Genetic and Functional Characterization of Cyclic Lipopeptide White-Line-Inducing Principle (WLIP) Production by Rice Rhizosphere Isolate \textit{Pseudomonas putida} RW10S2

Hassan Rokni-Zadeh, a Wen Li, a Aminaal Sanchez-Rodriguez, a Davy Sinneve, b Jef Rozenski, b José C. Martins, b and René De Mot a

Centre of Microbial and Plant Genetics, KU Leuven, Heverlee, Belgium; NMR and Structure Analysis Unit, Department of Organic Chemistry, Ghent University, Ghent, Belgium; and Laboratory of Medicinal Chemistry, Rega Institute for Medical Research, KU Leuven, Leuven, Belgium

The secondary metabolite mediating the GacS-dependent growth-inhibitory effect exerted by the rice rhizosphere isolate \textit{Pseudomonas putida} RW10S2 on phytopathogenic \textit{Xanthomonas} species was identified as white-line-inducing principle (WLIP), a member of the viscosin group of cyclic liponopeptide antibiotics. WLIP producers are commonly referred to by the taxonomically invalid name "\textit{Pseudomonas reactans}," based on their capacity to reveal the presence of a nearby colony of \textit{Pseudomonas tolaasii} by inducing the formation of a visible precipitate ("white line") in agar medium between both colonies. This phenomenon is attributed to the interaction of WLIP with a cyclic lipopeptide of a distinct structural group, the fungitoxic tolaasin, and has applications as a diagnostic tool to identify tolaasin-producing bacteria pathogenic to mushrooms. The genes encoding the WLIP nonribosomal peptide synthetases WlpA, WlpB, and WlpC were identified in two separate genomic clusters (\textit{wlpR-wlpA and wlpBC}) with an operon organization similar to that of the viscosin, massetolide, and entolysin biosynthetic systems. Expression of \textit{wlpR} is dependent on \textit{gacS}, and the encoded regulator of the LuxR family (WlpR) activates transcription of the biosynthetic genes and the linked export genes, which is not controlled by the RW10S2 quorum-sensing system PmrR/PmrI. In addition to linking the known phenotypes of white line production and hemolytic activity of a WLIP producer with WLIP biosynthesis, additional properties of ecological relevance conferred by WLIP production were identified, namely, antagonism against \textit{Xanthomonas} and involvement in swarming and biofilm formation.

\textbf{Plant root-associated fluorescent pseudomonads produce very diverse antagonistic secondary metabolites targeting competing microorganisms, including phytopathogens (18, 20). Synthesis of such molecules with antimicrobial properties is often triggered by the Gac/Rsm two-component signaling pathway, a global regulatory system in gammaproteobacteria (24). Lipopeptides (LPs) constitute a major group of antimicrobial molecules in the arsenal of \textit{Pseudomonas} used for this biological warfare (33, 36, 37, 43). Several strains accommodate large gene clusters in their genomes to encode the nonribosomal peptide synthetases (NRPSs) that generate such compounds. These multimodular megaenzymes sequentially recruit, activate, and stereospecifically condense amino acids to generate linear or cyclic LPs. A typical NRPS module is composed of three domains for consecutive adenylation, thiolation, and condensation of a building block, whereas a separate thioesterase (TE) domain is required for product release, often with concurrent cyclization (18). Many of the LPs from nonpathogenic plant-associated \textit{Pseudomonas} isolates exhibit antifungal activity and have adverse effects on oomycetes (37). As such, rhizosphere pseudomonads appear to be major players in suppressing plant diseases caused by these eukaryotic microorganisms (7, 29).

The inhibitory potential of \textit{Pseudomonas} LPs toward bacteria has not been explored to the same extent, but a number of studies indicate that some Gram-positive bacteria, such as \textit{Bacillus}, \textit{Staphylococcus}, and \textit{Mycobacterium}, are sensitive to some of these compounds (37). This was confirmed in a recent comparative study of five representative LPs (40). None of the Gram-negative strains tested (like \textit{Pseudomonas}, all belonging to the gammaproteobacteria) was significantly inhibited by massetolide A, orfamide A, anthrofactin, entolysin B, syringomycin E, or the atypical LP SB-253514 that is tailored with a rhamnosyl and cyclocarbamate moiety (40).

Apart from bacteriocins, few compounds mediating antagonism between \textit{Pseudomonas} and related bacteria have been identified. Promysalin, produced by \textit{Pseudomonas putida} RW10S1, specifically targets other \textit{Pseudomonas} organisms (26). Its amphipathic nature is reminiscent of LPs, but biosynthesis proceeds via a dedicated pathway, using salicylic acid in addition to an amino acid and a fatty acid as building blocks (26). Focusing on \textit{Xanthomonas}, a major genus of phylogenetically related phytopathogens (45), as a target, we identified anti-\textit{Xanthomonas} activity in \textit{P. putida} RW10S2 isolated from rice rhizosphere (51). This strain was previously identified as a potential LP producer by NRPS-directed PCR screening (41). Here, we show that the antibacterial activity of strain RW10S2 is mediated by white-line-inducing principle (WLIP), an LP of the viscosin group.

WLIP, the white-line-inducing principle, is produced by so-called "\textit{Pseudomonas reactans}" strains. When \textit{P. reactans} is confronted with \textit{Pseudomonas tolaasii} on solid medium, a white precipitate is produced between the colonies (54). This diagnostic test for the mushroom pathogen \textit{P. tolaasii}, named "white line in agar," relies on the interaction of WLIP with the fungitoxic lipo-
peptide tolasin generating the precipitation line. In this study, we identified the NRPS system for biosynthesis of WLIP by *P. putida* RW1052 and confirmed that it is required for the white-line reaction. A role for WLIP in biofilm formation and swarming, in addition to its antagonistic activity against *Xanthomonas*, is reported.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Bacterial strains, plasmids, and primers used for this study are listed in Tables S1 and S2 in the supplemental material. All *Pseudomonas* and *Xanthomonas* strains were grown in Trypticase soy broth (TSB) or agar (TSA) medium (BD Biosciences) at 30°C. Strains of *Pseudomonas aeruginosa*, *Klebsiella*, and *Staphylococcus* were cultured at 37°C in the same medium. Luria-Bertani (LB) medium was used to grow *Escherichia*, *Bordetella*, and *Salmonella* strains at 37°C and *Aeromonas*, *Burkholderia*, *Myxobacterium*, *Rhodococcus*, *Serratia*, *Sphingomonas*, *Varivorax*, and *Yersinia* strains at 30°C. Nutrient broth (NB) was used to culture *Bacillus megaterium* at 30°C and *Citrobacter*, *Enterobacter*, *Proteus*, and *Shigella* strains at 37°C. *Bacillus subtilis* was grown in TMY medium (5 g/liter peptone, 3 g/liter yeast extract) at 30°C. *Saccharomyces* and *Candida* were grown in YPD (10 g of Bacto Yeast, 20 g of Bacto Peptone, and 100 ml of 20% dextrose per liter) at 30°C. If needed, antibiotics were added at the following concentrations: kanamycin, 50 μg/ml; ampicillin, 50 μg/ml; and tetracycline, 20 μg/ml. Media were solidified with 1.5% agar. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl β-D-thiogalactoside (IPTG) (40 mg/liter; Duchefa Biochemie) were added to detect the presence of insert DNA cloned in vectors in *E. coli*.

**Mutant library construction, screening, and plasposon rescue.** Competent RW1052 cells were prepared for electrot transformation in MEB buffer as described by Fredlund (16). All procedures for electroporation, random pTnMod-OKm' mutagenesis, and plasposon rescue to clone mutated regions were performed as described by Parret et al. (34). Plasmid DNA was isolated using the Qiaprep Spin Miniprep kit (Qiagen). Routine DNA sequencing was performed at Macrogen Inc. (Seoul, South Korea). For the screening of mutants affected in antagonistic activity, mutants from the library were arrayed in a 96 (8 by 12)-spot pattern on a high-stringency solution (0.5 SSC, 0.5% sodium dodecyl sulfate, 2M NaCl, 0.1% SDS (3 min, room temperature) and twice in high-stringency solution (0.5% SSC, 0.1% SDS) (15 min, 65°C). Chemiluminescent detection was performed with Roche Applied Science. After hybridization at 45°C, the membrane was washed twice in low-stringency solution (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS (5 min, room temperature) and twice in high-stringency solution (0.5× SSC, 0.1% SDS) (15 min, 65°C). Chemiluminescent detection was performed with ready-to-use disodium 3-[4-methoxyphenyl][1,2-dioxetane-3,2-5-(chloro)tricyclo[3.3.1.1^{(7,3)}]decane]-4-yl]phenyl phosphate (CSPD) as recommended by Roche.

**Bioinformatic analyses.** NRPS domain analysis was performed with the PKS/NRPS Analysis web-based tool (2). Using NRPSpredictor2, the amino acid specificity of each A domain was analyzed (44). Homology searches were performed using the BLAST programs on http://www.ncbi.nih.nlm.gov/blast.cgi and http://www.pseudomonas.com. DNA or protein sequence alignments, phylogenetic analyses, and local BLAST searches were performed with Geneious Pro (version 5.5.3) (12).

**Complementation of regulatory mutants.** The RW1052 wlpB residue was PCR amplified using Platinum Pfx DNA polymerase (Invitrogen) and primer pair PGPRB-6551/PGPRB-6552 and cloned into the pJB3Tc20 vector (pCMPG6125) using the XbaI and EcoRI sites. Similarly, the RW1052 gacS gene was amplified using primer pair PGPRB-7317/PGPRB-7318 and cloned into the pJB3Tc20 vector (pCMPG6203) using HindIII and XbaI sites. Triparental conjugal was used to mobilize pCMPG6125 and pCMPG6203 from *Escherichia coli* DH5α to *P. putida* RW1052 mutants CMPG2173 and CMPG2134, respectively, with helper strain HB101 (pRK2013). Thirty microliters of each culture (optical density at 600 nm [OD600] of between 0.5 and 0.8) of *E. coli* DH5α containing the construct, *Pseudomonas*, and helper strain, was mixed and spotted on a TSA plate, followed by overnight incubation at 30°C. The mixture was resuspended in 1 ml of sterile TSB, and aliquots of different dilutions were spread on TSA supplemented with ampicillin (50 μg/ml, to countselect *E. coli*) and tetracycline (20 μg/ml). The same procedure was used to transfer the broad-host-range expression vector pHERD267 carrying the *P. putida* BW11M1 xirR gene (pCMPG6116) to mutant strain CMPG2172. Likewise, pCMPG6113, a pJB3Tc20 construct carrying RW1051 gacS (26), was introduced in mutant strain CAMP2134. The transconjugants were selected for further phenotypic analyses. Strains carrying a pHERD267 derivative were grown in the presence of t-arabinose (0.1%) to induce expression (35).

**Extraction and purification of WLIP.** A crude extract of WLIP produced by *P. putida* RW1052 and the reference WLIP producer *Pseudomonas* sp. strain LMG 2338 was obtained using the procedure reported by Mortishire-Smith et al. (31). Larger amounts of semipurified WLIP were obtained by solid-phase extraction (SPE) as previously described for viscosin (46). Five hundred milliliters of King’s B medium in 1-liter Erlenmeyer flasks was inoculated with 2.5 ml overnight bacterial culture. The cells were grown at 25°C for 4 days with shaking (200 rpm). To separate cells, cultures were centrifuged at 9,000 × g for 20 min (Beckman J-21M). The supernatant was filtered through Stericup membranes (0.22-μm pore size; Millipore), followed by acidification to pH 5 with HCl (1 M). The supernatant was kept at 4°C overnight to precipitate surface-active compounds. The SPE cartridge (C18 end capped, 25 ml/50 mg; Biotage) was conditioned and equilibrated with 15 ml methanol and Milli-Q water, respectively, using vacuum-accelerated elution (Vacmaster-20; Biotage). The cartridge was subsequently washed with 40%, 60%, and 80% (vol/ vol) acetonitrile (ACN) in water to remove impurities. To elute WLIP, 100% ACN was used. A white precipitate was obtained after drying the collected eluate in a vacuum concentrator. The SPE-extracted material was dissolved in methanol containing 0.7% formic acid.

**HPLC and mass spectrometry.** For high-pressure liquid chromatography (HPLC) experiments, a capillary chromatography system (CapiLC; Waters, Milford, MA) was directly connected to the mass spectrometer. The column dimensions were 0.32 by 150 mm (XTerra C18, 5 μm; Waters, Milford, MA), the injection volume was 0.1 μl, the flow rate was set to 5 μl/min and the samples were eluted with a water-ACN gradient. Spectra were taken every second in the m/z range of 400 to 1500.

Mass spectra were acquired in positive ionization mode on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-ToF-2; Micromass, Manchester, United Kingdom) equipped with a standard electrospray probe (Z-Spray; Micromass, Manchester, United Kingdom) and controlled by the MassLynx 3.4 software. The capillary voltage and cone voltage were set to 3 kV and 30 V, respectively. Fragment ion spectra were obtained with argon as the collision gas and with a collision energy of
40 eV. Samples were dissolved in ACN-water (1:1, vol/vol) and infused at 5 μl/min.

**NMR spectroscopy.** All nuclear magnetic resonance (NMR) measurements were performed either on a Bruker DRX spectrometer operating at 1H and 13C frequencies of 400.13 MHz and 100.61 MHz, respectively, or on a Bruker Avance II spectrometer operating at 1H and 13C frequencies of 500.13 MHz and 125.76 MHz, respectively, in both cases, a 1H, 13C, 15N 2D spectrum applying adiabatic 180° pulses. Standard pulse sequences as described by De Keersmaecker et al. (11). A platform of 96 polystyrene pegs (Nunc no. 445497) fitting into a microtiter plate (Nunc no. 269789) was used. Overnight bacterial culture was diluted using TSB to an OD600 of 0.5. This cell suspension was diluted further 100 times, and 200 μl of cells was loaded into a Nunc microtiter plate (eight repeats for each sample). The pegged lid was inserted into the plate, sealed with Parafilm, and incubated for 48 h at 30°C without shaking. To wash the cells, the lid was transferred to a new plate with 200 μl phosphate-buffered saline in each well. Pegs were then stained with 200 μl of 0.1% crystal violet in an isopropanol–methanol–phosphate-buffered saline solution (1:1:8) for 30 min. Subsequently, excess stain was rinsed off by placing the pegs in a 96-well plate filled with 200 μl distilled water per well. After the pegs were air dried (30 min), the dye bound to the adherent cells was extracted with 30% glacial acetic acid (200 μl) for 10 min, and then 135 μl of each extract was used to measure the absorbance at 570 nm (SynergyMX Multimode reader; BioTek).

**RNA extraction and reverse transcription-PCR (RT-PCR).** Total RNA was isolated from RW1052 and selected mutant cells after overnight culture in TSB, using the TRIzol Plus RNA purification system protocol (Invitrogen). In order to remove contaminating genomic DNA, RNA extracts were treated using a Turbo DNA-free kit (Ambion). Total RNA was quantified using the NanoDrop ND-1000 system (Thermo Scientific) and quality checked by running on a 1% agarose gel. A 1.5-μg amount of RNA was used to synthesize first-strand cDNA using Random Decamers (50 μM; Ambion), Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Westburg), and a deoxynucleoside triphosphate (dNTP) set (Westburg). cDNA was subjected to PCR with specific primers listed in Table S2 in the supplemental material. The 16S rRNA gene was used as an internal control. The primer pairs PGPRB-6231/PGPRB-6232, PGPRB-7319/PGPRB-7320, PGPRB-6233/PGPRB-6234, PGPRB-7321/PGPRB-7322, PGPRB-7323/PGPRB-7324, and PGPRB-6353/PGPRB-6354 were used to amplify cDNAs derived from transcripts corresponding to wlpA (849 bp), covering the adjacent gene pairs wlpB-wlpF (670 bp), wlpB-wlpC (801 bp), wlpC-wlpD (606 bp), and wlpD-wlpE (568 bp), and specific for the 16S rRNA gene (563 bp), respectively.

**Nucleotide sequence accession numbers.** The gene sequences have been submitted to GenBank under accession numbers JN982331 (gacS), JN982332 (wlpR and wlpA), JN982333 (wlpBC), and JN982334 (16S rRNA).

**RESULTS**

Genetic characterization of *P. putida* RW1052 mutants lacking *Xanthomonas* antagonism. Using a panel of phytopathogenic *Xanthomonas* strains, *P. putida* RW1052, an isolate from rice...
rhizosphere in Sri Lanka (51), showed significant antibacterial activity against several of these strains (Fig. 1). Differences in sizes of the growth inhibition halos indicated that sensitivities vary among Xanthomonas strains. To identify RW10S2 genes involved in production of anti-Xanthomonas activity, a plasposon mutant library (50,000 mutants) was constructed using pTn Mod-OKm. This library was screened for mutants lacking inhibitory activity against X. citri pv. malvacearum LMG 761, one of the most sensitive strains. By plasposon rescue, the flanking regions of the respective inserts were cloned and sequenced for selected null mutants. Taking advantage of a draft genome sequence of RW10S2 (unpublished data), the genes affected by plasposon insertion were identified (Fig. 2A and 3A).

Large numbers of independent mutants (three, five, and eight, respectively) with mutations in three NRPS genes were identified: CMPG2170 to CMPG2172 (NRPS1), CMPG2165 to CMPG2169 (NRPS2), and CMPG2120 to CMPG2127 (NRPS3) (Fig. 2A). Southern blot analysis confirmed the single-copy insertion sequence of RW10S2 (unpublished data), the genes affected by plasposon insertion were identified (Fig. 2A and 3A).

In addition to NRPS-related mutations, seven independent insertions in the RW10S2 gacS gene (see Table S1 in the supplemental material) also knocked out inhibitory activity against Xanthomonas (Fig. 3A). GacS is the sensor kinase member of the Gac/Rsm two-component system, a global regulatory system that controls the expression of many secondary metabolites in gamma-proteobacteria (24). The RW10S2 GacS protein is most similar (about 89% amino acid identity) to the P. putida GB-1 (PputGB1_1252) and P. entomophila (PSEEN1359) GacS proteins.

**In silico analysis of P. putida RW10S2 NRPS genes.** Analysis by the NRPS-PKS tool indicated that the first, second, and third encoded NRPSs consist of two, four, and three modules, respectively (Fig. 2A). Each module contains a condensation (C) domain, an adenylation (A) domain, and a thiolation (T) domain. The carboxy-terminal region of the third NRPS carries two tandem thioesterase (TE) domains. Alignments of the RW10S2 NRPS amino acid sequences with those of the similarly organized triple-NRPS systems for massetolide and viscosin biosyntheses (8, 9) revealed moderate homologies (60 to 65% amino acid identity) with MassA/ViscA (60.9%/60.6%), MassB/ViscB (64.2%/65.3%), and MassC/ViscC (61.8%/61.9%). The NRPSpredictor2 tool was used to analyze the presumed specificity of the nine A domains.

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**FIG 2** Organization of the wlp gene cluster in P. putida RW10S2 (A) and its transcriptional analysis (B). (A) The labeled circles show predicted NRPS domains encoded by wlpA (NRPS1), wlpB (NRPS2), and wlpC (NRPS3). A, condensation; T, thiolation; TE, thioesterase. The condensation domains with putative dual condensation/epimerization function are underlined. The condensation domain with predicted lipo-initiation activity, attaching a fatty acid (FA), is labeled C. For the nine modules, amino acid specificity based on *in silico* analysis of the respective A domains is indicated. The vertical bold lines mark the plasposon insertion sites leading to WLIP production deficiency. The mutants in bold were verified by Southern blot analysis (see Fig. S1 in the supplemental material) and phenotypically characterized. (B) The composite panel shows the amplicons obtained by RT-PCR transcript analysis using primer couples for the specified pairs of adjacent convergent genes and 16S rRNA (internal control). RNA was extracted from the wild type (WT) and from mutants CMPG2173 (wlpR) and CMPG2134 (gacS).
Assuming colinear biosynthesis, starting with NRPS1, continued by NRPS2, and ending with NRPS3, the putative peptide produced would have the sequence Leu-Glu/Asp-Thr-Val-Leu-Ser-Ile. Phylogenetic analysis of the A domains (see Fig. S2 in the supplemental material) supported this prediction. It pointed to Glu specificity of the second module and probable allo-Thr preference for the third module.

This amino acid sequence is very similar to those of members of the viscosin group, in particular to those of WLIP and viscosin (18). Both LPs contain a nonapeptide moiety but differ only by the configuration (D or L) of a leucine residue at the fifth position. According to Balibar et al. (4), the D configuration of amino acids in the Pseudomonas LP arthrofactin is generated by a specific condensation domain, with additional epimerization activity, acting on the amino acid loaded on the previous module. Such C/E domains constitute a distinct cluster separated from the clusters with regular condensation domains and the lipo-initiation domain cluster in phylogenetic analysis of their sequences (8, 41, 50). Application of this sequence comparison to the RW10S2 NRPSs strongly points to a system synthesizing a lipopeptide closely related to WLIP or, possibly, viscosin. The corresponding quantitative data are shown in Fig. 4 (biofilm formation) and in Table S3 in the supplemental material (antagonism, hemolysis, and swarming).

WLIP-linked phenotypes of P. putida RW10S2. Domain analysis of the RW10S2 NRPSs strongly points to a system synthesizing a lipopeptide closely related to WLIP or, possibly, viscosin. When a WLIP producer is grown close to P. tolaasii, a white-line precipitate will be formed, attributed to the interaction between WLIP and the LP tolaasin produced by P. tolaasii (54). Such a precipitate is not observed with a viscosin producer confronting P. tolaasii, indicating that the minor structural difference in viscosin compared to WLIP (L-Leu instead of D-Leu) affects the outcome of this diagnostic test (41). Strain RW10S2 generated a heavy white line when confronting P. tolaasii, while all mutants with a disrupted NRPS gene displayed a lack of white precipitate formation (Fig. 3B). The same phenotypic change was observed for the mutant with an inactivated NRPS-linked regulatory gene and the gacS mutants.

In a phenotypic variant of P. reactans LMG 5329, loss of white-line production coincided with disappearance of the capacity to lyse erythrocytes as visualized in blood agar (32). Using purified LPs, Lo Cantore et al. (28) demonstrated that purified WLIP from P. reactans NCPPB1311 causes red blood cell lysis through transmembrane pore formation, being a more potent hemolytic agent
than tolaasin I (38). Strong hemolysis was also observed for \textit{P. putida} RW10S2 cells grown on horse blood TSA (Fig. 3C; see Table S3 in the supplemental material). Notably, all mutants impaired in anti-\textit{Xanthomonas} antagonism and the white-line phenotype were also deficient in hemolytic activity (Fig. 3A to C; see Table S3 in the supplemental material).

Given the consistent correlation between gene activity and WLIP-related phenotypes in \textit{P. putida} RW10S2, the NRPS genes (encoding NRPS1, NRPS2, and NRPS3) and the NRPS1-linked LuxR family gene were designated \textit{wlpA}, \textit{wlpB}, \textit{wlpC}, and \textit{wlpR}, respectively. These genes are coexpressed as shown by RT-PCR analysis (Fig. 2B). Cotranscription was demonstrated for the \textit{wlpB-wlpC} genes (with overlapping stop-start codons) along with the downstream \textit{wlpD-wlpE} (\textit{maA-macB} homologues, also with overlapping stop-start codons). Likewise, cotranscription was observed for \textit{wlpE} (\textit{oprM} homologue) located downstream of \textit{wlpR} (Fig. 2B).

**Structural characterization of the \textit{P. putida} RW10S2 LP.** The module composition of the RW10S2 NRPS system and phenotypic characteristics of NRPS mutants suggested the LP produced by this strain to be very similar, if not identical, to WLIP. To resolve this issue, the LP was purified for chemical characterization. The mass spectrum of the RW10S2 LP extract contained an abundant protonated pseudo-molecular ion (\(\text{M} + \text{H}\)\(^+\)) at \(m/z\) 1126 (exact mass, 1,126.7 Da), corresponding to the molecular weight (1,125) of WLIP (31). HPLC analysis confirmed that the corresponding peak, displaying \textit{Xanthomonas}-antagonistic and hemolytic activities, was absent in extracts from a \textit{wlpC} mutant and a \textit{gacS} mutant (see Fig. S4 in the supplemental material). The mass fragmentation fingerprint of the RW10S2 compound was compared with its equivalent produced by strain LMG 2338 and found to be identical (see Fig. S5 in the supplemental material). Strain LMG 2338 (NCPPB 387) represents the original \textit{P. reactans} strain used to elucidate the structure of WLIP (31). The inferred identity with WLIP was further examined by NMR analysis of a solution of the RW10S2 LP in DMF-\(d_7\) (see Fig. S6 in the supplemental material). The presence of the 3-hydroxydecanoic acid (HDA) moiety and all the amino acid spin systems of WLIP were mental material. The presence of the 3-hydroxydecanoic acid (HDA) moiety and all the amino acid spin systems of WLIP were mental material. The presence of the 3-hydroxydecanoic acid (HDA) moiety and all the amino acid spin systems of WLIP were mental material.
Swarming capacity was regained by the wlpR-complemented mutant, but some difference in pattern (irregular swarm edge) was evident (Fig. 3D). This may be due to unbalanced plasmid-driven expression of a chromosomal regulatory gene participating in control of a complex phenotype that involves several other determinants. Interestingly, partial complementation was also achieved by introduction of the cloned NRPS-linked LuxR family gene xilR of the LP producer P. putida RW111M1 (sharing 66% amino acid identity [25, 41]). Biofilm formation by this heterologously complemented mutant attained about ~50% of the wild-type capacity, slightly lower than the partially restored biofilm phenotype of the homologously wlpR-complemented mutants (Fig. 4). The RW1052 gacS mutant could also be complemented to some extent by gacS from P. putida RW1051 (sharing 86% amino acid identity [26]). Introducing this heterologous gacS gene resulted in restored swarming capacity but with a modified dendritic pattern (Fig. 3D). Interestingly, RW1052 pmrR and pmrI mutants with a deficient N-acylhomoserine lactone-dependent quorum-sensing system (49) displayed wild-type WLIP-related phenotypes (data not shown), indicating quorum-sensing-independent regulation of WLIP production.

**DISCUSSION**

In this study, the antagonistic activity of P. putida RW1052 against pathogenic Xanthomonas species was investigated, which resulted in the isolation and biological characterization of the cyclic LP WLIP and identification of its biosynthetic and regulatory genes.

**Genetic backbone of WLIP biosynthesis.** Although the structure of WLIP has been known for more than 2 decades (31), genetic analysis of its biosynthesis was not yet reported. In contrast to the single-operon organization of several Pseudomonas multiple-NRPS systems (arthrofactin, orfamide, syringafactin, syringopeptin, and putisolvin [43]), we show here that three NRPS genes located in two regions of the RW1052 genome are responsible for WLIP biosynthesis. The wlpA gene, encoding an NRPS with the first two modules, is separated from the wlpBC operon, encoding 4- and 3-modular NRPSs, respectively. This type of spread gene organization has also been observed for synthesis of entolysin from P. entomophila L48 (50), massetolide A from P. fluorescens SS101 (8), and viscosin from P. fluorescens SBW25 (9). Massetolides and viscosin belong to the same structural group of lipononadepsipeptides, but entolysin is built from 14 amino acids. In the latter case, the isolated NRPS gene (etlA) also encodes the first two modules. The unlinked etlBC operon provides the remaining modules (8 and 4, respectively, compared to a 4-3 modular distribution for the WLIP, viscosin, and massetolide synthetases). The in silico analysis of A-domain specificity, using both the NRPS predictor tool (44) and a phylogenetic comparison with A domains of known Pseudomonas LPs, combined with Pseudomonas genomic synteny of the three NRPS genes and application of the colinearity rule in modular biosynthesis proved to be valuable for reliable prediction of the amino acid sequence and fatty acid modification of the secondary metabolite synthesized. In line with some previously noted exceptions to the “Balibar rule” (4, 8, 19), the prediction of the t-fold configurations in WLIP by phylogenetic analysis of C domains in its NRPSs was confirmed except for one residue.

The genomic sequence context of the mutated NRPS genes indicates the presence of three genes encoding a candidate export system for the secondary metabolite synthesized. Immediately downstream of wlpR, a coexpressed gene (wlpF) encoding a putative OprM-like outer membrane efflux protein (Pfam PF02321) is located (Fig. 2). Similarly, a coexpressed gene tandem downstream of wlpBC (wlpDE) encodes proteins related to MacA (Pfam PF12700) and MacB (Pfam PF12704) (Fig. 2). The Escherichia coli MacA-MacB-ToIC system is an ATP-dependent tripartite macrolide efflux transporter (30). Since OprM represents the functional and structural Pseudomonas equivalent of ToIC (1) and wlpF is cotranscribed with the RW1052 wlp genes, the three NRPS-linked genes likely encode an LP export system, WlpDEF. In several known Pseudomonas LP gene clusters, the oprM-like gene and macAB-like genes flank the initiating and terminating NRPS genes, respectively (8, 13, 50). Likewise, polycistronic transcription of the arthrofactin biosynthetic genes arfA, arfB, and arfC with the downstream exporter genes arfD (macA homologue) and arfE (macB homologue) was demonstrated in Pseudomonas sp. strain MIS38 (27). Mutational analysis of the Mac-like Pseudomonas genes downstream of the putisolvin (13) and arthrofactin (27) biosynthetic operons revealed reduced LP production, indicating a primary but not exclusive role in export of the corresponding product. This residual production level could explain why in our screening for mutants devoid of antagonistic activity, no insertions in these flanking genes were identified. The involvement of an OprM homologue in LP production by Pseudomonas has not yet been experimentally verified.

**Regulation of WLIP production.** As reported earlier for syringomycin (21), tolaasin (17), amphisin (22), and syringopeptin (52), WLIP production by P. putida RW1052 is dependent on a functional GacS, the sensor of the general regulatory system Gac/Rsm (24). It is not triggered by the RW1052 N-acylhomoserine lactone-mediated quorum-sensing system PmrR-PmrI (49). The LuxR family protein WlpR, encoded by a gacS-dependent wlpA-linked gene, apparently represents the cognate regulator of WLIP biosynthesis. Inactivation of wlpR abolished the expression of the other wlp genes, WLIP production, and WLIP-linked phenotypes. LuxR-type regulators are found in the flanking regions of most Pseudomonas LP-synthesizing NRPS genes (10). Among these homologues of wlpR, also located upstream of the respective initiating NRPS genes, several were also shown to be essential for production of syringafactin (syfR) (5), putisolvin (psorI) (13), viscosin (visCAR) (10), arthrofactin (arfF) (53), and entolysin (etlR) (50). A downstream-positioned luxR-type regulator gene (visBCR) is also involved in viscosin production, but inactivation of its coun-
Gram-negative bacteria, including an X. campestris that the growth of several other also observed by Lo Cantore et al. (28). We noticed, however, phaseoli indicator (strain ICMP3035), to LP inhibition was reported for purified tolaasin (28). Such an effect was not observed for purified WLIP on P. tolaasii pv. campestris was significantly more sensitive than tris cells caused by a concentrated pendent. Etchegaray et al. (15) demonstrated disintegration of cited by WLIP, but the severity appears to be species/strain dependent. The latter name is a phenotypic designation, referring to the properties of several LuxR-type regulators and encoded by regulatory genes clustered with the biosynthetic ones, constitutes a recurrent theme in LP production by Pseudomonas (37), reiterated in WLIP biosynthesis. In only few Pseudomonas strains, N-acylhomoserine lactone-dependent regulation has been reported (6, 14).

**Physiological role of WLIP.** The diagnostic tool introduced by Wong and Preece in 1979 to identify mushroom-pathogenic bacteria producing the toxin tolaasin has found wide application (54). It relies on the formation of a sharp precipitation line attributed to interaction of tolaasin with another LP, WLIP, secreted by juxta-positioned colonies of P. tolaassii and a WLIP-producing P. reactans. The latter name is a phenotypic designation, referring to the white-line reaction, for an ill-defined group of Pseudomonas strains. In this study, the P. putida strain RW1052 was identified as a WLIP producer, and characterization of defined biosynthetic and regulatory mutants lends support to the LP interaction hypothesis. Previously, Rainey and colleagues reported the lack of white-line production by P. tolaasii mutants with inactivated tolaasin biosynthetic enzymes (39). The ecological significance of this interaction remains unclear. Sequestering an opponent’s toxic LP may have a protective effect. In contrast to a previous report (38), an inhibitory effect on P. reactans growth was reported for purified tolaasin (28). Such an effect was not observed for purified WLIP on P. tolaasii (28), which was confirmed in our study. WLIP’s moderate antifungal activity and inhibition of Gram-positive bacteria were previously documented (28), with both properties being shared with many other Pseudomonas LPs (37, 40). The general recalcitrance of Gram-negative bacteria, including an X. campesiris pv. phaseoli indicator (strain ICMP3035), to LP inhibition was also observed by Lo Cantore et al. (28). We noticed, however, that the growth of several other Xanthomonas strains is inhibited by WLIP, but the severity appears to be species/strain dependent. Etchegaray et al. (15) demonstrated disintegration of Xanthomonas cells caused by a concentrated Bacillus subtilis LP extract containing iturin and surfactin. Xanthomonas campesiris pv. campesiris was significantly more sensitive than X. axonopodis pv. citri. The major rice pathogen X. oryzae pv. oryzae is not sensitive to WLIP produced by strain RW1052, which itself originates from the rhizosphere of rice plants. In line with the properties of several Pseudomonas LPs (37), our study further revealed that WLIP promotes solid-surface translocation and biofilm formation by its producer. These features may reflect an important role for WLIP in the colonization of structured environments.

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