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Nonribosomal Peptides, Key Biocontrol Components for *Pseudomonas fluorescens* In5, Isolated from a Greenlandic Suppressiv e Soil

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ABSTRACT Potatoes are cultivated in southwest Greenland without the use of pesticides and with limited crop rotation. Despite the fact that plant-pathogenic fungi are present, no severe-disease outbreaks have yet been observed. In this report, we document that a potato soil at Inneruulalik in southern Greenland is suppressive against *Rhizoctonia solani* Ag3 and uncover the suppressive antifungal mechanism of a highly potent biocontrol bacterium, *Pseudomonas fluorescens* In5, isolated from the suppressive potato soil. A combination of molecular genetics, genomics, and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) imaging mass spectrometry (IMS) revealed an antifungal genomic island in *P. fluorescens* In5 encoding two nonribosomal peptides, nunamycin and nunapeptin, which are key components for the biocontrol activity by strain In5 *in vitro* and in soil microcosm experiments. Furthermore, complex microbial behaviors were highlighted. Whereas nunamycin was demonstrated to inhibit the mycelial growth of *R. solani* Ag3, but not that of *Pythium aphanidermatum*, nunapeptin instead inhibited *P. aphanidermatum* but not *R. solani* Ag3. Moreover, the synthesis of nunamycin by *P. fluorescens* In5 was inhibited in the presence of *P. aphanidermatum*. Further characterization of the two peptides revealed nunamycin to be a monochlorinated 9-amino-acid cyclic lipopeptide with similarity to members of the syringomycin group, whereas nunapeptin was a 22-amino-acid cyclic lipopeptide with similarity to corpeptin and syringopeptin.

IMPORTANCE Crop rotation and systematic pest management are used to only a limited extent in Greenlandic potato farming. Nonetheless, although plant-pathogenic fungi are present in the soil, the farmers do not experience major plant disease outbreaks. Here, we show that a Greenlandic potato soil is suppressive against *Rhizoctonia solani*, and we unravel the key biocontrol components for *Pseudomonas fluorescens* In5, one of the potent biocontrol bacteria isolated from this Greenlandic suppressive soil. Using a combination of molecular genetics, genomics, and microbial imaging mass spectrometry, we show that two cyclic lipopeptides, nunamycin and nunapeptin, are important for the biocontrol activity of *P. fluorescens* In5 both *in vitro* and in microcosm assays. Furthermore, we demonstrate that the synthesis of nunamycin is repressed by the oomycete *Pythium aphanidermatum*. Overall, our report provides important insight into interkingdom interference between bacteria and fungi/oomycetes.

Agriculture in subarctic Greenland is possible without pesticides and with limited crop rotation despite the presence of plant-pathogenic fungi. For example, potatoes cultivated in a small field at the Agricultural Consulting Services in Qaqortoq for almost 50 years showed no severe incidence of diseases such as potato late blight (K. Hoegh, personal communication). One plausible explanation is that these Greenlandic soils are disease suppressive, i.e., they contain beneficial microbes that positively affect the growth and health of plants (1, 2). Suppressive soils have been described from several parts of the world, including *Fusarium* wilt-suppressive soils from Chateauenaund (France) and the Salinas Valley (United States), potato scab-suppressive soils from Washington (United States), and suppressive sugar beet soils (the Netherlands) (2, 3). Beneficial microorganisms responsible for suppression of fungal growth, the so-called biological control agents (BCAs), have been discovered among bacteria of several genera, e.g., *Bacillus*, *Alcaligenes*, *Burkholderia*, *Agrobacterium*, and *Pseudomonas* (1–4). Of these, fluorescent pseudomonads have been investigated in the greatest detail, and antifungal compounds, including phenazines, pyoluteorin, chlorogluconols, pyrrolnitrin, hydrogen cyanide (HCN), and nonribosomal peptides (NRPs), have been identified (5).
A *Pseudomonas* strain with high antifungal activity was previously isolated from a potato field at Inneruulalik, southern Greenland (6). This bacterium, *Pseudomonas fluorescens* In5, inhibited a wide range of pathogenic fungi and oomycetes in *vitro* in a temperature-dependent manner, i.e., showing higher activities at lower temperatures (6). Previous attempts to identify antifungal compounds such as phenazines, pyoluteorin, chlororogluconols, and pyrrolnitrin, or the genes encoding such compounds in *P. fluorescens* In5, were unsuccessful. The only putative antifungal compound identified was HCN, but genetic analysis indicated that an unknown NRP might also be involved in the antifungal activity of this strain (6, 7). The actual NRP, however, was never identified or isolated.

In this study, we unraveled the antifungal mechanism of the disease-suppressive *P. fluorescens* In5 strain using a combination of *in vitro* agar plate assays, soil microcosm experiments, molecular genetics, genomics, and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) imaging mass spectrometry (IMS) analyses. We show that the main biocontrol ability of *P. fluorescens* In5 is mediated by two lipopeptides with distinct fungal inhibition activities depending on the target fungus or oomycete.

**RESULTS**

**Potato soil from Inneruulalik in southern Greenland is disease suppressive.** Soil samples were collected from a potato field at Inneruulalik in southern Greenland. According to the farmer, potatoes were cultivated in the same plot for at least 10 consecutive years without any incidence of severe diseases. The disease-suppressive properties of the soil were investigated using *in vitro* soil microcosm experiments with seedlings of tomato (*Solanum*; family *Solanaceae*) and the plant-pathogenic basidiomycete *Rhizoctonia solani* Ag3 causing, e.g., stem rot and tuber black scurf of potatoes. Seedlings planted into the Inneruulalik soil inoculated with *R. solani* Ag3 mycelium did not show any damping-off symptoms, whereas a heat treatment of the soil (80°C for 1 h), followed by *R. solani* Ag3 inoculation, led to severely affected seedlings (Fig. 1).

In addition, the disease-suppressive properties of the heat-treated soil could be almost completely recovered by mixing in a small (10%) amount of the untreated soil (Fig. 1), which suggests that the Inneruulalik soil is suppressive against *R. solani* Ag3 and that the suppressive mechanism is of microbiological origin. Metagenomic DNA was isolated from the Inneruulik suppressive soil, and pyrosequencing generated a total of 30,747 high-quality sequences revealing members of the phyla *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Acidobacteria* to be the most abundant bacteria in the soil microbiome (see Table S1 in the supplemental material). Members of the bacterial genera *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Streptomycetes*, and *Burkholderia* have previously been described for their biocontrol activities (8–11); however, the culture-independent pyrosequencing analysis showed that these bacterial genera constituted only a small fraction of the total diversity in the Inneruulik soil (see Table S2). Previously, a potent antifungal pseudomonad, *P. fluorescens* In5, was identified from the Inneruulik potato soil using culture-based approaches (6); hence, this biocontrol bacterium was the subject of the subsequent studies.

**Molecular genetics of *P. fluorescens* In5 reveal an antifungal genomic island encoding nonribosomal peptides with distinct antifungal activities.** To identify genes in *P. fluorescens* In5 encoding antifungal compounds, a Tn5 transposon mutant library was constructed and the approximately 1,500 mutants were arranged in microtiter plate format and screened for loss of mycelial growth inhibition of *R. solani* Ag3 or *Pythium aphanidermatum* (an oomycete causing leak syndrome of potatoes) *in vitro*. One mutant, Tn1, failed to inhibit mycelial growth of *R. solani* Ag3, whereas inhibition of *P. aphanidermatum* was slightly increased compared to the level seen with wild-type strain In5 (Fig. 2A; see also Fig. S1 in the supplemental material). DNA sequence analysis of the insertion site showed that the transposon had inserted into an open reading frame (ORF), nunE, encoding a putative NRP (Fig. 3).

Interestingly, a closer examination of the whole-genome sequence of *P. fluorescens* In5 revealed that the genomic region around the transposon insertion site contained a number of ORFs with similarities to known NRP genes from *P. syringae* pv. *syringae* B728a, Cit7, and 642 and from *Pseudomonas* sp. SHC52 (Fig. 3; see also Table S3). The amino acid sequence encoded by nunE and that encoded by the adjacent nunD ORF showed high similarity to the products of the thanamycin synthesis gene cluster from *Pseudomonas* sp. SHC52 (60% identity [Id] to ThaA and 76% Id to ThaB, respectively) as well as similarity to those of the syringomycin synthesis gene cluster (syrE) from *P. syringae* pv. *syringae* B728a, Cit7, and 642 (approximately [approx.] 30% to 40% Id) (Fig. 3; see also Table S3). In addition to nunD and nunE, the nunB1 and nunB2 gene products showed high similarity to the thanC1 and thanC2 gene products from *Pseudomonas* sp. SHC52 (73% and 87% Id, respectively) and to syrB1 and syrB2 gene products from *P. syringae* strains B728a, Cit7, and 642 (approx. 70% to 88% Id) (Fig. 3; see also Table S3).

**FIG 1** Soil microcosm experiment. Presprouted tomato seedlings were planted in the Inneruulalik suppressive soil (S), in the Inneruulalik soil heat treated at 80°C (C, conducive), and in heat-treated soil mixed with the suppressive soil (9:1 [wt/wt]) (CS). Damping-off symptoms of infected seedlings caused by *R. solani* Ag3 were scored visually and expressed as percentages of disease incidence (means ± standard errors of the means [SEM], n = 4). The asterisk indicates a statistically significant difference from the suppressive soil (S) (*P < 0.05, Tukey’s HSD test).
encoding putative NRPs were present. Three ORFs denoted nupA, nupB, and nupC displayed similarity to the syringopeptin biosynthesis gene cluster from *P. syringae* pv. *syringae* B728a (between 36% and 47% Id) (Fig. 3; see also Table S3 in the supplemental material), whereas a corresponding gene cluster was not identified in *Pseudomonas* sp. SHC52 due to a truncated DNA sequence from this isolate. The putative peptide encoded by the nupA, nupB, and nupC gene cluster was denoted nunapeptin. In addition to the two nun and nup gene clusters, the antifungal genomic island in *P. fluorescens* In5 also contained the genes pseA to pseC (pseA-C), and pseE-F, which in *P. syringae* have been associated with regulation and export of peptides (Fig. 3; see also Table S3) (14). The gene order of the In5 antifungal genomic island was identical to that of *P. syringae* pv. *syringae* B728a, but the thaC1, thaC2, and thaD genes in *Pseudomonas* sp. SHC52 were located downstream of the major NRP genes thaA and thaB (Fig. 3).

Gene replacement mutants Gr1 (mutated in nunD) and Gr2 (mutated in nupB) as well as a double mutant, Tn1Gr2, carrying both the mutation in Tn1 (mutated in nunE) and that in Gr2, were constructed in *P. fluorescens* In5 (Fig. 3) and analyzed for antifungal activities in vitro on agar plates. Mutant Gr1 displayed activity similar to that seen with mutant Tn1, i.e., significantly reduced inhibition of *R. solani* Ag3 mycelial growth compared to that of
and expressed as percentages of disease incidence (means of inhibition against \textit{P. aphanidermatum} (column C) (Asterisks indicate statistically significant differences from the control treatment (column C). The results were compared to those seen with untreated control soil (column C), soil inoculated with \textit{P. fluorescens} in5 wild type containing both the nunamycin and nunapeptin peptides (NM+NP); 10 \mu g of the purified nunamycin (NM) or nunapeptin (NP) peptides was spotted on sterile pieces of filter paper in the center of the agar plate surrounded by four identical mycelium plugs. Inhibition of \textit{R. solani} Ag3 is indicated by white arrowheads, whereas inhibition of \textit{P. aphanidermatum} is indicated by black arrowheads.

FIG 5 Inhibition of \textit{R. solani} Ag3 (i) or \textit{P. aphanidermatum} (ii) mycelial growth by 10 \mu g crude peptide extract derived from the \textit{P. fluorescens} In5 wild type containing both the nunamycin and nunapeptin peptides (NM+NP); 10 \mu g of the purified nunamycin (NM) or nunapeptin (NP) peptides was spotted on sterile pieces of filter paper in the center of the agar plate surrounded by four identical mycelium plugs. Inhibition of \textit{R. solani} Ag3 is indicated by white arrowheads, whereas inhibition of \textit{P. aphanidermatum} is indicated by black arrowheads.

Wild-type strain In5, whereas inhibition of \textit{P. aphanidermatum} was slightly increased (Fig. 2A; see also Fig. S1 in the supplemental material). The biocontrol activity of the nun\textit{E} mutant, Tn1, was further studied in soil microcosm experiments, and results revealed a substantially reduced ability to protect tomato seedlings against \textit{R. solani} Ag3 compared to the mutants In5 wild type and \textit{P. fluorescens} In5 wild type containing both the nunamycin and nunapeptin peptides (NM+NP) (Fig. 2B and C). Inhibition of \textit{P. aphanidermatum} was scored visually and expressed as percentages of disease incidence (means \pm SEM, \textit{n} = 4). Asterisks indicate statistically significant differences from the control treatment (column C) (\textit{P} < 0.05, Tukey’s HSD test).

**Identification of nonribosomal peptides produced by \textit{P. fluorescens} In5 using MALDI-TOF imaging mass spectrometry.** In order to bypass laborious and time-consuming conventional peptide purification methods, we used matrix-assisted laser desorption–ionization time of flight (MALDI-TOF) imaging mass spectrometry (IMS) to identify the antifungal peptides encoded by the antifungal genomic island in \textit{P. fluorescens} In5. Ions of interest were selected by superimposing the false-colored ion distribution map from the IMS analysis onto an optical image of the biocontrolactivity of the nun\textit{E} mutant, Tn1, was further studied in soil microcosm experiments, and results revealed a substantially reduced ability to protect tomato seedlings against \textit{R. solani} Ag3 compared to the mutants In5 wild type and \textit{P. fluorescens} In5 wild type containing both the nunamycin and nunapeptin peptides (NM+NP) (Fig. 2B and C). Inhibition of \textit{P. aphanidermatum} was scored visually and expressed as percentages of disease incidence (means \pm SEM, \textit{n} = 4). Asterisks indicate statistically significant differences from the control treatment (column C) (\textit{P} < 0.05, Tukey’s HSD test).

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the structure due to a 24% increase in the M +2 peak due to the $^{35}$Cl isotope of chlorine. Analysis of the gene cluster also showed the presence of a chlorinase enzyme adjacent to an adenylation domain capable of loading a 4-Cl threonine residue (see Fig. S3 in the supplemental material). Analysis of the nunapeptin gene cluster combined with MS/MS metastatic mass losses for the lipid tail (Fig. 7; see also Fig. S4 in the supplemental material). Tandem MS analysis of these analogs showed identical to corpeptin and syringopeptin (15). The molecular formula for the m/z 473.272 fragment was consistent with a Thr-Ala-Dab-Ser-Ile cyclic peptide moiety (Fig. 7; see also Fig. S4), which is identical to the cyclic tail of corpeptin (15). The results of two-dimensional (2D) homo- and heteronuclear NMR experiments supported the mass spectral interpretation (see Table S4). In addition to the primary nunapeptin, three analogs were observed in the mass spectrum of In5 (m/z 2,023.22, m/z 2,037.24, and m/z 2,075.22) (Fig. S2). Tandem MS analysis of these analogs showed identical mass losses for the lipid tail (m/z 254.175) as well as for the terminal cyclic peptide (m/z 473.272).

**DISCUSSION**

In certain subarctic fields in Greenland, it is possible to cultivate potatoes with limited crop rotation and no use of pesticides, even though plant-pathogenic fungi are present in the soils. Using soil microcosms, we showed that a potato soil from Inneruulalik in southern Greenland was able to suppress the growth of the plant-pathogenic fungus *Rhizoctonia solani* Ag3. Such suppressive soils exist worldwide (2), but this report is the first to describe a disease-suppressive soil from a subarctic region. Antifungal microorganisms from suppressive soils have been shown to comprise several bacterial genera (2, 8–11), and an analysis of the Inneruulalik soil by 16S rRNA gene pyrosequencing identified the presence of organisms belonging to several of these genera, i.e., *Pseudomonas, Bacillus, Paenibacillus, Streptomycetes,* and *Burkholderia.* However, the frequency of organisms of these genera constituted in total less

FIG 6 Time course experiments with the *P. fluorescens* In5 wild type (A) and the mutant strains, *P. fluorescens* Tn1 (B) and *P. fluorescens* G1 (C), cultured on solid 1/5 PDA. Production of nunamycin (NM) (i.e., m/z 2,045.20 gave a clear sequence tag for 16 amino acid residues with the sequence Pro-Ala-Ala-Ala-Val-Ala-Dhb-Ser-Ile-Dha-Ala-Val-Ala-Val-Dhb-Thr-Ala-Dab-Ser-Ile, with strong similarity to corpeptin and syringopeptin (Fig. 7; see also Fig. S4 in the supplemental material). By high-resolution mass spectrometry, the primary ion at m/z 2,045.20 gave a clear sequence tag for 16 amino acid residues with the sequence Pro-Ala-Ala-Ala-Val-Ala-Dhb-Ser-Ile-Dha-Ala-Val-Ala-Val-Dhb-Thr-Ala-Dab-Ser-Ile. In addition, two larger fragments at m/z 254.175 and m/z 473.272 appeared in the mass spectrum. The molecular formula of the m/z 254.175 fragment correlated with a Dhb residue attached to 3-OH dodecanoic acid (Fig. 7; see also Fig. S4), which is similar to the structure of syringopeptin and corpeptin (15). The molecular formula for the m/z 473.272 fragment was consistent with a Thr-Ala-Dab-Ser-Ile cyclic peptide moiety (Fig. 7; see also Fig. S4), which is identical to the cyclic tail of corpeptin (15). The results of two-dimensional (2D) homo- and heteronuclear NMR experiments supported the mass spectral interpretation (see Table S4). In addition to the primary nunapeptin, three analogs were observed in the mass spectrum of In5 (m/z 2,023.22, m/z 2,037.24, and m/z 2,075.22) (Fig. S2). Tandem MS analysis of these analogs showed identical mass losses for the lipid tail (m/z 254.175) as well as for the terminal cyclic peptide (m/z 473.272).

**FIG 7** The proposed structures of nunamycin from *P. fluorescens* In5 and the analogous peptides thanamycin, cornycin A, and syringomycin (A) and of nunapeptin from *P. fluorescens* In5 and the analogous peptides corpeptin A and syringopeptin (B). The abbreviations are as follows: (3-OH)Asp, 3-hydroxyaspartic acid; (4-Cl)Thr, 4-chlorothreonine; Dab, 2,4-diaminobutyric acid; Dhb, dehydrobutyrine; Dha, dehydroalanine; Orn, ornithine; Hse, homoserine. Consensus amino acids are denoted in gray-lined boxes.
than 1% of all phylotypes, which led us to the assumption that the bacteria present in the disease-suppressive *Innereulilik* soil must be potent inhibitors of fungal growth. An effective biocontrol bacterium, *P. fluorescens* In5, was previously isolated from the *Innereulilik* soil and was found to encode genes for the synthesis of antifungal compounds, including hydrogen cyanide and a hitherto-uncharacterized nonribosomal peptide (6, 7).

Nonribosomal peptides isolated from several pseudomonads using conventional methods such as extraction of active compounds in organic solvents followed by high-pressure liquid chromatography (HPLC) fractionation and structure resolution by NMR and MS have been described previously (16–18). In this study, we demonstrated that molecular genetics combined with genomics and MALDI-TOF IMS may facilitate the process of identification and purification of NRPs and in addition assist in verifying the presence of the desired compounds within the different extraction and purification steps. Furthermore, this approach facilitated expression studies of the individual NRPs in live bacterial-fungal cocultures both spatially and temporally. MALDI-TOF IMS analysis of *P. fluorescens* In5 revealed the presence of two antifungal NRPs in the inhibition zone of *R. solani* AG3 and *P. aphanidermatum*, respectively. These NRPs, denoted nunamycin and nunapeptin, showed structural resemblance to syringomycin and syringopeptin from *P. syringae* and to cornycin A and corpeptin A from *P. corrugata* (15) as well as to thanamycin from *Pseudomonas* sp. SH-C52 (19). Nunamycin was found to be a chlorinated cyclic lipopeptide composed of 9 amino acid residues, Ser-Dab-Gly-Hse-Dab-Thr-Thr-(3-OH)Asp-(4-Cl-Thr), with a molecular mass of 1,137.54 Da, while nunapeptin was produced as a family of cyclic lipopeptides of 22 amino acid residues, Dhb-Pro-Ala-Ala-Ala-Val-Ala-Dhb-Ser-Val-Ile-Dha-Ala-Val-Ala-Dhb-Thr-Ala-Dab-Ser-Ile, with molecular masses ranging from 2,023 to 2,075 Da. The structures of nunamycin and nunapeptin contained some uncommon amino acids, such as (3-OH)Asp (3-hydroxyaspartic acid), (4-Cl)Thr (4-chlorothreonine), Dab (2,4-diaminobutyric acid), Dhb (dehydrobutyrine), and Dha (dehydroalanine). However, these amino acids are present in a range of nonribosomal peptides, including antimicrobial peptides of the syringomycin, tolaasin, and syringopeptin families (15).

While the biosynthesis of nunamycin is catalyzed by three NRPs (NupA, NupB, and NupC) in a collinear manner, the biosynthesis of nunapeptin, catalyzed by the three NRPs, NunB1/NunB2, NunD, and NunE, does not respect the collinearity. For example, a split-module phenomenon was found in the ninth module in the nunamycin NRP, NunE; the C and T domains were present, but an A domain was not, which instead was present in NunB1, as also described for the syringomycin synthase (15). Furthermore, an additional A domain was lacking in module 5 of the nunamycin NRP, NunD. However, chemical analysis showed that Dab was present as the fifth amino acid in the nunamycin peptide chain; thus, we speculate that the A domain in module 2 could function twice. Such module shuffling has been documented before in NRP-catalyzed peptide synthesis (20).

Transposon and gene replacement mutants, i.e., Tn1, Gr1, Gr2, and Tn1Gr2, constructed in *P. fluorescens* In5 confirmed that nunamycin and nunapeptin are key components for the biocontrol ability observed by this strain. However, the two peptides showed distinct inhibition activities. Nunamycin was found to play a role primarily in *R. solani* AG3 mycelial growth inhibition, whereas nunapeptin was essential for *P. aphanidermatum* inhibition. However, when a nunapeptin NRP knockout mutant (Gr2, mutated in *nupB*) was analyzed in coculture, this mutant was defective in growth inhibition of both *P. aphanidermatum* and *R. solani* AG3, although the nunamycin biosynthesis was intact. A closer examination of the *nup* operon showed the presence of six genes, *nupD*, *pseA-C*, and *pseE-F*, possibly involved in the efflux of NRPs (14, 21). Thus, it is likely that the knockout of the *nupB* gene in *P. fluorescens* In5 negatively influences not only the synthesis of nunapeptin but also the efflux system located downstream of the *nup* genes. If expression of the *pse* genes is affected, this could inhibit efflux of both the nunapeptin and nunamycin and consequently also affect the inhibition activity against both *P. aphanidermatum* and *R. solani* AG3. This hypothesis was supported by MALDI-TOF MS analysis, which showed no production of nunapeptin or nunamycin by this mutant strain.

MALDI-TOF IMS enabled us to study the expression of nunamycin and nunapeptin simultaneously and in relation to interactions between *P. fluorescens* In5 and *R. solani* AG3 or *P. aphanidermatum*. Nunamycin and nunapeptin were both expressed in cocultures of *P. fluorescens* In5 with *R. solani* AG3. However, only nunapeptin was present in cocultures with *P. aphanidermatum*, suggesting that the synthesis of nunamycin in *P. fluorescens* In5 was inhibited by *P. aphanidermatum*. Regulation of lipopeptides and other secondary metabolites in pseudomonads by other organisms has been reported in several cases. For example, the production of secondary metabolites in *P. aeruginosa* was found to be affected by a halogenated furanone compound produced by the Australian macroalga *Delisea pulchra* (22), and sugar beet extracts were shown to affect lipopeptide synthesis in *Pseudomonas* sp. DSS73 (23). The exact mechanism by which *P. aphanidermatum* inhibited the biosynthesis of nunamycin in *P. fluorescens* In5 in this study, however, still needs to be elucidated.

MALDI-TOF MS analysis similarly permitted a study of the expression of nunamycin and nunapeptin by *P. fluorescens* In5 wild-type and mutant strains and allowed a time course analysis of nunamycin and nunapeptin synthesis by *P. fluorescens* In5. Nunamycin and nunapeptin were first detected 12 h after inoculation, with signals for the peptides reaching a maximum after 48 to 72 h of incubation and subsequently decreasing after 7 days of incubation. This expression profile is different from other lipopeptide synthesis profiles. For example, in *P. fluorescens* strain SS101, masatolide A biosynthesis initiates between 12 h and 16 h of incubation and peaks after 24 h of incubation (24). In *Bacillus amyloliquefaciens*, synthesis of surfactin and fengycin displayed maximal production after 10 to 40 h and decreased after 60 h, whereas bacillomycin D showed maximal intensity after 40 and 60 h (25). The transient production of nunamycin, however, has previously been observed with the analogous molecule thanamycin from *Pseudomonas* sp. SH-C52 (19).

The results presented in this report demonstrate the powerful approach of combining in vitro inhibition experiments with molecular genetics, genomics, and MALDI-TOF IMS analyses. Using this combination of tools, we were able to unravel the key biocontrol factors of a Greenlandic bacterium, *P. fluorescens* In5, isolated from a disease-suppressive potato soil in subarctic Greenland, to elucidate the structure and function of two antifungal lipopeptides, and to document that the effective biocontrol ability of *P. fluorescens* In5 requires the synthesis of both peptides.
TABLE 1 Bacteria, fungi, and plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid or bacterial or fungal strain</th>
<th>Relevant genotype and (or) characteristic(s)*</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>ppiKmLacZ</td>
<td>Km'-InZ cassette; pTnMod-based; mobilizable</td>
<td>26</td>
</tr>
<tr>
<td>pRK213</td>
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<td>pEX100T</td>
<td>oriT, bla, sacB</td>
<td>28</td>
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</table>

| Bacteria                            |                                              |           |
|-------------------------------------|                                              |           |
| Escherichia coli DH5a               | endA1 hisD17 supE44 thi-1 recA1 U169 deoR   | 34        |
| Pseudomonas fluorescens In5         | WT                                           | 6         |
| Pseudomonas fluorescens In5-Tn1     | ΔnunE                                        | 6         |
| Pseudomonas fluorescens In5-Gr1     | ΔnunD                                        | This study|
| Pseudomonas fluorescens In5-Gr2     | ΔnupB                                        | This study|
| Pseudomonas fluorescens In5-Tn1Gr2  | ΔnunE ΔnupB                                  | This study|

| Fungus or oomycete                  |                                              |           |
|-------------------------------------|                                              |           |
| Rhizoctonia solani Ag3              | Basidiomycete                                | 6         |
| Pythium aphanidermatum               | Oomycete                                     | 6         |

* Km, kanamycin; WT, wild type.

MATERIALS AND METHODS

Strains and culture conditions. Bacterial strains (Table 1) were routinely grown in liquid or on solid (1.5% agar) Luria-Bertani (LB) medium at 28°C. Escherichia coli [for transposon mutagenesis] and Plasmid or bacterial or fungal strain Relevant genotype and (or) characteristic(s)* Reference

Plasmids

<table>
<thead>
<tr>
<th>Plasmid or bacterial or fungal strain</th>
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<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>ppiKmLacZ</td>
<td>Km'-InZ cassette; pTnMod-based; mobilizable</td>
<td>26</td>
</tr>
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Fungus or oomycete

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Soil sampling, DNA isolation, and pyrosequencing analysis of the Inneruulalik potato soil. For details on soil sampling, DNA isolation, and pyrosequencing analysis of the Inneruulalik potato soil, see Materials and Methods in the supplemental material.

Soil microcosm assays. The Inneruulalik soil was sieved (0.5-cm-pore-size mesh) to remove plant debris and small stones. Tomato seeds (cv. Marmande Super) were presprouted for 4 days at 20°C in petri dishes on wetted filter paper.

For determination of soil suppressiveness, four presprouted tomato seedlings were placed randomly in petri dishes filled with 40 g of soil with an initial moisture content of 14.8% (vol/wt). The various soil treatments were suppressive soil (S), suppressive soil heat-treated at 80°C (S*), suppressive soil heat-treated at 80°C in an oven (S**), or gentamicin (10 µg·ml⁻¹), or ampicillin (50 µg·ml⁻¹), or kanamycin (50 µg·ml⁻¹), or gentamicin (10 µg·ml⁻¹). The basidiomycete R. solani Ag3 and the oomycete P. aphanidermatum (Table 1) were used as target pathogens in vitro inhibition assays and soil microcosm experiments. The fungus or oomycete was maintained on standard potato dextrose agar (PDA; BD/Difco, USA) medium at 20°C.

To determine the ability of the different strains and the purified nunamycin and nunapeptin to inhibit R. solani, PDA agar plates supplemented with either kanamycin and ampicillin or kanamycin and ampicillin plus gentamicin (10 µg·ml⁻¹) were prepared, and 1/5 PDA medium was added to the plates. As a control, standard PDA without antibiotic was used. The plates were incubated in a growth chamber at 15°C or 20°C with a 16-h:8-h light:dark cycle. Plates were watered as described above. Four replicates (i.e., plates) were made for each soil treatment, and the experiment was repeated twice. After 10 and 6 days of incubation at 15°C and 20°C, respectively, the percentage of infected tomato seedlings showing damping-off symptoms was scored.

Screening of the transposon mutant library. Transposon mutagenesis of P. fluorescens In5 was performed using a mobilizable plasposon, pTnModOKm (26), as previously described (27). Strain In5 transconjugants with transposons integrated into the chromosome were selected on LB agar plates supplemented with kanamycin and ampicillin. Approximately 1,500 mutants were screened for lack of antifungal activity. Mutants were grown O/N in P69 Microwell plate (Nunc) (in 150 µL LB with ampicillin [50 µg·ml⁻¹ per well] and kanamycin [50 µg·ml⁻¹ per well]). Overnight cultures (1.5 µL) were transferred by a multichannel pipette to OmniTrays (Nunc) containing PDA at a strength of 1/5 (1/5 PDA) previously inoculated with a suspension of blended fungal hyphe of R. solani Ag3 or P. aphanidermatum. Transposon rescue of selected mutants using XhoI or SacII was performed as previously described (6).

Homologous recombination. Gene knockout by homologous recombination in the m/z 1,138 and m/z 2,060 NRP gene clusters was carried out using a gene replacement vector, pEX100T (28). Approximately 2,000 2,300 bp of the m/z 1,138 and m/z 2,060 NRP gene clusters containing one or two SalI sites were amplified with primers NRPS13a (5'-CGCATAAC CGGACACTG-3') and NRPS13b (5'-CTGGAAACAGTGCTGTCG-3') and primers 2 F (5'-TGTCAAGGCGTCCGCGCAC-3) and 2 R (5'-CG CGGAAGCGGTGATCC-3), respectively. The m/z 1,138 and m/z 2,060 NRP gene fragments were amplified using Phusion blunt-end polymerase (annealing temperature, 63°C), according to the manufacturer’s instructions. The blunt-end-amplified fragments were ligated into Smal-digested pEX100T. The pEX100T vectors with insertions were digested with Sall, and a Sall-digested gentamicin resistance (Gm') gene and a promoter from plasmid Tn7gfp2 (29) were inserted into the NRP gene clusters. The pEX100T vectors with NRP gene fragments disrupted by the Gm' gene were transformed into electrocompetent P. fluorescens In5 cells together with the pUX-BF13 helper plasmid as previously described (30). Strain In5 transconjugants with a site-directed knockout in one of the two NRP gene clusters were selected on LB agar plates supplemented with ampicillin and gentamicin.

Fungus/oomycete inhibition assays on agar plates. Antifungal activity of the different strains and the purified nunamycin and nunapeptin was tested on 1/5 PDA against R. solani Ag3 or P. aphanidermatum. Ten
Heteronuclear multiple-bond correlation (HMBC) spectra. 2D 1H-13C analysis of 1D proton, 2D COSY double-quantum filter (Dqf-COSY), 2D 1H-13C with a 1.7-mm-diameter Microbiology CryoProbe at 298 K, with standard NMR spectra were recorded on a Bruker Avance iii 600-Mhz spectrometer was inoculated in the center of the agar plates. Sterile MilliQ purified H2O was added to 10 microliters of O/N bacterial cultures or 10

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microliters of O/N bacterial cultures or 10 μg of the crude peptide extract or purified nunamycin and nunapeptin peptides on sterile filter papers was inoculated in the center of the agar plates. Sterile MilliQ purified H2O on filter paper was used as a control for the peptide extract and purified peptides. Four fungus/oomycete mycelium plugs were placed 2 cm from the center of the plate around the colony or filter paper. Plates were made in triplicate and incubated at 15°C (P. aphanidermatum) or 20°C (R. solani Ag3). Following 48 to 240 h of coculturing, depending on the fungus/oomycete strain and incubation temperature used, all plates were visually inspected and the inhibitory effect of the bacterial strains was evaluated as percent inhibition of radial growth (PIRG) as previously described (31).

MALDI-TOF imaging mass spectrometry analysis. P. fluorescens In5 or mutant strain Tn1 or Gr1 was cocultured with R. solani Ag3 or P. aphanidermatum on thin 1/5 PDA plates (i.e., 10 ml of medium in a standard petri dish, resulting in a thickness of medium of about 1.5 mm). Once the desired time point for growth was reached, areas of agar media containing the microbial colonies were excised from the petri dish and transferred to the MALDI stainless steel target plate. The subsequent matrix application was performed using a 53-μm-pore-size test sieve; dry matrix was deposited evenly across the sample surface until the desired matrix thickness was achieved. The sample was dried O/N followed by MALDI-TOF IMS analysis using a Bruker Microflex mass spectrometer. Data were collected from 50 to 3,000 m/z at 80-Hz laser frequency at a spatial resolution of 400 μm using a random-walk shot pattern. Data were analyzed using the Compass 1.2 software suite (FlexImaging 2.0, FlexControl 3.0, and FlexAnalysis 3.0; Bruker Daltonics) as previously described (32). The resulting mass spectrum was binned at 0.5-m/z increments and manually inspected for masses of interest. Masses were visualized using false coloring on an optical image of the original sample plate.

Peptide extraction. Plates with 1/5 PDA medium were inoculated with P. fluorescens In5. After incubation for 48 h at room temperature, the agar was sliced into small pieces and extracted with acidified deionized water (pH 2) for 1 h at 28°C and 225 rpm in a 2.8-liter Erlenmeyer flask followed by addition of n-butanol and incubation for 18 h at 28°C. The n-butanol extract was separated from agar by cheesecloth filtration and subsequently from cell debris by centrifugation (6,440 g, 10 min). n-Butanol was removed with a rotovaporator (Buchi R-200). The crude peptide extract was resuspended in 1 ml methanol and analyzed by MALDI-TOF mass spectrometry (MS). The resuspended extract was centrifuged for 2 min at 16,000 × g, and the supernatant was placed on a methanol-equilibrated Sephadex LH20 column (30 cm length). The peptide extract was separated with a methanol mobile phase at a flow rate of 0.4 ml/min. Fractions of 7.5 ml were collected, lyophilized, and resuspended in 200 μl methanol. Gel filtration fractions were analyzed by MALDI-TOF MS for peptide content. Fractions of the peptide-containing gel filtration were combined and concentrated by lyophillization. The peptides were further purified by reversed-phase high-pressure liquid chromatography (HPLC) using an Agilent 1260 system equipped with a Phenomenex Kinetex C18 column (4.6 mm by 15 cm). The peptides were eluted from the column using a gradient of 10% acetonitrile containing 0.1% formic acid:90% water containing 0.1% acetonitrile containing 0.1% formic acid:10% water containing 0.1% formic acid over 45 min at a flow rate of 1.0 ml/min. Peaks for nunamycin and nunapeptin were collected manually (24.6 min for nunamycin and multiple peaks between 35 and 40 min for the nunapeptin family of compounds), after which they were lyophilized and stored at −80°C.

NMR and tandem MS analysis. About 1 mg of nunapeptin at m/z 2,023 to 2,075 and about 100 μg of nunamycin at m/z 1,138 were dissolved in 40 μl of deuterated methanol (CD3OD) for NMR data acquisition. NMR spectra were recorded on a Bruker Avance iii 600-MHz spectrometer with a 1.7-mm-diameter Microbiology CryoProbe at 298 K, with standard pulse sequences provided by Bruker. Structures were confirmed by analysis of 1D proton, 2D COSY double-quantum filter (Dqf-COSY), 2D 1H-13C heteronuclear single-quantum coherence (HSQC), and 2D 1H-13C heteronuclear multiple-bond correlation (HMBC) spectra. 2D 1H-13C HMB spectra were recorded with delays corresponding to a 2) or 3) H-C coupling constant of 8 Hz and a 1) H-C coupling constant of 145 Hz. 2D 1H-13C multiplicity-edited HSQC spectra were recorded, with delays corresponding to a 1) H-C coupling constant of 140 Hz. Spectra were referenced to the methanol methyl resonance at 3.31 ppm.

Tandem mass spectrometry experiments were performed on a Thermo LTQ fault-tolerant (FT) mass spectrometer (Thermo Electron Corp., Bremen, Germany) equipped with a Diversa Nanomate electrospray source. Aliquots from solutions of purified nunapeptin and nunamycin compounds were diluted in acetonitrile:0.1% formic acid (70:30 [vol/vol]). Electrospray parameters included a spray voltage of 1.40 kV, heated inlet temperature of 200°C, nebulizing gas pressure of 40 lb/in2 N2, collision-induced dissociation energy of 25 V, and collision time of 500 ms. The instrument was operated in FT mode (peak resolution, 50 k) for detection of parent (MS) and daughter (MS/MS) ion peaks.

MALDI-TOF mass spectrometry. MALDI-TOF MS was used to detect target peptides in the crude peptide extract and fractions from gel filtration and HPLC as well as peptide production over time in the time course experiment. The sample was mixed 1:1 with a saturated solution of Universal MALDI matrix—70% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and spotted on a Bruker MSP 96 anchor plate. The target peptide containing the samples was dried and inserted into a Microflex Bruker Daltonics mass spectrometer. External calibration was done as described for MALDI-TOF IMS. Mass spectra were obtained with the FlexControl method as used for MALDI-TOF IMS and a single-spot acquisition of 200 shots. Single-spot MALDI-TOF MS data were analyzed by FlexAnalysis software.

Time course experiment of peptide production. Cultures of the P. fluorescens In5 wild type and the mutant strains, P. fluorescens Tn1 and Gr1, were inoculated on solid 1/5 PDA at 20°C and sampled at specific time points. Samples for dry-droplet analysis of peptide production (m/z 1,138 and 2,060) by MALDI-MS were taken after 6, 12, 24, 36, 48, 72, and 96 h and after 7 days. A scrape of bacterial colonies was dissolved in 100 μl of sterile deionized water. The bacterial suspension was mixed 1:1 with a saturated solution of Universal MALDI matrix in 70% acetonitrile containing 0.1% TFA and spotted on a Bruker MSP 96 anchor plate. MALDI MS analysis was carried out as described above.

Statistical analysis. Tukey’s honestly significant difference (HSD) test was used in conjunction with analysis of variance (ANOVA) in order to evaluate the data from the in vitro inhibition and microcosm experiments to identify means with significant differences. The tests were carried out with R version 2.10.0 (33).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl doi:10.1128/mBio.00079-15/-/DCSupplemental.

Text S1, DOC file, 0.01 MB.

Figure S1, PDF file, 1 MB.

Figure S2, PDF file, 0.2 MB.

Figure S3, PDF file, 0.2 MB.

Figure S4, PDF file, 0.3 MB.

Table S1, PDF file, 0.2 MB.

Table S2, PDF file, 0.1 MB.

Table S3, PDF file, 0.1 MB.

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REFERENCES


