Identification of the Biosynthetic Gene Cluster for 3-Methylarginine, a Toxin Produced by *Pseudomonas syringae* pv. *syringae* 22d/93[∇]

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The epiphyte *Pseudomonas syringae* pv. *syringae* 22d/93 (Pss22d) produces the rare amino acid 3-methylarginine (MeArg), which is highly active against the closely related soybean pathogen *Pseudomonas syringae* pv. *glycinea*. Since these pathogens compete for the same habitat, Pss22d is a promising candidate for biocontrol of *P. syringae* pv. *glycinea*. The MeArg biosynthesis gene cluster codes for the *S*-adenosylmethionine (SAM)-dependent methyltransferase MrsA, the putative aminotransferase MrsB, and the amino acid exporter MrsC. Transfer of the whole gene cluster into *Escherichia coli* resulted in heterologous production of MeArg. The methyltransferase MrsA was overexpressed in *E. coli* as a His-tagged protein and functionally characterized (K_m , 7 mM; k_{cat} , 85 min⁻¹). The highly selective methyltransferase MrsA transfers the methyl group from SAM into 5-guanidino-2-oxo-pentanoic acid to yield 5-guanidino-3-methyl-2-oxo-pentanoic acid, which then only needs to be transaminated to result in the antibiotic MeArg.

Microbial plant pathogens cause severe losses in agriculture each year (1). For example, the plant pathogen *Pseudomonas syringae* pv. *glycinea* is responsible for bacterial blight of soybean, a leaf spot disease of great economic impact. Besides chemical treatment, biocontrol agents that antagonize microbial plant pathogens are gaining increasing importance in fighting plant diseases (6, 11, 27). In a screening for possible biocontrol strains, an epiphytic bacterium showing a strong and selective activity against the pathogen *P. syringae* pv. *glycinea* was isolated from soybean leaves (29). The strain was characterized as *Pseudomonas syringae* pv. *syringae* 22d/93 (Pss22d). The antagonism of Pss22d against *P. syringae* pv. *glycinea* has been demonstrated successfully *in vitro* and *in planta* under greenhouse and field conditions (19, 29). In order to identify the molecular basis of the antagonism of Pss22d against *P. syringae* pv. *glycinea*, we focused on its secondary metabolites. Besides the well-known lipodepsipeptides syringomycin and syringopeptin (3), Pss22d produces the rare amino acid 3-methylarginine (MeArg) (5). As little as 20 nmol of MeArg strongly and selectively inhibits *P. syringae* pv. *glycinea* but no other pseudomonads *in vitro* (29). Since the inhibition can be compensated for by L-arginine supplementation but not by any other essential amino acid, it is likely that the toxin acts as an inhibitor of the arginine biosynthesis pathway or an arginine-dependent pathway, such as nitric oxide formation (13, 16). Feeding experiments and Tn5 transposon mutagenesis suggested that MeArg is produced by an *S*-adenosyl methionine (SAM)-dependent methyltransferase (5) converting the enol of 5-guanidino-2-oxo-pentanoic acid to 5-guanidino-3-methyl-2-oxo-pentanoic acid. An analogous reaction is known to occur

with the methyltransferases GlmT, DptI, and LptI, which form 3-methylglutamate from α -ketoglutarate (18). On the way to MeArg, only a transaminase catalyzing the formation of MeArg from 5-guanidino-3-methyl-2-oxo-pentanoic acid and an amino acid exporter to secrete the toxin would be needed.

Here, we describe the identification and functional characterization of the MeArg biosynthesis gene cluster from the epiphyte Pss22d.

MATERIALS AND METHODS

Strains, plasmids, and cultivation conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *P. syringae* strains were cultured and maintained on King's B (KB) medium (17). The Pss22d mutants were cultivated on selective KB medium containing spectinomycin (25 μ g/ml). The complemented strain *P. syringae* pv. *syringae* 22d/93.1C (Pss22d.1C) was grown on KB agar containing kanamycin (25 μ g/ml). *Escherichia coli* strains were maintained on Standard 1 medium (Merck). The *E. coli* strains 3150 (Ec3150) and 2795 (Ec2795) were cultivated on Standard 1 agar containing ampicillin (25 μ g/ml).

Agar diffusion assay. In order to test for MeArg production by *P. syringae* pv. *syringae* strains and Pss22d mutants, agar diffusion assays with *P. syringae* pv. *glycinea* 1a/96 (Psg1a) as the indicator strain were performed (5). Briefly, Psg1a was cultured on KB agar plates overnight at 28°C. Single colonies of Psg1a were used to prepare a suspension of approximately 4×10^8 CFU/ml in sterile water. For preparation of test plates (diameter, 135 mm), 50 ml of 5b agar medium (10) was warmed to 50°C and supplemented with 2 ml of the Psg1a suspension. The strains to be tested were inoculated onto the agar plates, and the plates were incubated at 28°C for 24 h. To determine the relative toxin concentration, a standard curve using purified MeArg was generated (4). Levels of syringomycin and syringopeptin production were determined using agar diffusion assays against *Geotrichum candidum* and *Bacillus megaterium* as indicator strains, respectively (26).

Tn5 mutagenesis. Transposon mutagenesis of Pss22d was performed by two-parent matings using *E. coli* S17- λ pir containing the plasmid pUT/mini-Tn5 Sm/Sp (30) as the donor strain. Plate matings were carried out on Standard 1 agar at 28°C overnight. Pss22d mutants were selected on MG agar (15) containing spectinomycin (25 μ g/ml) as the selection agent. The methyltransferase *mrsA* transposon mutant *P. syringae* pv. *syringae* 22d/93.1 (Pss22d.1) (Table 2) was identified by screening for the loss of antibiotic activity against *P. syringae* pv. *glycinea* in the agar diffusion assay, followed by liquid chromatography-mass spectrometry (LC-MS) analysis (5). The growth rates of Pss22d and the mutant Pss22d.1 in HSC medium (21) at 28°C were determined by measuring the optical density at 578 nm (OD₅₇₈).

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
<i>Pseudomonas syringae</i> pv. <i>syringae</i> strains		
Pss22d	Wild type, isolated from soybean	46
Pss22d.1	Sp ^r , transposon mutant of Pss22d, Tn5 insertion in methyltransferase gene <i>mrsA</i>	6
Pss22d.1C	Sp ^r Km ^r , Pss22d.1 harboring plasmid pB3150 for complementation of MeArg production	This study
<i>Pseudomonas syringae</i> pv. <i>glycinea</i> strain		
Psg1a	Wild type, isolated from soybean	46
<i>Escherichia coli</i> strains		
Ec2795	DH5α containing plasmid pG2795	This study
Ec3150	DH5α containing plasmid pG3150	This study
DH5α	<i>supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	17
BL21(DE3)	F ⁻ <i>dcm ompT lon hsdS_B(r_B⁻ m_B⁻) gal λ (DE3)</i>	Novagen
S17-λpir	<i>recA thi pro hsdR M⁺, RP4:2-Tc:Mu:Km Tn7, λpir, Tp^r Sm^r</i>	48
Plasmids		
pMKm	Source of Km ^r cassette	33
pGEM-T Easy	Cloning vector, Ap ^r	Promega
pJet 1.2	Cloning vector, Ap ^r	Fermentas
pBBR1MCS	Cloning vector, Cm ^r	27
pET28b(+)	Cloning vector, Km ^r	Novagen
pUT/mini-Tn5 Sm/Sp	Sm ^r Sp ^r Ap ^r , contains mini-Tn5	10
pG2795	Ap ^r , contains a 2,795-bp fragment carrying the MeArg biosynthesis cluster of Pss22d without the exporter gene <i>mrsC</i> in pGEM-T Easy	This study
pJ2795	Ap ^r , contains a 2,795-bp fragment carrying the MeArg biosynthesis cluster without the exporter gene <i>mrsC</i> in pJet 1.2	This study
pG1342	Ap ^r , contains a 1,343-bp adaptor PCR fragment carrying a part of the exporter gene <i>mrsC</i> of Pss22d in pGEM-T Easy	This study
pJ3150	Ap ^r , contains a 3,150-bp PCR fragment carrying the MeArg biosynthesis cluster of Pss22d in pJet 1.2	This study
pG3150	Ap ^r , contains a 3,150-bp PCR fragment carrying the MeArg biosynthesis cluster of Pss22d in pGEM-T Easy	This study
pB3150	Cm ^r , contains a 3,150-bp PCR fragment carrying the MeArg biosynthesis cluster of Pss22d in pBBR1MCS	This study
pET28b(+)-MrsA	Km ^r , contains a 1,053-bp PCR fragment carrying the methyltransferase gene <i>mrsA</i> of Pss22d	This study

Cloning and sequencing of the 3-methylarginine biosynthesis cluster. Genomic DNA was isolated following standard procedures (24). Preparation of plasmid DNA was performed using a Qiagen plasmid mini kit (Qiagen, Hilden, Germany). The insertion sites of Tn5 in the Pss22d mutants were determined by cloning the insertion loci into the plasmid pBBR1MCS and sequencing of the insert (5). A 265-bp fragment flanking the Tn5 insertion in the MeArg-deficient

mutant Pss22d.1 showed high homology to SAM-dependent methyltransferases and was identified as part of the MeArg biosynthesis gene cluster (5). The noncoding region upstream of the methyltransferase gene was used to design the reverse primer MT_{rev} (5'-ATCAGGAATGCGGCACTACA-3'). Analysis of the sequences surrounding the methyltransferase gene in the genome sequence of *P. syringae* pv. *syringae* B728a (PssB728a) revealed the presence of two open

TABLE 2. Characterization of Pss22d, Pss22d.1, and the complemented mutant Pss22d.1C as well as *E. coli* strains Ec2795 and Ec3150 containing 3-methylarginine biosynthesis genes^a

Strain	3-Methylarginine concn (μg/ml) at 24 h ^a		Growth (OD ₅₇₈) at 24 h	SM ^c	SP ^d
	Without arginine	With arginine ^b			
<i>Pseudomonas syringae</i> pv. <i>syringae</i> strains					
Pss22d (wild type)	74.7 ± 13.0	0.0 ± 0.0	7.3 ± 0.1	+	+
Pss22d.1 ^f	0.0 ± 0.0	0.0 ± 0.0	7.3 ± 0.1	+	+
Pss22d.1C	61.1 ± 5.2	0.0 ± 0.0	8.2 ± 0.1	+	+
<i>Escherichia coli</i> strains					
Ec2795	0.0 ± 0.0	0.0 ± 0.0	2.9 ± 0.1	—	—
Ec3150	4.3 ± 0.0	0.0 ± 0.0	3.0 ± 0.1	—	—

^a Concentrations were estimated by a standard curve (6).

^b 5b agar medium (10) supplemented with 0.1 mM L-arginine.

^c SM, syringomycin halo (+, like that of the wild type; —, no production).

^d SP, syringopeptin halo (+, like that of the wild type; —, no production).

^e Values for 3-methylarginine concentrations and growth levels are given as means ± standard deviations.

^f The predicted function of the disrupted gene of mutant Pss22d.1 was that of a SAM-dependent methyltransferase.

reading frames (ORFs) showing high similarity to genes coding for an amino-transferase and an amino acid exporter. The genome sequence of PssB728a was used to design the forward primer LT _{fwd} (5'-TTGGATTCGCGGTTTCGA-3'), binding a sequence in the amino acid exporter gene. A 2,795-bp fragment was PCR amplified from genomic DNA of Pss22d using the primers LT _{fwd} and MT _{rev} and cloned into pJET 1.2 (Fermentas, St. Leon-Rot, Germany), yielding plasmid pJ2795, which was sequenced using standard primers from the pJET 1.2 kit. Additionally, the 2,795-bp fragment was cloned into pGEM T-easy (Promega, Mannheim, Germany), yielding plasmid pG2795. To clone the complete biosynthesis cluster, an adaptor PCR was performed (22). Genomic DNA of Pss22d was digested with the restriction enzyme BclI and subsequently ligated with the BclI adaptor (5'-GATCCCTATAGTGAGTCGTATTAAC-3' [restriction site underlined]) overnight. Using the adaptor primer (5'-CCCTATAGTGAGTCGTATTAAC-3') and primer LT _{rev} (5'-ATTCCGTGGCTCTGAAGT-3'), derived from the sequence of the 2,795-bp fragment in pJ2795, a 1,342-bp fragment was PCR amplified from genomic DNA of Pss22d and cloned into pGEM T-easy, yielding plasmid pG1342. The primers MeArg _{fwd} (5'-GTGAATCAGCCTCGAAG-3') and MeArg _{rev} (5'-GGAGAGTCTGAATTTTTCGCG-3') were deduced from the sequences of pJ2795 and pG1342 and used to PCR amplify the complete MeArg biosynthesis gene cluster of Pss22d. The amplified 3,150-bp fragment was cloned into pJET 1.2, yielding plasmid pJ3150, which was sequenced using standard primers from the pJET 1.2 kit. For complementation of MeArg-deficient mutants, the 3,150-bp fragment was first cloned into pGEM T-Easy, yielding plasmid pG3150. Subsequently, a 3,150-bp XhoI-XbaI fragment cut from pJ3150 was ligated into XhoI-XbaI-digested pBBR1MCS, yielding plasmid pB3150.

In silico analysis of the 3-methylarginine biosynthetic genes. The nucleotide sequence of the putative MeArg biosynthetic gene cluster was translated into the corresponding amino acid sequences and analyzed using the BLASTP program from the National Center for Biotechnology Information. The fragment cloned in pJ3150 was compared to the genome sequence of PssB728a using VectorNTI software (Invitrogen, Karlsruhe, Germany), ClustalX2 (28), and TreeViewX (20). Promoter site prediction was performed with BPROM software (Softberry Inc., Mount Kisco, NY).

Cloning of the SAM-dependent methyltransferase MrsA. The SAM-dependent methyltransferase MrsA was PCR amplified from genomic DNA of Pss22d using the primers MT _{fwd} (5'-CATATGAATCTGCTTGACTCTATAAA-3') and MT _{rev} (5'-CTCGAGTCATGCCCTCCGACGAGATA-3') (the start and stop codons are shown in bold, and the NdeI and XhoI restriction sites are underlined). The amplified 1,060-bp fragment was digested with NdeI and XhoI and cloned into NdeI-XhoI-digested pET28b(+) (Novagen), yielding plasmid pET28b(+)-MrsA. The identity of the insert was confirmed by sequencing (JenaGen, Jena, Germany).

Overproduction and purification of MrsA. To overproduce the SAM-dependent methyltransferase MrsA, the plasmid pET28b(+)-MrsA was transformed into *E. coli* BL21(DE3). One liter of Standard 1 medium containing kanamycin (25 µg/ml) was inoculated with 20 ml of an overnight culture and incubated at 180 rpm at 37°C. At an OD₅₇₈ of 0.9 to 1.3, cultures were induced with 0.2 ml of 1 M isopropyl-β-D-thiogalactopyranoside (IPTG) and then incubated at 16°C to an OD₅₇₈ of 1.8 to 2.0. The cells were harvested by centrifugation. After resuspension of the cells in 50 ml binding buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.9, 5 mM imidazole, 10% glycerol), the cells were broken using a French pressure cell press (SIM-AMNCO; Spectronic Instruments Inc., Rochester, NY). The cell lysate was centrifuged (8,000 × g, 4°C, 40 min), and the supernatant was loaded onto Ni²⁺-nitrilotriacetate resin (Qiagen, Hilden, Germany) preequilibrated with binding buffer. The column was washed with 10 ml binding buffer and 4 ml wash buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.9, 60 mM imidazole, 10% glycerol). To elute the methyltransferase, 6 ml of elution buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.9, 200 mM imidazole, 10% glycerol) was added. Imidazole was removed by buffer exchange to 100 mM NaCl, 50 mM HEPES, pH 7.5, using Vivaspin-6 concentrators (30,000 molecular weight cutoff; Vivascience, Hannover, Germany) (6,000 × g, 4°C, 60 min). The purity of the methyltransferase MrsA was determined by SDS-PAGE and electrospray ionization mass spectrometry (ESI-MS).

Generation of 5-guanidino-2-oxo-pentanoic acid. Fifty milligrams of D-arginine in 5 ml phosphate buffer (30 mM, pH 8.3) was incubated with 46 U of D-amino acid oxidase from porcine kidney (Sigma, Deisenhofen, Germany) and 29,500 U of catalase from bovine liver (Sigma, Deisenhofen, Germany) for 24 h. After precipitation of the enzyme by acidification (pH 1.0) and vortexing with 5 ml CH₂Cl₂, the aqueous layer was concentrated and subjected to medium-pressure liquid chromatography (MPLC) (Büchi, Flawil, Switzerland) purification using RP-18 resin and gradient elution (solvent A, H₂O; solvent B, methanol [MeOH]). For LC-MS analysis, an Agilent HP1100 high-pressure liquid chro-

matography (HPLC) system (Agilent, Waldbronn, Germany) hooked to an LTQ mass spectrometer (Thermo Fisher, Egelsbach, Germany) fitted with a Luna NH₂-HPLC column (150 mm by 2 mm, 5 µm; Phenomenex, Aschaffenburg, Germany) under hydrophobic interaction chromatography (HILIC) (5) conditions was used. The details of the HPLC program are as follows: 3 min at 100% solvent A (MeCN, 0.1% acetic acid [AcOH]), followed by 27 min at 100% solvent B (H₂O, 0.1% AcOH) (flow rate, 0.2 ml/min). High-resolution electrospray ionization tandem mass spectrometry (HR-ESI-MS-MS) experiments were performed by direct injection of purified samples into an LTQ Orbitrap (Thermo Fisher, Egelsbach, Germany).

5-Guanidino-2-oxo-pentanoic acid eluted at 23.0 min; its mass spectral data are as follows: HR-ESI-MS [M+H]⁺ measured 174.08748, calculated 174.08787; ESI-MS-MS of [M+H]⁺ 60 (2), 70.06532 (41), 114.05496 (100), 130.09769 (8), 156.07690 (12).

Functional characterization of MrsA. In order to determine the methyltransferase activity required for the conversion of 2-oxo acids (5-guanidino-2-oxo-pentanoic acid, pyruvate, phenylpyruvate, and α-ketoglutarate) to 3-methyl-2-oxo acids, approximately 0.6 µM of the methyltransferase was mixed with 2-oxo acids (1.6 mM) and S-adenosylmethionine (SAM) (1.6 mM), with 100 µl reaction buffer (glycine buffer, 30 mM, pH 8.5) (145-µl final volume). The reaction mixture was incubated at 28°C for 40 min. Control reactions were performed without enzyme, SAM, or 2-oxo acids. All incubations were stopped by addition of 10 µl HCl (10 M) and 50 µl CH₂Cl₂ and vortexing. The conversion of 5-guanidino-2-oxo-pentanoic acid to 5-guanidino-3-methyl-2-oxo-pentanoic acid at different pH and temperature values was analyzed by LC-ESI-MS-MS comparing the peak ratios of 2-oxo acid to those of 3-methyl-oxo acid (for HPLC conditions, see above). The pH optimum of the methyltransferase (3.5 µg) was studied over a pH range of 1.0 to 11.0 using 100 µl citrate buffer (30 mM, pH 1 to 5), 100 µl phosphate buffer (30 mM, pH 5 to 8.5), and 100 µl glycine buffer (30 mM, pH 8.5 to 11) (145-µl final volume). The temperature dependence of the enzyme was analyzed from 10°C to 60°C (30 mM phosphate buffer, pH 8.0).

For nuclear magnetic resonance (NMR) analysis of 5-guanidino-3-methyl-2-oxo-pentanoic acid, 4 mg 5-guanidino-2-oxo-pentanoic acid (23 mmol), 10 mg SAM (23 mmol), and 60 µg enzyme were incubated in phosphate buffer (30 mM, pH 8.3) overnight. After precipitation of the enzyme by vortexing with CHCl₃, the sample was centrifuged, and the aqueous layer was concentrated *in vacuo* and subjected to MPLC separation followed by HPLC purification (see above). NMR measurements were performed with a Bruker DRX 500 spectrometer (Bruker, Rheinstetten, Germany) using the D₂O signal for calibration (D₂O, 4.68 ppm).

The product of the MrsA reaction eluted at 22.0 min; its mass spectral and NMR data are as follows: HR-ESI-MS [M+H]⁺ measured 188.10320, calculated 188.10352; ESI-MS-MS of [M+H]⁺ 60 (0.2), 84.08094 (11), 128.07075 (100), 144.11314 (1.5), 170.09250 (7.7); ¹H NMR (500 MHz, D₂O) δ 0.85 (d, J = 6.85, 3H), 1.51 to 1.61 (m, 1H), 1.93 to 2.01 (m, 1H), 2.25 to 2.34 (m, 1H), 3.28 to 3.36 (m, 1H), 3.44 to 3.51 (m, 2H).

Kinetics of MrsA. Kinetic data for MrsA were determined by LC-MS using the peak area of the base peak ions of the MS-MS spectra of 5-guanidino-2-oxo-pentanoic acid (*m/z* = 114) and 5-guanidino-3-methyl-2-oxo-pentanoic acid (*m/z* = 128) to determine their amounts at different time points (0.5, 1, 3, 5, 10, 15, 30, 45, and 60 min) using 3.3 µM MrsA and the substrates 5-guanidino-2-oxo-pentanoic acid and SAM both at the same concentration (0.4, 1.2, 2.0, 4.6, or 7.7 mM) per assay, with 100 µl phosphate buffer (30 mM, pH 9) at 45.0°C (150-µl final volume). *K_m* was determined after calculation of the reaction rates at the different substrate concentrations by use of the Lineweaver-Burk plot.

RESULTS

Characterization of Pss22d Tn5 mutants with altered 3-methylarginine production. To identify the genes involved in MeArg biosynthesis, mini-Tn5 mutagenesis was carried out. Out of 2,468 Tn5 mutants, 21 were affected in MeArg production, but only the mutant Pss22d.1 did not produce MeArg although its lipopeptide formation was comparable to that of the wild type. Sequencing of the DNA flanking the mini-Tn5 insert from Pss22d.1 revealed that the insertion was located in a gene with high similarity to genes coding for SAM-dependent methyltransferase from PssB728a (Pssr_0118; 96% identity, GenBank accession no. AAY35192) and *Acidovorax avenae* subsp. *citrulli* (Aave_3704; 75% identity, GenBank accession no. ABM34252)

FIG. 1. 3-Methylarginine biosynthesis gene cluster from *P. syringae* pv. *syringae* 22d/93, i.e., *mrsA* (GenBank accession no. FJ788104), *mrsB* (GenBank accession no. FJ788104), and *mrsC* (GenBank accession no. FJ788104) genes, coding for a SAM-dependent methyltransferase, an aminotransferase, and a 3-methylarginine exporter, respectively. The lines below the physical map represent conserved domains identified by a BLASTP search (Psyr_0116, AAY35190; Psyr_0117, AAY35191; Psyr_0118, AAY35192; Aave_3705, ABM34253; Aave_3704, ABM34252). The insertion site of the mini-Tn5 in the *mrsA* gene of the Pss22d.1 mutant is indicated (▼). Predicted sites for putative promoters were analyzed with BPROM software and are also indicated (◄). The sizes and homologies of the ORFs are given.

(Fig. 1). Due to the high homology of the disrupted gene to SAM-dependent methyltransferases, the mutant Pss22d.1 was a promising candidate for a MeArg biosynthesis mutant because this enzyme function is necessary to produce MeArg (5).

Analysis of the 3-methylarginine biosynthesis gene cluster. Analysis of the sequences surrounding the methyltransferase gene in PssB728a revealed the presence of two ORFs showing high similarity to genes coding for aminotransferases and amino acid exporters. Thus, the sequences of PssB728a were used to design primers to PCR amplify the putative MeArg biosynthesis gene cluster from genomic DNA of Pss22d. Because no significant similarities were found in the sequences downstream of the exporter gene, adaptor PCR was used to identify the downstream sequence in Pss22d. Finally, two primers, MeArg_{fwd} and MeArg_{rev}, were designed to amplify a 3,150-bp fragment from Pss22d containing the complete MeArg biosynthesis cluster. The three ORFs were designated *mrsA*, *mrsB*, and *mrsC* (for 3-methylarginine synthesis). Analysis of the deduced amino acid sequences indicated high degrees of similarity between the proteins from Pss22d and PssB728a (93 to 96% identity) (Fig. 1). Moreover, MrsA and MrsB showed significant sequence homology to a putative methyltransferase (Aave_3704; 75% identity, GenBank accession no. ABM34252) and a putative aminotransferase (Aave_3705; 66% identity, GenBank accession no. ABM34253), respectively, from *Acidovorax avenae* subsp. *citrulli* AAC00-1. However, no homologue of MrsC was found in *A. avenae* subsp. *citrulli* (Fig. 1).

MrsA was predicted to be a SAM-dependent methyltransferase with a conserved SAM domain belonging to the type 12 family (6). MrsB contains the motifs typical of an aminotransferase of family I (15) (Fig. 2A). From the multiple sequence alignment with members of aminotransferase family I, we deduced that MrsB belongs to the subfamily Iy (14). This sub-

family contains aminotransferases catalyzing the formation of different amino acids, e.g., lysine and tyrosine (Fig. 2B) (9, 14). MrsC shows high similarity to a putative lysine transporter from PssB728a (8) and belongs to the LysE superfamily. A detailed motif analysis revealed that MrsC groups to the LysE subfamily (2) (Fig. 3).

A precise analysis of the sequences of *mrsA* and *mrsB* revealed an overlapping region in the start/stop sequences (*mrsA*-ATGA-*mrsB*) (Fig. 1). This suggests that *mrsA* and *mrsB* are translationally coupled, which was supported by the finding of only one putative promoter region (Fig. 1). Downstream of the MeArg biosynthesis cluster, a gene with high similarity to a putative transposase from PssB728a was found. Upstream of the MeArg biosynthesis cluster, an ORF with similarity to a gene coding for a mannuronan C-5 epimerase from *Pseudomonas syringae* pv. *tomato* (41% amino acid identity, GenBank accession no. AAO57541) was identified.

Complementation of the mutant Pss22d.1 and heterologous expression of the entire MeArg biosynthesis gene cluster in *E. coli*. The MeArg-deficient mutant Pss22d.1 was complemented with the plasmid pB3150, harboring the genes *mrsA*, *mrsB*, and *mrsC*. This complementation completely restored MeArg production in Pss22d.1, resulting in a growth inhibition of Psg1a in the agar diffusion assay similar to that of the wild-type strain Pss22d (Table 2; Fig. 4). Moreover, the high-copy-number plasmid pG3150, containing the MeArg biosynthesis genes *mrsA*, *mrsB*, and *mrsC* from Pss22d, was introduced into *E. coli* DH5α. The resulting strain, Ec3150, produced MeArg. In HSC liquid medium Ec3150 generated only small amounts of MeArg (Table 2), but on 5b agar plates Ec3150 produced amounts of MeArg similar to those produced by the parent strain Pss22d, causing comparable inhibition zones in the agar diffusion assay against *P. syringae* pv. *glycinea* (Fig. 4). The inhibition zones in the agar diffusion assay caused by Ec3150

FIG. 2. (A) Sequence alignment for members of aminotransferase superfamily I. Abbreviations (GenBank or SwissProt accession numbers): Pss22d AT, *P. syringae* pv. *syringae* 22d/93 aminotransferase MrsB (FJ788104); PssB728a AT, *P. syringae* pv. *syringae* B728a aminotransferase Psyr_117 (YP_233229); Aac AT, *A. avenae* subsp. *citruilli* aminotransferase (YP_972027); Eco TyrB, *E. coli* aromatic aminotransferase (X03628); Eco HisC, *E. coli* imidazole acetol phosphate (IAP) aminotransferase (P06986); Rme AspA, *Rhizobium meliloti* aspartate aminotransferase A (L05064); Rme AspB, *Rhizobium meliloti* aspartate aminotransferase B (L12149). (B) Dendrogram of the class I aminotransferase superfamily, generated with the ClustalX multiple-alignment program. The primary function(s) of each aminotransferase is indicated (right). Abbreviations (GenBank or SwissProt accession numbers): Rme Tyr, *Rhizobium meliloti* aromatic aminotransferase (L05065); Pae PhhC, *Pseudomonas aeruginosa* aromatic aminotransferase (M88627); Eco AspC, *E. coli* aspartate aminotransferase (X03629); Eco TyrB, *E. coli* aromatic aminotransferase (X03628); Msa Asp2, *Medicago sativa* aspartate aminotransferase (AAB46611); Alf Asp1, alfalfa aspartate aminotransferase 1 (P28011); Rat AspM, rat mitochondrial aspartate aminotransferase (M18467); Rat AspC, rat cytosolic aspartate aminotransferase (J04171); Sty HisC, *Salmonella enterica* serovar Typhimurium HisC (J01804); Eco HisC, *E. coli* IAP aminotransferase (P06986); Bsu HisH, *Bacillus subtilis* IAP aminotransferase (P17731); Zmo HisH, *Zymomonas mobilis* IAP aminotransferase (L36343); Hvo HisH, *Haloferax volcanii* IAP aminotransferase (M33161); Lla HisC, *Lactococcus lactis* IAP aminotransferase (M90760); Sce His5, *Saccharomyces cerevisiae* IAP aminotransferase (X05650); Cgl His, *Corynebacterium glutamicum* histidine biosynthesis (AY238320); Rme AspA, *Rhizobium meliloti* aspartate aminotransferase A (L05064); Rme AspB, *Rhizobium meliloti* aspartate aminotransferase B (L12149); Bsp Asp, *Bacillus* sp. aspartate aminotransferase (P23034); Sso Asp, *Sulfolobus solfataricus* aspartate aminotransferase (X16505); Cgl 2877, *Corynebacterium glutamicum* purine synthesis (AY238316); Rat Tyr, rat tyrosine aminotransferase (X02741); Cgl DapC, *Corynebacterium glutamicum* lysine biosynthesis (AY170830); Cgl IlvE, *Corynebacterium glutamicum* IlvE (AF424637).

were completely reversed after addition of 0.1 mM arginine (Fig. 4). Furthermore, the production of MeArg was verified by LC-MS-MS.

Heterologous expression and functional characterization of the SAM-dependent methyltransferase MrsA. In order to characterize the key enzyme in the biosynthesis of 3-methylarginine, we overexpressed the SAM-dependent methyltrans-

ferase MrsA as a His-tagged protein in *E. coli* BL21(DE3) using plasmid pET28b(+)-MrsA. Overexpression and enzyme purification yielded 7 mg/ml pure protein, as can be seen by SDS-PAGE (Fig. 5A). ESI-MS analysis confirmed the identity of the protein, with the determined protein mass minus methionine (M-Met) of 40,766 Da being in very good accordance with the calculated mass of 40,765 Da (Fig. 5B).

RhtB subfamily			
Pss22d Exp	17	VVPGPSFAYA	77 IGCAYLVAL
PssB728a Exp	17	VVPGPSFAYA	77 IGCTYLVAL
Ec YeaS	21	LVPGEITLFV	81 LGAFYLLYL
Pa YcaR	20	LIPGPNLSFV	80 LGAAFLFYL
Ec RhtC	16	MSPGPDFFV	76 GGGYLLCWM
Ps CmaU	12	LMPGPENTLL	72 ASMAFLFKT
LysE subfamily			
Ah YggA	16	IFIGAQNAFV	60 GGVFLFLWF
Ec YggA	16	LPLGQNAFV	74 GGVVFLWY
		motif I	motif II
			motif III

FIG. 3. Multiple sequence alignment for RhtB and LysE amino acid transporters. Both subfamilies are members of the LysE superfamily and differ in the shown motifs. Abbreviations of the exporters (GenBank accession numbers): Pss22d Exp, *P. syringae* pv. *syringae* 22d/93 MrsC (FJ788104); PssB728a Exp, *P. syringae* pv. *syringae* B728a lysine exporter protein LysE/YggA (YP233228); Ec YeaS, *E. coli* neutral amino acid efflux system protein (NP416312); Pa YcaR, *Pseudomonas aeruginosa* leucine export protein (NP253445); Ec RhtC, *E. coli* threonine efflux system protein (YP026264); Ps CmaU, *Pseudomonas syringae* biosynthesis of coronamic acid protein (AAC46034); Ah YggA, *Aeromonas hydrophila* putative amino acid transporter YggA (SwissProt no. P52047); Ec YggA, *E. coli* arginine exporter protein (NP289490).

The methyltransferase MrsA efficiently converts 5-guanidino-2-oxo-pentanoic acid to 5-guanidino-3-methyl-2-oxo-pentanoic acid in the presence of SAM (Fig. 6). Control reactions without enzyme or without one of the substrates did not show any product formation. The enzyme has its pH optimum at pH 9. The catalytic activity decreases 3-fold at pH 7 and 10-fold at pH 10. At 45°C maximal conversion was achieved, whereas at the physiological temperature of 28°C the enzyme activity decreased 3-fold compared to the optimum. The methylation of 5-guanidino-2-oxo-pentanoic acid is fast, with a K_m of 7 mM and a k_{cat} of 85 min⁻¹.

Use of other 2-oxo acids, such as pyruvate, α -ketoglutarate, and phenylpyruvate, as the substrate did not result in any 3-methyl-2-oxo acid formation.

DISCUSSION

By use of Tn5 transposon mutagenesis, the 3-methylarginine-deficient mutant Pss22d.1 was obtained and served to identify a 3-kb gene cluster responsible for the biosynthesis of MeArg. The gene cluster contained three ORFs designated *mrsA*, *mrsB*, and *mrsC*. MrsA showed high homology to SAM-dependent methyltransferases, MrsB was identified as a putative aminotransferase belonging to the subfamily Iy, and MrsC was assigned to code for a 3-methylarginine exporter due to its similarity to amino acid exporters (2) (Fig. 1). The SAM-dependent methyltransferase MrsA methylates 5-guanidino-2-oxo-pentanoic acid to 5-guanidino-3-methyl-2-oxo-pentanoic acid. Subsequently, the putative aminotransferase MrsB transaminates 5-guanidino-3-methyl-2-oxo-pentanoic acid to result in 3-methylarginine, which is secreted via the MeArg exporter MrsC (Fig. 7).

The involvement of the three genes in MeArg biosynthesis was proven by complementation of the mutant Pss22d.1 with the plasmid pB3150 harboring the entire MeArg biosynthesis gene cluster (*mrsA*, *mrsB*, and *mrsC*). The complemented mutant showed a clear inhibition of *P. syringae* pv. *glycinea* in the agar diffusion assay. In addition, *E. coli* DH5 α bearing the plasmid pG3150 was shown to produce MeArg by use of the agar diffusion assay and LC-MS for detection. In contrast, *E. coli* DH5 α bearing plasmid pG2795, which harbors only the genes *mrsA* and *mrsB*, did not cause an inhibition zone in the agar diffusion assay, which suggests the function of MrsC as a MeArg exporter (Fig. 4). The complementation experiments are in line with the high homology of MrsC to LysE exporters, which serve to export the basic amino acids L-lysine and L-arginine (7).

The deduced genes from the MeArg biosynthesis cluster of Pss22d showed a high similarity to genes from PssB728a. However, in spite of PssB728a containing a similar set of genes, this strain did not produce MeArg. A possible reason for this is a

FIG. 4. Agar diffusion bioassays (5b agar medium [10]) performed with the indicator strain Psg1a (sensitive to 3-methylarginine) without L-arginine (A) and with 0.1 mM L-arginine (B), which compensates for the toxicity of 3-methylarginine (5). Pss22d, *Pseudomonas syringae* pv. *syringae* wild-type strain; Pss22d.1, methyltransferase Tn5 mutant; Pss22d.1C, methyltransferase Tn5 mutant complemented with the plasmid pB3150 harboring the genes *mrsA*, *mrsB*, and *mrsC*; Ec2795, *Escherichia coli* DH5 α complemented with the plasmid pG2795 harboring the genes *mrsA* and *mrsB*; Ec3150, *E. coli* DH5 α complemented with the plasmid pG3150 harboring the genes *mrsA*, *mrsB*, and *mrsC*; Psg, *Pseudomonas syringae* pv. *glycinea* wild-type strain.

FIG. 5. SDS-PAGE (A), ESI-MS (B), and deconvoluted mass (C) of the SAM-dependent methyltransferase MrsA, involved in 3-methyl-arginine biosynthesis.

lack of function of the genes (96% identity to *mrsA*, 93% identity to *mrsB*, and 94% identity to *mrsC* at the protein level) or the possibility that PssB728a does not express the corresponding proteins under the growth conditions used. Alternatively, another related product may be produced by PssB728a.

In order to study the MeArg biosynthesis cluster of Pss22d in more detail, we heterologously expressed its key enzyme, the methyltransferase MrsA, as a His-tagged protein in *E. coli*. The purified enzyme converted 5-guanidino-2-oxo-pentanoic acid

to 5-guanidino-3-methyl-2-oxo-pentanoic acid in the presence of SAM as the methyl group donor (K_m , 7 mM; k_{cat} , 85 min⁻¹) (Fig. 6). The K_m of MrsA is about 100 times higher than that of the related GlmT, a SAM-dependent methyltransferase that produces (2S,3R)-3-methylglutamate, and the k_{cat} of MrsA is 850 times higher than that of GlmT (0.11 min⁻¹) (18). Similarly to other related methyltransferases, such as GlmT, DptI, and LptI, which convert α -ketoglutarate to 3-methylglutamate in a highly selective manner (18), MrsA is specific for

FIG. 6. Conversion of 5-guanidino-2-oxo-pentanoic acid into 5-guanidino-3-methyl-2-oxo-pentanoic acid by the SAM-dependent methyltransferase MrsA. Reaction scheme (A), LC-MS ion traces at m/z 174 and m/z 188 (B), and ESI-MS-MS (C) for 5-guanidino-2-oxo-pentanoic acid and 5-guanidino-3-methyl-2-oxo-pentanoic acid. I/%, relative intensity (percent).

FIG. 7. Suggested biosynthetic pathway of 3-methylarginine formation in Pss22d.

its substrate and did not accept any other substrates (pyruvate, α -ketoglutarate, or phenylpyruvate). Unfortunately, our attempts to overexpress the aminotransferase MrsB in *E. coli* were not successful, so far preventing us from producing larger amounts of MeArg *in vitro*.

The trio of *mrs* genes is sufficient for the epiphyte Pss22d to produce and export 3-methylarginine. Thus, Pss22d exhibits a remarkable efficiency to generate with such a small set of genes a potent toxin against the plant pathogen *P. syringae* pv. *glycinea*. Most other natural amino acid toxins require more enzymatic steps for their formation, e.g., the well-known arginine analogue canavanine, which is produced by the jack bean (*Canavalia ensiformis*) (23). Even though natural products from microorganisms make use of rare amino acids as constituents of non-ribosomally produced peptides, only a few free nonproteinogenic amino acids, such as MeArg, are known to come from microorganisms (25). Free rare amino acids appear to be more common among plants and often serve as defense compounds (12).

Although the mode of action of MeArg still has to be investigated, we suspect MeArg to act as an inhibitor of the arginine biosynthesis pathway or an arginine-dependent pathway (5). MeArg probably also interferes with the formation of the signal compound nitric oxide, because 5-(2-methylisothioureido)-2-amino-3-methylpentanoic acid, a synthetic analogue closely related to MeArg, strongly inhibited mammalian nitric oxide synthases, which are important targets for treating diseases such as diabetes, septic shock, and various neurodegenerative diseases (13).

In summary, the genes responsible for the production of MeArg by the epiphyte Pss22d were identified. MeArg is synthesized from 5-guanidino-2-oxopentanoic acid, which is methylated by the SAM-dependent methyltransferase MrsA. The resulting 5-guanidino-3-methyl-2-oxopentanoic acid is likely transaminated by the putative aminotransferase MrsB to result in 3-methylarginine. The toxin is secreted via the amino acid exporter MrsC (Fig. 7). Identification of the MeArg biosynthesis gene cluster may provide the basis for its large-scale biotechnological production in order to test its potential for control of the soybean pathogen *P. syringae* pv. *glycinea* or its potential for pharmacological applications.

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