Function and Distribution of a Lantipeptide in Strawberry Fusarium Wilt Disease–Suppressive Soils

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Streptomyces griseus S4-7 is representative of strains responsible for the specific soil suppressiveness of Fusarium wilt of strawberry caused by *Fusarium oxysporum* f. sp. *fragariae*. Members of the genus *Streptomyces* secrete diverse secondary metabolites including lantipeptides, heat-stable lanthionine-containing compounds that can exhibit antibiotic activity. In this study, a class II lantipeptide provisionally named grisin, of previously unknown biological function, was shown to inhibit *F. oxysporum*. The inhibitory activity of grisin distinguishes it from other class II lantipeptides from *Streptomyces* spp. Results of quantitative reverse transcription-polymerase chain reaction with *lanM*-specific primers showed that the density of grisin-producing *Streptomyces* spp. in the rhizosphere of strawberry was positively correlated with the number of years of monoculture and a minimum of seven years was required for development of specific soil suppressiveness to Fusarium wilt disease. We suggest that *lanM* can be used as a diagnostic marker of whether a soil is conducive or suppressive to the disease.

Fusarium oxysporum is a complex species comprising ubiquitous soil-borne plant pathogens with more than 150 host-specific forms or forma speciales (Baayen et al. 2000). These pathogens invade the root epidermis and extend into the vascular tissue, in which they colonize the xylem vessels and produce mycelium and conidia (Beckman 1987). The characteristic wilt symptoms result from severe water stress, mainly due to vessel clogging (Beckman 1987), and are characterized by yellowish leaves, irregular leaf formation, and wilted plants. There may be 30 to 40% yield loss, which can increase to 80% under favorable weather conditions (Kapoor 1988).

As a consequence of the intensive use of fungicides, fungicide-resistant subpopulations of *Fusarium* spp. and other pathogens have emerged and are a serious problem in crop production (De Cal et al. 2005; Liu et al. 2007). Preplant soil

fumigation with methyl bromide (MB) or mixtures of MB and chloropicrin has been standard practice for controlling soil-borne pathogens (De Cal et al. 2005), but soil fumigation is not completely efficient and is discouraged as environmentally unsound. In contrast, biological control mediated by *Fusarium* disease-suppressive soils has been recognized for decades (Alabouvette et al. 1993; Mazurier et al. 2009; Scher and Baker 1980, 1982; Sneh et al. 1984; Weller et al. 2002). More recently, biological control based on phytobiome information has become an exciting and rapidly developing research area with important implications for plant productivity. In one such example, Cha et al. (2016) reported control of the strawberry wilt pathogen *F. oxysporum* f. sp. *fragariae* by a *Streptomyces* isolate selected from the microbiome of strawberry grown in a 15-year continuous monoculture field that had become suppressive of wilt disease. In that report, *S. griseus* S4-7 was both necessary and sufficient to protect against the strawberry wilt pathogen.

Members of the genus *Streptomyces* are widely regarded as plant probiotics that protect against a wide range of plant pathogens (Bakker et al. 2010; Zivković et al. 2010). They are characterized as having a complex filamentous-type structure and sporulation and produce lytic enzymes, including chitinases, proteases, and cellulases, as well as numerous antibiotics and secondary metabolites. To date, nearly 75% of known antimicrobial compounds are derived from this genus. These include important pharmacophores and medical antibiotic derivatives such as vancomycin, erythromycin, tobramycin, capreomycin, daptomycin, chloroeremomycin (precursor for oritavancin), and echinocadin B (Baltz 2008). These compounds are produced by nonribosomal peptide synthetase (NRPS) or polyketide synthase clusters, and antibacterial compounds consistently have originated via these pathways. Also recently reported are lantipeptides, ribosomally synthesized and posttranslationally modified microbial secondary metabolites, many of which have antimicrobial activities (Dufour et al. 2007; Hofmann et al. 2012; Lubelski et al. 2008). For example, lantipeptides are known for their antimicrobial or bacteriocin-like activity (Foulston and Bibb 2010) and they kill by either binding to the cell-wall precursor lipid II, by forming pores in cell membranes, or both, leading to the efflux of small molecules and dissipation of the membrane potential (Breukink et al. 1999). When lantipeptides (structural terminology) are confirmed to have antibiotic function, they are termed lantibiotics. Lantipeptide biosynthesis operons are widely distributed among the actinobacteria, suggesting that they play an important ecological role (Yu et al. 2013; Zhang et al. 2015), but

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the biological functions of few of the lantipeptides produced by *Streptomyces* spp. are known.

Lantipeptides can be subdivided based on their structure and mode of posttranslational modification (Zhang et al. 2012). Class I lantipeptides exhibit a linear secondary structure, are positively charged upon synthesis, and are modified by two distinct LanB and LanC synthetase enzymes. LanB is required for the selective dehydration of Ser or Thr residues in the LanA core peptide to Dha or Dhb. Class II lantipeptides, conversely, exhibit a globular structure, are noncharged (Garg et al. 2013), and the core peptide is modified by a single bifunctional LanM enzyme of 900 to 1,200 amino acids, with an N-terminal dehydratase domain bearing no homology to LanB and a C terminal cyclase domain with only about 25% sequence identity to LanC, including a conserved zinc-binding motif critical for activity (Li and van der Donk 2007). LanM enzymes share little sequence similarity with one another. Two additional lantipeptide classes also are known. Class III lantipeptides are heat-sensitive molecules with a molecular weight greater than 30 kDa (Breukink et al. 1999) and class IV lantipeptides, discovered in 2010 by genome analysis of a cryptic gene cluster in *S. venezuelae*, are cyclic structures synthesized via a novel three-domain LanL synthetase (Knerr and van der Donk 2012).

In this study, we identified six lantipeptide biosynthesis gene clusters in the genome of strain S4-7. The class II lantipeptide *lanM* was found to affect antifungal activity and was developed as a molecular-geographical marker for the distribution of S4-7 in strawberry fields. The results of these surveys revealed that the number of years of strawberry monoculture was strongly correlated with the density of grisin producers in the rhizosphere. Here, we report the successful characterization of the geoeology and biological functions of this novel lantipeptide in the Fusarium wilt disease-suppressive soil system.

RESULTS

Resequencing the *S. griseus* S4-7 genome and putative antifungal metabolites.

The complete genome sequence of *S. griseus* S4-7, isolated from the strawberry rhizosphere as a Fusarium wilt-suppressive agent (Cha et al. 2016), was determined via the PacBio RS II single-molecule real-time (SMRT) sequencing platform. De novo assembly of the SMRT reads was undertaken and a genome circular map was generated by analysis with BLAST Ring Image Generator (BRIG) v 0.95. We executed SMRT analysis to produce subreads by trimming and filtering the raw reads utilizing the following parameters: minimum read length = 16,571, read score = 0.84, and minimum length = 11,818. Genome coverage was 83.48x.

The genome consisted of a single linear chromosome of 7,697,387 base pairs with no plasmids (Fig. 1). Genes were annotated by using the RAST pipeline, and the strain had 6,935 protein coding genes, six ribosomal RNA operons, and G+C content of 71.5%. Thirty secondary metabolite gene clusters were predicted by using antiSMASH. These clusters predicted coding for known or unknown secondary metabolites, including four lantipeptides, two bacteriocins, two siderophores, two butyrolactones, three NRPSs, six clusters encoding terpene, two ectoines, three polyketide synthetases, four NRPSs linked to polyketide synthetases, one melanine cluster, and a lasso-peptide cluster (Supplementary Table S1).

Lantipeptide biosynthesis genes in *S. griseus* S4-7.

AntiSMASH, RAST, and National Center for Biotechnology Information (NCBI) databases were employed to predict the lantipeptide biosynthesis genes in the S4-7 genome (Table 1).

Fig. 1. *Streptomyces griseus* S4-7 genome circular map and genome comparison with *S. griseus* NBRC 13350. **A**, The innermost rings show GC content (black) and open reading frame fragments outside this ring are the alternating bars. The draft genome was analyzed by using BRIG (BLAST Ring Image Generator) v 0.95. **B**, For the comparison genome sequence, the shaded blocks indicate regions of DNA similarity.

Table 1. Lantipeptide biosynthesis gene clusters in *Streptomyces griseus* S4-7^a

AntiSMASH	RAST	NCBI	Class
Cluster 12–Lan A	Lan A	Lanthionine precursor peptide LanA	III
Cluster 12–Lan L-2	Lan L-2	Lanthionine biosynthesis protein LanL	III
Cluster 12–Lan L-1	Hypothetical protein	Hypothetical protein	III
Cluster 17–Lan B	Lan B	Lanthionine biosynthesis protein LanB	I
Cluster 20–Lan M	Lan M	Lanthionine biosynthesis protein LanM	II
Not detected	Lan C	Lanthionine biosynthesis protein LanC	I

^a RAST = rapid annotation using subsystem technology, NCBI = National Center for Biotechnology Information.

With antiSMASH, the genome included three lantipeptide clusters and one thiopeptide-lantipeptide cluster. Two lantipeptide clusters and the thiopeptide-lantipeptide cluster contained lantipeptide biosynthesis genes; cluster 12 included *lanA*, *lanL-1*, and *lanL-2*; cluster 17, the thiopeptide-lantipeptide, included *lanB*; and cluster 20 contained *lanM*. *lanC* was observed in one RAST cluster (Fig. 2). RAST annotation results including *lanA*, *lanB*, *lanC*, *lanM*, and *lanL-2* were similar to those of antiSMASH, while the *lanL-1* gene differed from the results of antiSMASH analysis. Finally, the prokaryotic genome automatic annotation pipeline in the NCBI database detected *lanA*, *lanL-2*, *lanB*, *lanM*, and *lanC* (Table 1). Taken together, there were minor differences among the results of the three different annotation pipelines. We concluded from the collective results that *S. griseus* S4-7 includes a total of six lantipeptide biosynthesis genes, which we grouped according to lantipeptide class, with two class I (*lanB*, *lanC*), one class II (*lanM*), three class III groups (*lanA*, *lanL-1*, *lanL-2*), and no class IV group genes. Genes *lanA*, *lanB*, and *lanC* already have been described as having involvement in aerial hyphae formation or antibacterial activity, respectively (McAuliffe et al. 2001; Ueda et al. 2002). However, no biological function of *lanM* has been reported previously in *Streptomyces* spp.

Biological function of grisin, the LanM lantipeptide.

Based on the genome information, we then created six lantipeptide biosynthesis gene deletion mutants (*lanA*, *lanB*, *lanC*, *lanL-1*, *lanL-2*, and *lanM*). The mutants showed differential antagonistic activity against *F. oxysporum* f. sp. *fragariae*. Four mutants (*lanC*, *lanL-1*, *lanL-2*, and *lanB*) retained their antifungal activity against the pathogen, but two mutants in *lanA* and *lanM* exhibited dramatically reduced antifungal activity on PDK (10 g of potato dextrose broth, 10 g of peptone, 20 g of agar, 1 liter of distilled water) agar after 5 days (Fig. 3). Sporulation of the *lanM* mutant was similar to that of the wild type and spore color varied from white (at the initiation stage) to gray (at the maturation stage) on PDK media (Fig. 3). LanM

was not expressed in the $\Delta lanM$ mutant (Supplementary Fig. S1), and the function of *lanA* already has been related to antimicrobial activity and sporulation (Ueda et al. 2002).

Correlation between density of grisin produced and years of strawberry monoculture.

The *Streptomyces* species-specific amplicon primers Fofra qF and Fofra qR, which target a 512-bp intergenomic region between the transposable elements Han and Skippy (Supplementary Table S2) and *lanM* gene-specific primer sets targeting a 1,033-bp amplicon were used to screen DNA from 25 different *Streptomyces* isolates, three from strawberry, one from turfgrass, 11 from kiwifruit, and 10 from various soil sources (Supplementary Table S3). All isolates gave a positive reaction with the *Streptomyces*-specific primers, but the *lanM* gene was only detected in the strains from strawberry. Not surprisingly, comparison of the LanM protein sequence from *S. griseus* S4-7 with LanM sequences from various other bacteria showed that the S4-7 protein differed significantly from previously reported LanM sequences (Supplementary Fig. S2).

DNA samples were extracted from the rhizosphere soil of strawberry plants from 30 different commercial fields located in Sugok-myeon, Sancheong-gun, and Hadong-gun counties in the Republic of Korea and were subjected to quantitative real-time polymerase chain reaction (qRT-PCR) with the *lanM* and *F. oxysporum* f. sp. *fragariae*-specific primers. The results showed that the intergenomic fragment of the pathogen was detected at more than 10^5 copies per gram of soil regardless of the geographical origin of the soils (Fig. 4A). Interestingly however, the presence and density of the *lanM* gene varied among the samples (Fig. 4B), and only fields with seven or more years of strawberry monoculture attained levels more than 10^5 gene copies of *lanM* per gram of rhizosphere soil (Fig. 4C). Additionally, Fusarium wilt disease incidence and severity presented a negative correlation with the number of years of monoculture (Fig. 4D; Supplementary Table S4).

DISCUSSION

Lantipeptides comprise a growing family of over 90 structurally diverse ribosomally synthesized and posttranslationally modified natural products. Historically, these compounds were discovered via antimicrobial screens, but recent advances in genomics and bioinformatics have revealed hundreds of taxonomically distant bacterial strains with putative lantipeptide biosynthetic enzymes that may ultimately yield novel

Fig. 2. Gene organization and function of the lantipeptide clusters in S4-7. **A**, Cluster 12; **B**, cluster 17; **C**, cluster 20; and **D**, LanC protein in RAST cluster. Black arrows indicate locations of homologs knocked out by the pKC1132 suicide vector.

Fig. 3. Antagonism of *Fusarium oxysporum* f. sp. *fragariae* by wild-type *Streptomyces griseus* S4-7 and lantipeptide deletion mutants on PDK agar (10 g of potato dextrose broth, 10 g of peptone, 20 g of agar, 1 liter of distilled water) incubated for 5 days at 28°C.

structures and biological activities of potential pharmacological value. These peptides represent a new type of antibiotic produced by *Streptomyces* spp. In this study, we characterized LanM, a novel lantipeptide synthetase from the *Fusarium* wilt-suppressive bacterium *S. griseus* S4-7. Genomic sequence analysis revealed that strain S4-7 contains six lantipeptide biosynthesis genes. Of these, genes *lanA* and *lanL*, located in cluster 12, are perfectly matched with *amfS* and *amfB*, which have been identified as regulators for aerial mycelium formation in *S. griseus* (Ueda et al. 2002). Genus *Streptomyces* is characterized as having a complex filamentous-type structure and sporulation, both of which relate to the production of

antibiotics. Therefore, the *lanA* and *lanL* genes were excluded from the major antifungal functions in this study.

Cluster 17 includes a different lantipeptide class synthesis gene, *lanB*. This gene is identical to *tpaL* from *Nocardiopsis* sp. strain TFS65-07 (Engelhardt et al. 2010). Antibiotic production by this strain involves the genes *tpaC*, *tpaD*, *tpaE*, *tpaF*, *tpaL*, *tpaH*, and *tpaK* (Engelhardt et al. 2010). Cluster 20 of strain S4-7 has a unique single class II lantipeptide biosynthesis gene, *lanM*, encoding for a lantipeptide with strong antifungal activity that we have provisionally named grisin. Like other class II synthetases, the predicted enzyme includes an N-terminal dehydratase domain and a C-terminal cyclase domain with a

Fig. 4. Quantitative real-time polymerase chain reaction analysis of the absolute copy number of *Fusarium oxysporum* f. sp. *fragariae* and *lanM*-producing genes in soil, illustrating the relationship between *lanM* gene concentration and years of strawberry monoculture. Relationship between years of strawberry monoculture and Fusarium wilt disease index. Locations of soil sampling include Sugok-myeon (SG), Sancheong-gun (SC), and Hadong-gun (HD). **A**, *F. oxysporum* f. sp. *fragariae* population density in soil samples. **B**, *lanM* gene detection in strawberry field soil samples. **C**, Correlation between *lanM*-producing *Streptomyces* population and years of strawberry monoculture. **D**, Correlation between years of strawberry monoculture and Fusarium wilt disease index.

zinc-binding site (Goto et al. 2010). However, as it exists in the genus *Streptomyces*, this type of lantipeptide has not yet been studied. Individual mutants (*lanA* and *lanM*) lost antifungal activity against *F. oxysporum* f. sp. *fragariae*. The S4-7 LanM protein sequence showed no match with previously reported lantipeptide sequences in *Streptomyces* spp. and, to our knowledge, this is the first study to reveal evidence of LanM biological function. Cha et al. (2016) reported previously that conprimycin has a critical role in the antifungal activity of strain S4-7, and the strain also produces caryolan-1-ol, a volatile compound with significant inhibitory activity (Cho et al. 2017). Grisin is the third compound active against *F. oxysporum* f. sp. *fragariae*, suggesting that the pathogen may be unable to overcome the arsenal of metabolites produced by this natural probiotic agent in the rhizosphere. The kinetics of synthesis of these compounds is currently under investigation.

Regardless of the cropping system, agricultural soils harbor countless microbes, and long-term monoculture can lead to coevolution between a plant and members of its microbiome, leading to the selection of strains that function as probiotics to overcome certain pathogens. Soils that have undergone this phenomenon are said to exhibit specific suppressiveness and are characterized by their ability to limit disease caused by particular pathogens. In such soils, the disease does not occur because the target pathogen is suppressed by the biocontrol agent (Weller et al. 2002). In take-all decline, the classic example of specific soil suppressiveness, the suppressive agent *Pseudomonas brassicacearum* produces the antibiotic 2,4-diacetylphloroglucinol (DAPG) and must reach a threshold population on roots of at least 10^5 CFU per gram of rhizosphere soil to provide protection against the take-all pathogen (Raaijmakers and Weller 1998). In general, the transition to suppressiveness in that system requires four to six years of wheat monoculture (Kwak and Weller 2013), and the DAPG biosynthesis locus can be detected by PCR in soils in decline (Mavrodi et al. 2007). Similarly, we detected the *lanM* gene in *S. griseus* isolates from soils cropped to strawberry but not in isolates recovered from other host plants or from non-agricultural soils. These results suggested that *lanM* can be used to detect specific grisin-producing *Streptomyces* strains in the rhizosphere. Accordingly, we developed a unique PCR-based method to evaluate the relationship between the cell density of strains harboring *lanM* and the number of years of

monoculture, and we found that the two were positively correlated. Bacterial densities of up to 10^5 CFU per gram of rhizosphere soil were detected in strawberry fields after seven or more years of monoculture, even though the *Fusarium* wilt pathogen population was present at a similar level in all soils regardless of the number of years of monoculture history. These results are consistent with the classic model of specific soil suppressiveness. Moreover, using the approach described here, we have shown that *lanM* gene detection combined with knowledge of the number of years of strawberry monoculture can provide a potential basis for deciding whether or not the soil will be suppressive of strawberry wilt disease.

To conclude, we have discovered a lantipeptide synthase encoded by a *lanM* gene in *S. griseus* S4-7 that contributes to the ability of the bacteria to suppress strawberry wilt disease caused by *F. oxysporum* f. sp. *fragariae*. The product of LanM has antifungal activity and is the first class II lantipeptide from genus *Streptomyces* reported to have a biological function. This novel peptide provides antifungal activity to strains with the capacity to produce it, and a marker for the specific suppressiveness of soil in long-term strawberry cultivation systems. We propose that *S. griseus* S4-7 can act effectively against *Fusarium* wilt because the bacterium produces the major lantibiotic-type antibiotic LanM, and we suggest that detection of *lanM* is predictive of whether or not a soil will have the ability to limit *Fusarium* wilt disease in many cropping systems. To become suppressive against *Fusarium* wilt disease in strawberries does, however, require a minimum 7-year monoculture strategy. This finding is critical not only in phytobiome research and practical plant-probiotic interactions but also for strawberry production.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

Strains and plasmids used in this study are listed in Table 2. For the preparation of *Streptomyces* spore stock, a single colony was inoculated into 5 ml of PDK broth and was shaken at 30°C for 5 days. The cells were collected by centrifugation at $5,000 \times g$ for 15 min, were suspended in 1 ml of PDK broth, were spread on MS agar (20 g of mannitol, 20 g of soya, 20 g of agar, 1 liter of distilled water) and were incubated at 30°C for 5 days (Kieser et al. 2000). Then, 1 ml of PDK broth was sprayed onto the MS agar and the surface of the plate was scraped to detach

Table 2. Bacterial strains and plasmids used in this study

Strains/plasmids	Description ^a	Reference
<i>Escherichia coli</i>		
DH5α	F [−] , Φ80dlacZAM15, (lacZYA-argF)U169, deoR, recA1, endA1, hsdR17 (rk [−] ,mk ⁺), phoA, supE44, λ [−] , thi-1, gyrA96, relA1	Bierman et al. 1992
ET12567/pUZ8002	dam-13::Tn9 dcm-6 hsdM; carries RK2 derivative with defective oriT for plasmid mobilization, Kan ^r	Bierman et al. 1992
<i>Streptomyces griseus</i> S4-7		Cha et al. 2016
S4-7, <i>lanA</i>	S4-7Δ <i>lanA</i> ::apr	This work
S4-7, <i>lanB</i>	S4-7Δ <i>lanB</i> ::apr	This work
S4-7, <i>lanC</i>	S4-7Δ <i>lanC</i> ::apr	This work
S4-7, <i>lanL-1</i>	S4-7Δ <i>lanL-1</i> ::apr	This work
S4-7, <i>lanL-2</i>	S4-7Δ <i>lanL-2</i> ::apr	This work
S4-7, <i>lanM</i>	S4-7Δ <i>lanM</i> ::apr	This work
Plasmid/cosmid		
pKC1132	Suicide vectors for <i>Streptomyces</i> containing oriT RK2 for conjugation from <i>E. coli</i> to <i>Streptomyces</i> spp.	Kieser et al. 2000
pKC, <i>lanA</i>	pKC 1132 containing <i>lanA</i> region	This work
pKC, <i>lanB</i>	pKC 1132 containing <i>lanB</i> region	This work
pKC, <i>lanC</i>	pKC 1132 containing <i>lanC</i> region	This work
pKC, <i>lanL-1</i>	pKC 1132 containing <i>lanL-1</i> region	This work
pKC, <i>lanL-2</i>	pKC 1132 containing <i>lanL-2</i> region	This work
pKC, <i>lanM</i>	pKC 1132 containing <i>lanM</i> region	This work

^a Kan^r = kanamycin-resistant.

the spores from the mycelia. The spore suspension was filtered through sterile cotton in a syringe (15 ml), and the filtrate (800 µl) and 800 µl of 20% glycerol were mixed together in an Eppendorf tube and were stored at -20°C.

S4-7 genome sequencing (PacBio) and annotation.

Bacterial cells were grown in TSB broth containing 20% sucrose. Genomic DNA (1 µg) isolated by the cetyltrimethylammonium bromide (CTAB) method was used to amplify the lantipeptide genes. Sequencing was carried out by Pacific Biosciences. Sequencing employed the RSII system, a process enabling SMRT technology assembly methods, consisted of nonhybrid approaches, including the Hierarchical Genome Assembly Process (HGAP) 2 for both preassembly and de novo assembly (PacBio).

The BRIG program served to help create the genome circular map (Alikhan et al. 2011). Annotation was carried out using RAST version 2.0 and the classic RAST pipeline, release70 FIGfam (Aziz et al. 2008). For antiSMASH version 3.0.5, sequences were analyzed with the cluster finder algorithm, minimum cluster size coding sequence of five, minimum cluster finder probability score of 0.6, and the remaining options set automatically (Medema et al. 2011). The *Streptomyces griseus* S4-7 sequence is available at NCBI as GenBank assembly accession GCA_000932225.1, WGS Project JYBE01.

Generation of lantipeptide mutants.

Genomic DNA (1 µg) isolated by the CTAB method was used to amplify the six lantipeptide genes. To generate knock-out mutants of the lantipeptide genes, left arm (LA) and right arm (RA) fragments were synthesized with primer sets (Supplementary Table S5), were linked by PCR, and were amplified with LA F and RA R primers, yielding the cloned gene with flanking sequences. PCR was carried out in an MJ Research PTC-200 thermal cycler (Bio-Rad). The resulting linked constructs were cloned into the pGEM T vector (Promega), were transformed into *Escherichia coli* DH5α, and were identified by blue or white selection on Luria Bertani (LB) agar containing ampicillin (100 µg/ml) and X-gal (40 µg/ml). After color selection, the inserted DNA in pGEM-T was cut by restriction enzymes *Xba*I and *Eco*RV (NEB). Next, the insert was ligated into plasmid pKC1132 and was transferred to the *Streptomyces* conjugation strain *E. coli* ET12567, with selection on LB agar containing kanamycin (30 µg/ml), chloramphenicol (25 µg/ml), and apramycin (50 µg/ml).

To generate mutants, *E. coli* ET12567 containing the cloned lantipeptide fragments in pKC1132 was mixed into a S4-7 spore stock and was spread on MS agar. Sixteen hours later, apramycin (50 µg/ml) and nalidixic acid (25 µg/ml) were overlaid onto the MS agar plates. After 7 days, the resulting colonies were streaked sequentially three times, on MS agar containing apramycin (50 µg/ml), to obtain single crossovers (Kieser et al. 2000). To obtain double cross-over mutants, the colonies were transferred onto MS agar without antibiotics (Bierman et al. 1992).

Antagonism tests.

Ten microliters of a bacterial suspension of strain S4-7 and its mutants at an optical density at 595 nm of 2.0 was streaked with a wooden stick in two lines 3 cm long onto a plate of PDK agar. After 3 days, an agar block of a culture of the fungal pathogen was inoculated at the center of the plate, 2 cm from each of the bacterial streaks. Five days later, antifungal activity was assessed based on the size of the inhibition zone and was denoted as follows: no inhibition, + (very weak); 0 to 1 mm, ++ (weak); >1 to 5 mm, +++ (moderate); >5 to 10 mm, ++++ (strong); >10 to 20 mm, +++++ (very strong) (Nguyen et al. 2012).

Total RNA extraction and qRT-PCR.

Total RNA was extracted three times, at vegetative hyphal formation (24 h), aerial hyphae formation (48 h), and after formation of aerial hyphae (72 h). RNA was isolated by the TRIzol method and was purified with an RNeasy Mini kit (Qiagen), according to manufacturer protocols, with liquid nitrogen grinding (Van Dessel et al. 2004). The extracted RNA was stored at -80°C. qRT-PCR was performed relative to expression of a set of housekeeping genes. RNA (1 µg) was used to amplify a cDNA library with the ReverTraAce-α-cDNA synthesis kit (Toyobo). Each reaction included primers for *lanM* and the housekeeping genes *hrdA*, *gryA*, and *recA*.

qRT-PCR of rhizosphere DNA from soil samples.

A total of 30 soil samples, each with three biological replications, was collected during strawberry cultivation from three geographically different regions, including Sugok-myeon, Sancheong-gun, and Hadong-gun. For extraction, soil (0.5 g) was homogenized in 978 µl of sodium phosphate buffer and 122 µl of MT buffer (MP Biomedicals) with a bead beater for 5 min at maximum speed. The Fast DNA spin kit (QBiogene, MP Biomedicals) served to extract the soil DNA, which was stored at -80°C. A specific fragment between the transcription factors Han and Skippy was used to detect *F. oxysporum* f. sp. *fragariae* with primers FofraF (5'-CAGACTGGGGTGCTTAAAGTT-3') and FofraR (5'-AACGCTAGGGTCGTAA CAAA-3') (Hong et al. 2018; Suga et al. 2013). Primers StrepB (5'-GTAAAACGACGGCCAGT-3') and StrepE (5'-AACAGC TATGACCAT-3') were used to detect *Streptomyces* spp. (Rintala et al. 2001). For qRT-PCR, soil DNA was diluted 10-fold with double distilled (dd)H₂O, and 5 µl was amplified with 25 µl of SYBR green mixture (Toyobo) and ddH₂O up to 50 µl. A CFXConnect OpticsModule Real-Time PCR system (Bio-Rad) was used for qRT-PCR.

Evaluation of disease incidence and severity of Fusarium wilt in fields.

Five plots were randomly designated in each field and each plot had 50 to 55 strawberry plants. The plants with Fusarium wilt symptoms were evaluated. The disease severity was evaluated on a disease index scale from 0 to 5: 0 = healthy, 1 = one to three leaves yellowed and rolled, 2 = more than three leaves rolled and deformed, 3 = chlorosis and early plant wilting, 4 = necrosis and entire plant wilting, and 5 = dead or nearly so.

Statistics analysis.

The data were analyzed using SigmaPlot (Windows ver. 11.0; System Software Inc.). analysis of variance was used to examine differences between means. The dot plot graph was visualized by ggplots version 3.0.1 and ggplot2 version 2.1.0 (R package).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

antiSMASH program: <https://antismash.secondarymetabolites.org>
 BRIG (BLAST Ring Image Generator) software:
<http://courseforge.net/projects/brig>
 NCBI website: <https://www.ncbi.nlm.nih.gov>
 RAST annotation service: <http://rast.nmpdr.org>