%) sequence identity to members of the other classes. The GvgI-like group comprises GvgI homologues from *gvg* and *gvg*-related clusters, which are widely distributed across several bacterial taxa.[23] Two smaller groups that branch off of the main GvgI-like group may comprise other amino acid *N*-formyltransferases that are involved in natural product biosynthesis. Interestingly, GvgI does not group with PvdF from pyoverdine biosynthesis[24] or the formylation domain of LgrA, the nonribosomal peptide synthetase from linear gramicidin biosynthesis.[25] PvdF formylates the side chain of hydroxyornithine, and LgrA formylates the *N*-terminus of valine. The lack of grouping of these amino acid formylating enzymes suggests their sequence divergence.

The result from the *gvg*(−*DE*) partial cluster expression complements work on a different FVG-producer, *Pantoea ananatis* BRT175.[26] The *gvg*-like gene cluster in the BRT175 strain contains orthologues of putative FVG biosynthetic genes *gvgA*, *gvgC*, *gvgF*, *gvgH*, and *gvgI* as well as the transcriptional regulator *gvgR* and the transporter *gvgJ*, but it does not contain orthologues of the amidinotransferase *gvgD* or the transporter


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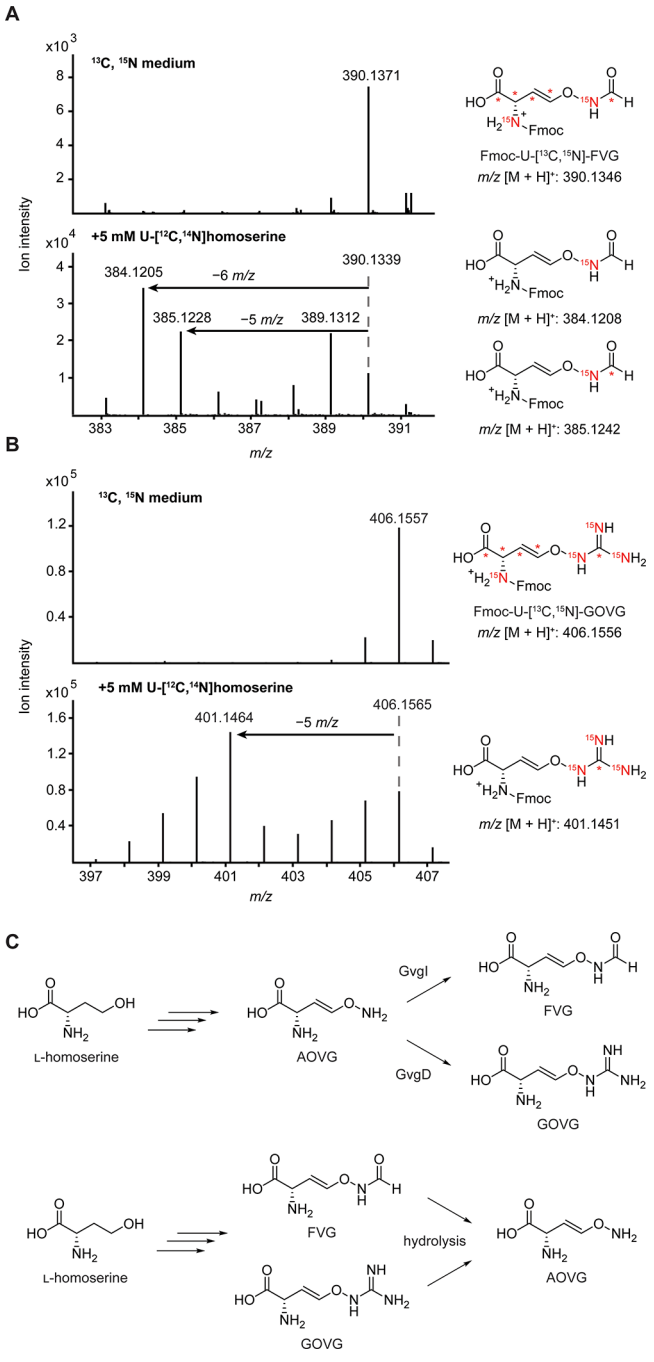


**Figure 4.** (A) Mass spectra of FVG from the inverse feeding experiment. Relevant ions are shown to the right. (Top) Mass spectrum of Fmoc-FVG extracted from cultures grown in fully $^{13}C$- and $^{15}N$-labeled medium. (Bottom) Isotope distribution of Fmoc-FVG with 5 mM unlabeled homoserine fed to cultures grown in $^{13}C$ and $^{15}N$ medium. Data are representative of two independent experiments with biological triplicates. (B) Detection of Fmoc-GOVG in the same experiment described in panel A. Errors between expected and observed *m/z* for all labeled ions are less than 7 ppm. (C) Two possible biosynthetic pathways of natural products from the *gvg* cluster, where AOVG is either a pathway intermediate (top) or a degradation product of FVG and GOVG (bottom).

*gvgE*.[27] BRT175 has been shown to produce FVG,[26] but the absence of *gvgD* suggests that this strain cannot produce GOVG. It is unknown whether BRT175 also produces AOVG. While WH6 Δ*gvgI* retains antimicrobial activity against *E. amylovora*,[4] this activity was abolished in transposon mutants of the *gvgI* homologue in BRT175, which is likely due to its inability to produce GOVG. These observations suggest that GOVG is also bioactive.

At least four different biosynthetic gene clusters have been discovered for oxyvinylglycines. AMB biosynthesis uses an L-glutamic acid precursor in a thiotemplated nonribosomal peptide synthetase pathway.[13] AVG and rhizobitoxine both come from a homoserine precursor but use different biosynthetic pathways.[10,11] We show that the biosynthesis of FVG, GOVG, and AOVG also uses homoserine as a precursor, which is more similar to that of AVG and rhizobitoxine than AMB. Genes in the *gvg* cluster differ substantially from those involved in the biosynthesis of rhizobitoxine or AVG, however, which suggests that different chemistry is involved in the formation of the vinyl alkoxyamine moiety of FVG and related compounds. The remaining steps of FVG biosynthesis are subject to further investigation. Elucidation of the full *gvg* biosynthetic pathway will answer key questions about the enzymatic chemistry in making these unique oxyvinylglycines.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.2c00374.

Materials and methods; primer table; NMR data; additional LC−MS and tandem MS chromatograms; GvgI rate plots; diagrams for heterologous expression of partial gene clusters; and formyltransferase SSN (PDF)

### Accession Codes

GvgI (PFWH6_5257): E2XYY4_PSEFL (UniProtKB).

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### Notes

The authors declare no competing financial interest.

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