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# Protozoan-Induced Regulation of Cyclic Lipopeptide Biosynthesis Is an Effective Predation Defense Mechanism for *Pseudomonas fluorescens*[▽]

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**Environmental bacteria are exposed to a myriad of biotic interactions that influence their function and survival. The grazing activity of protozoan predators significantly impacts the dynamics, diversification, and evolution of bacterial communities in soil ecosystems. To evade protozoan predation, bacteria employ various defense strategies. Soil-dwelling *Pseudomonas fluorescens* strains SS101 and SBW25 produce the cyclic lipopeptide surfactants (CLPs) massetolide and viscosin, respectively, in a quorum-sensing-independent manner. In this study, CLP production was shown to protect these bacteria from protozoan predation as, compared to CLP-deficient mutants, strains SS101 and SBW25 exhibited resistance to grazing by *Naegleria americana* in vitro and superior persistence in soil in the presence of this bacterial predator. In the wheat rhizosphere, CLP-producing strains had a direct deleterious impact on the survival of *N. americana*. In vitro assays further showed that *N. americana* was three times more sensitive to viscosin than to massetolide and that exposure of strain SS101 or SBW25 to this protozoan resulted in upregulation of CLP biosynthesis genes. Enhanced expression of the *massABC* and *viscABC* genes did not require physical contact between the two organisms as gene expression levels were up to threefold higher in bacterial cells harvested 1 cm from feeding protozoans than in cells collected 4 cm from feeding protozoans. These findings document a new natural function of CLPs and highlight that bacterium-protozoan interactions can result in activation of an antipredator response in prey populations.**

The composition and activity of biological communities are influenced by a myriad of biotic and abiotic factors in the ecosystem of interest. In soil, interactions among the biological members of the ecosystem have an important effect on the ultimate structure of the microbial community. Plants have a dominant impact on the overall microbial activity as the rhizosphere is a site of high substrate accessibility due to the secretion of an array of exudates that are available in readily metabolized forms. In this habitat, larger microbial populations subsequently lead to increases in the populations and feeding activities of their predators (42). Thus, rhizosphere bacteria are exposed to increased predation pressure as bacteriovorous free-living nematodes, as well as protozoans, are present at levels that are up to 30-fold higher than those in bulk soil (18).

The grazing activity of bacteriovores not only is an instrumental component contributing to the cycling of nitrogen in soil ecosystems but also has a significant impact on shaping the bacterial community structure (7, 40). Preferential feeding has been reported for protozoans in soil systems, and bacterial genera such as *Pseudomonas* are favored over genera such as *Streptomyces* and *Bacillus* (9, 45). As reviewed by Matz and Kjelleberg (33), bacteria employ various defense strategies to evade predation by protozoans. The preingestion adaptations to elude predation include development of an altered morphology that results in an inedible form (21, 25), enhanced motility that confers evasion capabilities (32), and altered membrane properties that lead to diminished cell recognition by protozoan predators (46). The postingestion modes of bacterial resistance to protozoan grazing include the production and release of bioactive compounds. These metabolites may play a significant role in limiting bacterial susceptibility to the predatory soil microfauna, including protozoans (23, 31). Indeed, the phenolic antifungal compound 2,4-diacetylphloroglucinol (23) and the pigment violacein (31), produced by *Pseudomonas fluorescens* CHA0 and *Chromobacterium violaceum* CV0, respectively, were capable of inhibiting the growth of protists and inducing encystment and cell lysis. In general, production of these metabolites is regulated in a population-dependent manner (31) rather than via a pathway involving predator perception.

Diverse bacterial genera, including *Bacillus*, *Pseudomonas*, and *Streptomyces*, produce a variety of bioactive peptides via nonribosomal peptide synthesis (3, 30, 38, 41). Cyclic lipopeptide surfactants (CLPs) have received considerable attention because of their antimicrobial activities and role in bacterial virulence (38, 39). CLPs disrupt cell membrane function, leading to lysis of various microbial life stages, including zoospores of oomycete plant pathogens (10, 14) and trypomastigotes of the human protozoan pathogen *Trypanosoma cruzi* (8). The natural functions of CLPs in a bacterium's life history have received little attention; however, CLPs were shown to play an important role in bacterial virulence, swarming motility, and biofilm formation (15, 39).

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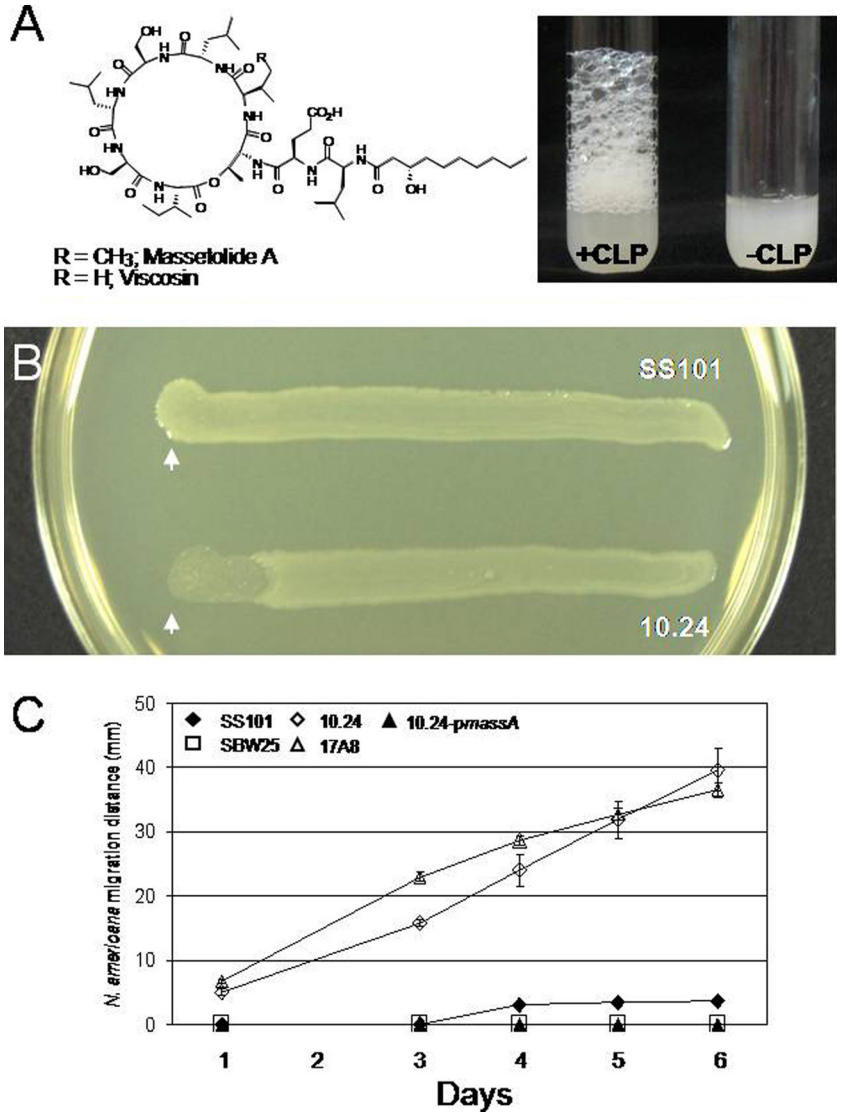


FIG. 1. (A) Structures of the CLPs massetolide and viscosin produced by *P. fluorescens* strains SS101 and SBW25, respectively (left panel) and detergent-like properties of these CLPs visualized by shaking suspensions of strains SS101 and SBW25 (+CLP) and their CLP-deficient mutants (−CLP) (right panel). (B) In vitro assessment of the effect of CLP production on bacterial resistance to protozoan grazing. After 3 days of incubation, migration of *N. americana* from the inoculation point (indicated by arrows) can be discerned by the clearing of confluent growth of the CLP-deficient mutant 10.24 of *P. fluorescens* SS101, resulting in an obvious feeding margin, which is absent with the CLP-producing wild-type strain SS101. (C) Migration distances for *N. americana* along confluent growth of CLP-producing strains SS101 and SBW25 and the corresponding CLP-deficient mutants, 10.24 and 17A8, cultured on 0.2× NBY agar. Genetic complementation of mutant 10.24 with p*massA* (10.24/p*massA*) restored massetolide production and resistance to feeding by *N. americana*.

*P. fluorescens* strains SBW25 and SS101, which were used in this study, produce two structurally related CLPs, viscosin and massetolide, respectively (Fig. 1A). Production of these CLPs is governed by three nonribosomal peptide synthetase (NRPS) genes designated *viscA*, *viscB*, and *viscC* in strain SBW25 (10) and *massA*, *massB*, and *massC* in strain SS101 (11). Although the CLPs viscosinamide, tensin, and putisolvin are produced in a cell density-dependent manner in the late exponential or stationary phase (29, 37), massetolide and viscosin are produced by SS101 and SBW25 in the early exponential growth phase and are not regulated by *N*-acylhomoserine lactone-based quorum sensing (11). For strain SS101, in vitro studies showed that expression of the *massABC* genes is directly accompanied by massetolide production (11); for strain SBW25, a similar relationship was found between *viscABC* gene expression and viscosin production in vitro (12).

In this study, we conducted in vitro feeding assays as well as soil experiments to assess the function of bacterial CLP production as a defense mechanism against protozoan predation. Reporter gene assays and quantitative PCR analyses were performed to determine whether active bacterial perception of predation regulates the expression of this potential defense mechanism. The results show that CLPs of *P. fluorescens* provide protection against protozoan grazing both in vitro and in situ and that *Naegleria-Pseudomonas* interactions lead to enhanced transcription of CLP biosynthesis genes.

## MATERIALS AND METHODS

**Organisms and cultivation.** *P. fluorescens* SS101 and *P. fluorescens* SBW25 were originally isolated from the rhizosphere of wheat and the phyllosphere of sugar beet, respectively (4, 14). Spontaneous rifampin (rifampicin) -resistant derivatives of these strains were used in this study. A CLP-deficient derivative of SS101, strain 10.24, is a Tn*5* mutant with the transposon inserted in *massA* (11). The position and orientation of the transposon insertion in mutant 10.24 were mapped, and the transposon was found to be located downstream of the *massA* promoter region (11). Transcription of the promoterless *lacZ* gene located on the transposon is under the control of the *massA* promoter. As described by De Bruijn et al. (11), the mutation in strain 10.24 was complemented by introduction of the p*massA* plasmid, which confers tetracycline resistance, resulting in strain 10.24/p*massA*. A CLP-deficient derivative of SBW25, strain 17A8, contains a Tn*Mod* plasposon insertion in *viscA* (10). All bacterial strains were routinely cultured on King's medium B (KB) (28) agar at 28°C.

The bacteriovorous amoeba flagellate *Naegleria americana* C1 (9) was utilized as the protozoan predator in these studies. Amoebae were propagated by cultivation with heat-killed *Escherichia coli*. Bacterial cells (~$10^{10}$ cells) were spread evenly over a water agar surface in a 9-cm-diameter petri plate, which was inoculated with 200 μl of a suspension containing $2 \times 10^2$ *N. americana* cysts $\mu l^{-1}$ and overlaid with 3 ml of Page's modified Neff's amoeba saline (PAS). The plates were sealed with Parafilm and incubated at 20°C, and 2 ml PAS was added to the plates at 7-day intervals.

**Feeding assay.** Inocula containing individual CLP-producing bacterial strains and corresponding CLP-deficient bacterial strains prepared on KB agar were streaked across the surface of 0.2× nutrient broth-yeast extract (NBY) (44) agar at a width of 4 mm using a transfer loop. After overnight incubation at 24°C, 3.5 μl of a suspension containing 300 *N. americana* cysts $\mu l^{-1}$ was spotted at one end of the linear bacterial growth, and the plates were incubated at 24°C; there were 15 replicates for each strain at the temperature used. Migration of *N. americana* trophozoites along the confluent bacterial growth was monitored over a period of 6 days.

**CLP antiprotozoan activity.** The CLPs massetolide A and viscosin were extracted from cultures of strains SS101 and SBW25 and purified by reverse-phase high-performance liquid chromatography using the method described previously (11, 14). The individual purified surfactants were resuspended in PAS to obtain final concentrations of 1, 10, 25, 100, and 500 μg $ml^{-1}$. A 5-μl aliquot of an *N. americana* trophozoite suspension was added to 50 μl of a CLP suspension, and the amoebae were monitored microscopically (magnification, ×100) for up to 1 h.

**Effect of *N. americana* feeding on CLP gene transcription.** Initial assays employed *N. americana* and mutant strain 10.24. The feeding assay was conducted as described above using agar plates supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to visualize β-galactosidase activity in the bacterial cells. The protozoan predator (5 μl of a suspension containing 100 cysts $\mu l^{-1}$) was spotted at one end of the linear confluent growth of the bacterium. Cultures were examined after 72 h of incubation at 28°C for development of a blue color indicative of transcription of the *lacZ* gene.

In subsequent assays, bacterial cells were prepared to monitor CLP gene expression by real-time quantitative PCR (Q-PCR) as previously described (11). CLP-producing strains SS101 and SBW25 were cultured in linear bands on 0.2× NBY agar using four replicate 4-mm-wide bands per agar plate. Cultures were incubated at 28°C for 3 h, and then a 5-μl spot of an *N. americana* suspension (100 cysts $\mu l^{-1}$) was placed at one end of each bacterial band. After 48 h of incubation at 28°C, bacterial cells were collected from the entire 4-mm-wide bacterial culture using an RNase-free pipette tip at locations 0, 1, and 4 cm from the *N. americana* trophozoite feeding margin. RNA was extracted from cells using a Qiagen RNAeasy kit according to the manufacturer's protocol or as described by De Bruijn et al. (11). cDNA was synthesized as previously described (11). The primers used in Q-PCR are listed in Table 1. Q-PCR analysis was performed in duplicate (technical replicates) for three independent RNA isolations (biological replicates) and repeated for three sets of feeding assays. Transcript levels of the CLP biosynthesis genes were corrected for the transcript levels of the housekeeping gene *rpoD* by using the formula $\Delta C_T = C_T(\text{CLP gene}) - C_T(rpoD)$ and were expressed relative to the transcript level of cells obtained 4 cm from the feeding front by using the formula fold change = $2^{-[\Delta C_T(0 \text{ or } 1 \text{ cm}) - \Delta C_T(4 \text{ cm})]}$, where $C_T$ is the cycle threshold. Statistically significant differences were determined for log-transformed fold change values by analysis of variance ($P < 0.05$), followed by Bonferroni post hoc multiple comparisons.

**Effect of CLP production on bacterium-protozoan interaction in soil.** Bacterial cultures were scraped from the surface of a KB agar plate and resuspended in sterile distilled water, and the concentration was adjusted to approximately $5 \times 10^9$ CFU $ml^{-1}$. Cells were dispensed into the chamber of a 50-ml chromatography sprayer and applied as an atomized mist to individual 1.4-kg samples of soil (Adkins very fine sandy loam; pH 7.6) that had been pasteurized by exposure to steam (100°C) for 3 h prior to inoculation. *N. americana* cysts were collected from water agar plates, and the concentration was adjusted to 200 cysts $\mu l^{-1}$ in PAS. The cyst suspension was applied to the soil using a 50-ml spray bottle. Numbers of *N. americana* were estimated using the most-probable-number method (1), and the concentration was adjusted to obtain an initial protozoan population of approximately $10^4$ organisms $g^{-1}$ soil.

TABLE 1. Primers used in Q-PCR analysis

| Gene | Orientation | Primer sequence |
|---|---|---|
| *massA* | Forward | 5′-GCTGTACAACATTGGCGGCT-3′ |
| | Reverse | 5′-GGTATGCAGTTGAGTGCGTAGC-3′ |
| *massA2* | Forward | 5′-GCGCGATCAAGGTTTCCA-3′ |
| | Reverse | 5′-CGCCTCGTTGTAGACGCAAT-3′ |
| *massB* | Forward | 5′-AACAACGACCGGAGATGCC-3′ |
| | Reverse | 5′-AAGGTGTGCAGCAAGTGATGG-3′ |
| *massC* | Forward | 5′-GTCGACCCTCAACGCGTCT-3′ |
| | Reverse | 5′-CCACCGACAGTTGGTCAAGC-3′ |
| *viscA* | Forward | 5′-CCGGATGGCAATCTTGAGTTT-3′ |
| | Reverse | 5′-GTGACTCGATTTCCCCCAGTT-3′ |
| *viscB* | Forward | 5′-ATCCGTGGCCTGCGTATC-3′ |
| | Reverse | 5′-CCTTGACCGATGCGTGTTC-3′ |
| *viscC* | Forward | 5′-CGGACCTCTTGAGCTTTATCGA-3′ |
| | Reverse | 5′-AGAATCACTGCGTCGTGACAAC-3′ |
| *rpoD* | Forward | 5′-GCAGCTCTGTGTCCGTGATG-3′ |
| | Reverse | 5′-TCTACTTCGTTGCCAGGGAATT-3′ |

Treated soils were decanted into tapered tubes (20.5 cm by 4 cm [top diameter]) using 100 g of soil per tube and 12 replicates per treatment arranged in a complete randomized design. Assays were conducted in environmental growth chambers with a 16-h photoperiod and with day and night temperatures of 24 and 18°C, respectively, for 2 or 4 weeks. At weekly intervals a 1-g soil sample was collected at a depth of 3 to 5 cm from six tubes for each soil treatment. For determination of populations of SS101, 10.24, SBW25, and 17A8, the 1-g sample was resuspended in 10 ml sterile distilled water and vortexed for 60 s, and serial dilutions were plated onto KB agar amended with rifampin (100 μg $ml^{-1}$) and cycloheximide (75 μg $ml^{-1}$). For 10.24/p*massA*, serial dilutions were plated on the same medium supplemented with tetracycline (10 μg $ml^{-1}$).

Alternatively, soils were treated as described above and decanted into conical tubes, and two wheat seeds (*Triticum aestivum* cv. Eltan) were planted in each tube, using 12 replicates per soil treatment arranged in a complete randomized design. The wheat rhizosphere populations of the introduced bacterial strain and *N. americana* were determined at 2-week intervals. Six tubes were randomly selected for each bacterial treatment. Wheat plants were removed and shaken to remove loosely attached soil from the roots, and two 6-cm-long root segments were excised from one plant. Root segments were placed in 10 ml of sterile PAS and vortexed for 60 s. Numbers of bacteria were determined by plating serial dilutions on selective media as described above. To estimate the numbers of *N. americana* in the same samples, three 1-ml aliquots of each serial dilution were combined with 0.02× Trypticase soy broth in 24-well microtiter plates. After 7 days of incubation at 20°C, the contents of each well were examined microscopically at a magnification of ×100 for the presence of *N. americana*, and the numbers of *N. americana* were estimated by the most-probable-number method. Bacterial rhizosphere populations were determined by plating serial dilutions as described above. Root dry weight was determined after processing, and the numbers of bacteria and *N. americana* per gram of root were recorded.

Data were analyzed using SigmaStat (version 3.1; Systat Software Inc., Point Richmond, CA). Soil and rhizosphere population data were $\log_{10}$ transformed before an analysis of variance was conducted, and mean separation was performed using the Student-Newman-Keuls method.

## RESULTS

**Growth of *N. americana* on *P. fluorescens*.** *N. americana* trophozoites exhibited limited or no migration along linear growth of wild-type CLP-producing strains SS101 and SBW25 (Fig. 1A and 1B). *N. americana* exhibited enhanced feeding on

TABLE 2. Time to lysis of *N. americana* trophozoites in response to the CLPs viscosin and massetolide

| Concn of CLP (μg/ml) | Time to cell lysis (min) with[a]: | |
|---|---|---|
| | Viscosin | Massetolide |
| 1 | NL | NL |
| 10 | 14.4 | NL |
| 25 | 3.5 | 9.4 |
| 100 | 1.1 | 3.5 |
| 500 | 0 | 0 |

[a] NL, no cell lysis within 1 h; 0, immediate cell lysis.

CLP-deficient mutants 10.24 and 17A8 compared to the feeding on the corresponding wild-type strains, and trophozoites migrated more than 35 mm along confluent growth of 10.24 and 17A8 during 6 days of incubation (Fig. 1C). Genetic complementation of the *massA* mutation in strain 10.24 by introduction of the stable pME6031-based plasmid p*massA* restored resistance to feeding by *N. americana*, and no trophozoite migration was observed on confluent growth of 10.24/p*massA* (Fig. 1C). At 6 days postinoculation, *N. americana* cysts were typically detected at the point of inoculation on confluent growth of SS101, 10.24/p*massA*, or SBW25 but were never detected at the point of inoculation on confluent growth of CLP-deficient strains 10.24 or 17A8.

**Response of *N. americana* to bacterial CLPs.** When trophozoites were exposed to purified viscosin, lysis of the trophozoites occurred rapidly and in a concentration-dependent manner (Table 2). With 500 μg $ml^{-1}$ viscosin, cyst germination was observed intermittently, and cell lysis followed immediately. Also, purified massetolide (Table 2) or cell suspensions of SS101 (Fig. 2A to D) caused death of *N. americana*, but cell lysis was delayed compared to that observed with viscosin (Table 2). Trophozoite lysis was not detected at a massetolide concentration of 10 μg $ml^{-1}$; however, protozoan feeding ceased after 27 min and encystment was initiated. Collectively, these results show that both CLPs have lethal effects on *N. americana* and indicate that small structural differences between these two CLPs (e.g., differences in the fourth amino acid of the peptide moiety [Fig. 1A]) affect their antiprotozoan activities.

**Exposure to *N. americana* results in enhanced expression of the *mass* and *visc* genes.** In mutant 10.24, transcription of the promoterless *lacZ* gene located on the transposon is under the control of the *massA* promoter. Hence, mutant 10.24 can be used as a reporter strain for *massA* gene expression. The results of the *Naegleria-Pseudomonas* plate assays showed that there were increased levels of β-galactosidase activity in mutant 10.24 at the feeding margin (Fig. 3A) but not at other edges or in any other part of the bacterial colony where protozoans were not present, including the opposite end (margin) of the linear bacterial growth approximately 4 cm from the feeding margin. To further investigate whether interaction with actively feeding *N. americana* trophozoites triggers CLP gene expression, Q-PCR analysis was performed with cells of wild-type strains SS101 and SBW25 collected at different distances from the protozoan feeding margin. Simultaneously, the presence of *N. americana* along the bacterial colony was determined microscopically (magnification, ×100), and the results showed that trophozoites were present at the feeding margin (0 cm) but were not present at the 1-cm sampling point or at the 4-cm sampling position, which included the opposite edge of the linear bacterial culture. Quantitation of transcript levels by Q-PCR demonstrated that the feeding activity of *N. americana* indeed modulated expression of all three NRPS genes governing CLP biosynthesis in *P. fluorescens* strains SS101 (Fig. 3B) and SBW25 (Fig. 3C). In both bacterial systems, NRPS gene expression was significantly higher in cells that were collected closest to *N. americana* (Fig. 3B and 3C). Bacterial cells collected at the trophozoite feeding margin exhibited *mass* and *visc* expression levels that were up to 3.5-fold higher than those in corresponding cells collected at 4 cm, where no protozoans were detected; similar results were obtained in two independent experiments. Cells collected at a point 1 cm from the protozoan feeding front exhibited lower and more variable levels of gene expression than cells collected at 0 cm, but the levels were higher than those in cells collected at 4 cm (Fig. 3B and 3C).

**Effect of CLP production on survival of *P. fluorescens* and *N. americana* in soil.** In the absence of *N. americana*, soil populations of mutant 10.24 were not significantly different from parental strain SS101 soil populations (Fig. 4A). In the presence of *N. americana*, however, the survival of mutant 10.24 was compromised compared to that of the parental strain; SS101 persisted in soil at a significantly ($P = 0.005$) higher density than 10.24, and the density was nearly an order of magnitude higher after 4 weeks (Fig. 4A). In assays conducted in the presence of *N. americana*, the complemented strain 10.24/p*massA* persisted in soil at densities similar to those of

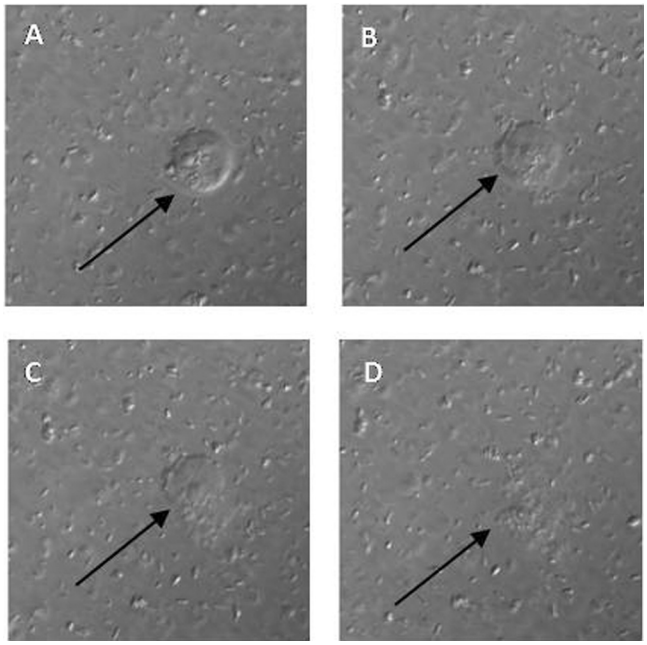


FIG. 2. Video microscopic frames (magnification, ×400) demonstrating *N. americana* trophozoite lysis in the presence of *P. fluorescens* SS101 ($10^8$ CFU $ml^{-1}$). Lysis is shown in a series of frames in sequence from panel A to panel D and occurred within a period of 10 min. A similar response was observed in the presence of strain SBW25 or purified CLPs massetolide and viscosin.

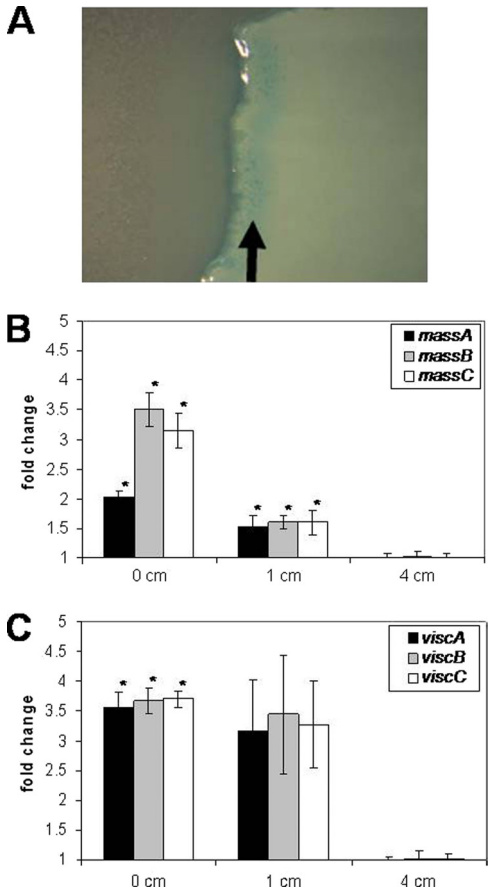


FIG. 3. In vitro assay conducted with *N. americana* and *P. fluorescens* 10.24. In mutant 10.24, transcription of the promoterless *lacZ* gene is under the control of the *massA* promoter. (A) Assay conducted on 0.2× NBY agar plate supplemented with X-Gal to visualize β-galactosidase activity in the bacterial cells. *N. americana* was spotted on confluent growth of the bacterium and grazed from left (cleared) to right. The enhanced blue color just behind the protozoan feeding margin (arrow) is indicative of transcriptional activity of the *lacZ* gene. Elevated β-galactosidase activity was observed only at the edge of the colony where the protozoans were feeding and not at other edges or in other parts of the colony where no protozoans were present. (B) Transcript levels of *massA*, *massB*, and *massC* in cells of wild-type strain SS101 collected at locations 0, 1, and 4 cm from the feeding front of *N. americana* trophozoites incubated at 24°C on 0.2× NBY agar for 72 h. (C) Transcript levels of *viscA*, *viscB*, and *viscC* in strain SBW25. Transcript levels of the CLP biosynthesis genes were corrected for the transcript levels of the housekeeping gene *rpoD* by using the formula $\Delta C_T = C_T(\text{CLP gene}) - C_T(rpoD)$ and were expressed relative to the transcript level of cells obtained 4 cm from the feeding front by using the formula fold change $= 2^{-[\Delta C_T(0 \text{ or } 1 \text{ cm}) - \Delta C_T(4 \text{ cm})]}$. For each distance, mean values are given for two biological replicates, each performed in duplicate. The error bars represent the standard errors of the means. An asterisk indicates a statistically significant ($P < 0.05$) difference from results for samples taken at 4 cm.

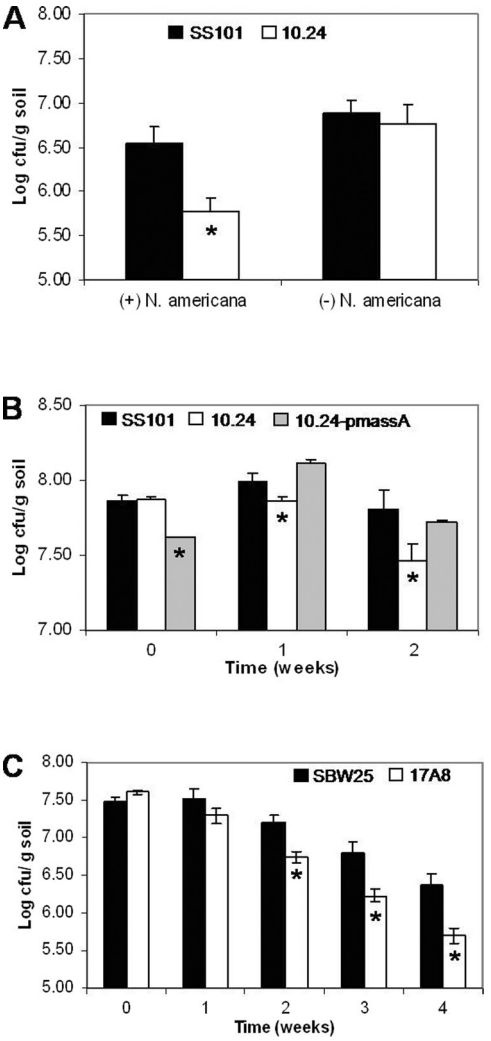


FIG. 4. Interactions between *N. americana* and *P. fluorescens* SS101, *P. fluorescens* SBW25, CLP-deficient mutants 10.24 and 17A8, and genetically complemented mutant 10.24/p*massA*. *N. americana* and bacterial strains were established in soil at initial population densities of approximately $10^4$ cysts $g^{-1}$ soil and $3 \times 10^7$ CFU $g^{-1}$ soil, respectively. (A) Soil populations of *P. fluorescens* SS101 and its CLP-deficient mutant in the presence [(+) *N. americana*] and absence [(−) *N. americana*] of *N. americana* 4 weeks after inoculation of the soil. (B) Population dynamics of *P. fluorescens* SS101, its CLP-deficient mutant 10.24, and the genetically complemented mutant 10.24/p*massA* in soil enriched with *N. americana*. (C) Populations of *P. fluorescens* SBW25 and its CLP-deficient mutant 17A8 in soil enriched with *N. americana*. The error bars represent the standard errors of the means. For each sampling time, an asterisk indicates a mean value significantly ($P < 0.05$) different from the value for the wild-type strain.

SS101 and significantly higher than those of mutant 10.24 at both the 1-week ($P = 0.027$) and 2-week ($P = 0.011$) sampling times (Fig. 4B). Similarly, wild-type strain SBW25 persisted at densities that were significantly higher than those of mutant 17A8 in *N. americana*-inoculated soils (Fig. 4C).

Bacterial populations in the wheat rhizosphere exhibited trends that were consistent with those observed in soil. In the presence of *N. americana*, CLP-producing strain SS101 persisted in the rhizosphere at densities significantly ($P < 0.05$) higher than those of mutant 10.24 at both the 2- and 4-week sampling times (Fig. 5A). When established at densities of $10^6$ to $10^7$ CFU $g^{-1}$ root, the introduced bacterial strains had differential effects on the persistence of *N. americana* in the rhizosphere of wheat. The numbers of *N. americana* in the wheat rhizosphere were significantly lower in the presence of strain SS101 than in the presence of mutant 10.24 (Fig. 5B). The *N. americana* populations in the rhizosphere of wheat

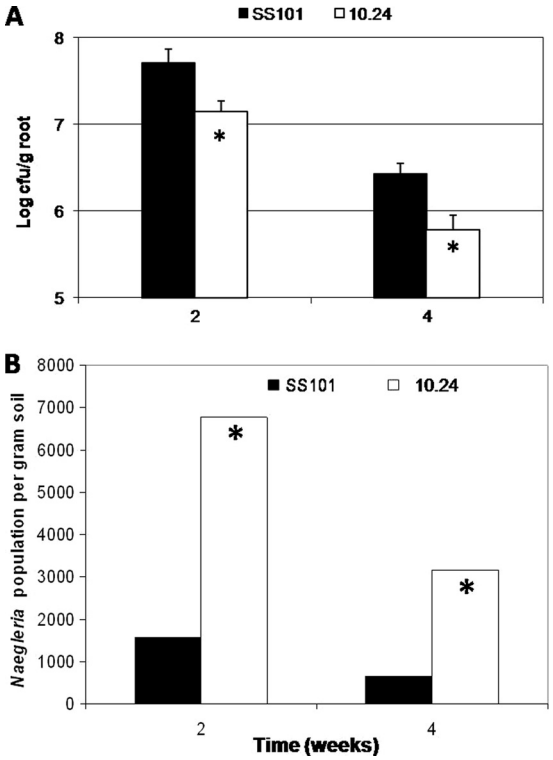


FIG. 5. Interaction between *N. americana* and *P. fluorescens* SS101 or CLP-deficient mutant 10.24 in the rhizosphere of wheat. *N. americana* and the bacterial strains were established in soil at initial population densities of approximately $10^4$ cysts $g^{-1}$ soil and $3 \times 10^7$ CFU $g^{-1}$ soil, respectively. (A) Rhizosphere populations of *P. fluorescens* SS101 and its CLP-deficient mutant in the presence of *N. americana* at 2 and 4 weeks after introduction. (B) Populations of *N. americana* in the wheat rhizosphere in the presence of SS101 or CLP-deficient mutant 10.24. For each sampling time, an asterisk indicates a mean value significantly ($P < 0.05$) different from the value for the wild-type strain.

were significantly smaller in the presence of SBW25 than in the presence of mutant 17A8 at both the 2- and 4-week sampling times, and the magnitude of the difference was similar to that observed for SS101 and its CLP-deficient mutant, strain 10.24 (data not shown).

## DISCUSSION

Protozoan predation puts significant pressure on bacterial survival and imposes an important selective force on bacterial community dynamics, structure, and evolution in soil ecosystems (6). Bacteria employ various strategies to evade protozoan predation, including production of secondary exometabolites possessing antibiotic and cytotoxic properties (23, 24, 31). However, a role for specific individual metabolites of *Pseudomonas* in attenuating protozoan grazing in soil habitats has not been demonstrated previously. Furthermore, the role of predation-induced activation of genes controlling the production of antiprotozoan compounds is largely unexplored. Our results show that CLPs of soil-dwelling *P. fluorescens* strains provide protection against protozoan grazing and demonstrate that protozoan-*Pseudomonas* interactions can lead to enhanced transcription of CLP biosynthesis genes.

Compared to the corresponding CLP-deficient mutants, *P. fluorescens* strains SS101 and SBW25 exhibited resistance to protozoan grazing in vitro and superior persistence in soil when they were confronted with the bacterivorous protozoan *N. americana*. The enhanced survival of *P. fluorescens* CHA0 in the presence of *Acanthamoeba castellanii* compared to the survival of an isogenic *gacS* mutant of this bacterium was attributed to increased susceptibility of the regulatory mutant to protozoan predation (24). However, *gacS* mutations affect a myriad of biosynthetic pathways, and CHA0 mutants that do not produce exometabolites were shown to have a compromised capacity to colonize the rhizosphere even in the absence of the protozoan predator (24, 26). In contrast, our findings demonstrate that the enhanced survival of SS101 and SBW25 compared to the survival of the corresponding CLP biosynthesis mutants in situ is a direct consequence of a superior ability to avoid predation rather than simply diminished colonization capacity of the mutant strains. This conclusion is supported by observations in this study that CLP-producing and CLP-deficient strains typically survived at similar densities in assays conducted in the absence of *N. americana*. These results suggest that the production of CLPs is targeted primarily toward predators and is less important in competition with other (micro)organisms in soil and rhizosphere environments. This observation is consistent with previous work (35) which showed that strain SS101 and the CLP-deficient mutant 10.24 exhibited similar levels of activity against indigenous root-infecting *Pythium* species, indicating that massetolide did not play a major role in toxicity against this group of coexisting soil inhabitants.

The ecological relevance of CLP production by SS101 and SBW25 in interactions with susceptible bacterivores such as *N. americana* is likely to differ in a temporal manner from the ecological relevance of other bacterial toxins or antibiotics. For *P. fluorescens* strain CHA0, it was suggested that interactions with the protozoan predator *A. castellanii* are of minor importance during early stages of rhizosphere colonization (24). For *gacS*-regulated secondary metabolite production (20, 27), the temporal nature of this response would be expected. In contrast, CLP production in *P. fluorescens* strains SS101 and SBW25 occurs in the early exponential growth phase (11, 12), and results of this study showed that the difference in persistence between the wild-type strains and corresponding CLP-deficient mutants in soil did not tend to expand beyond the second sampling period at 2 weeks. Thus, it appears that the temporal nature of the contribution of bacterial exometabolites as a survival mechanism, and specifically as a means to evade protozoan predation, may vary in a metabolite-specific manner.

In soil and rhizosphere assays, the population densities of the introduced bacterial strains were approximately $10^6$ to $10^7$ CFU per g of root or soil, which are comparable to the densities of indigenous populations of fluorescent pseudomonads. With regard to CLP concentrations and in situ production levels, there are some inherent technical difficulties in detecting massetolide and viscosin in complex soil environments (43). In vitro, massetolide and viscosin are produced at concentrations of approximately 0.2 μg per $10^7$ cells. Work by Nielsen and Sørensen (36) on structurally related CLPs (amphisin and tensin) showed similar levels of production in soil (approxi-

mately 0.3 μg per g of soil at a *Pseudomonas* population density of $10^7$ cells $g^{-1}$ soil). Also, work by Asaka and Shoda (2) on *Bacillus* showed in situ production levels of the CLPs iturin A and surfactin of 0.5 to 4.4 μg $g^{-1}$ soil at bacterial densities of $10^7$ to $10^8$ cells per g of soil. Collectively, these results suggest that the cell densities used in our soil assays are biologically relevant and that, based on studies with structurally related CLPs, these metabolites are produced in situ. Whether massetolide and viscosin reach in situ concentrations high enough to kill protozoans depends on the spatial heterogeneity of the bacterial populations; at microsites where bacterial densities are high, CLPs may also reach concentrations high enough for protection against protozoan predators.

Based on genome and biochemical analyses (5, 10, 15, 19, 38), the production of CLPs appears to be a common trait in soil-inhabiting *Pseudomonas* and *Bacillus* species. The CLPs produced by these bacterial genera exhibit considerable structural diversity, including differences in the number, nature, and configuration (L or D) of the amino acids in the macrocyclic peptide ring, as well as in the type and length of the fatty acid tail (16, 38, 39). How these differences in structural features affect the activity against protozoan grazing or determine the spectrum of target protozoan species remains to be addressed. Nevertheless, previous studies by Gerard et al. (17) showed that the antimycobacterial activities of viscosin and massetolide differed and that viscosin was twofold less active against *Mycobacterium tuberculosis* and *Mycobacterium avium-Mycobacterium intracellulare* than massetolide A. Conversely, in this study, the in vitro antiprotozoan activities of these two CLPs varied, and *N. americana* was more sensitive to viscosin than to massetolide.

Although this study was confined to examination of a single predator species, our findings may have implications for ecosystem function and overall plant health. Protozoans have direct and indirect effects on N mineralization (13), root proliferation (7, 22), and microbial community composition and function (6). Introduction of CLP-producing rhizobacteria for disease control could have the undesired effect of diminishing N availability, particularly in production systems relying on organic N sources, resulting in diminished plant productivity. Alternatively, protozoan predation may trigger enhanced production of CLPs, thereby indirectly promoting plant growth via enhanced activity against deleterious soilborne pathogenic fungi and oomycetes.

Exposure to *N. americana* resulted in altered gene expression in both *P. fluorescens* strains, leading to upregulation of genes involved in the biosynthesis of metabolites that confer protection against predation. Consistent with recent work on amoeba-induced transcriptional changes in the human pathogen *Pseudomonas aeruginosa* (34), our study documents that soil-dwelling bacterium-protozoan interactions can result in activation of an antipredator response in prey populations. Potential signals resulting in upregulation of CLP genes in *P. fluorescens* were not determined; however, this does not appear to require physical contact as bacterial cells 1 cm from *N. americana* also exhibited enhanced gene expression. Thus, the potential outcome of the *Pseudomonas-Naegleria* interaction does not inevitably require intimate contact, as reported previously for the killing effect of biofilm-associated *P. aeruginosa* with *A. castellanii* (34). Considering the importance of CLPs in bacterial motility and biofilm formation, our study highlights the functional versatility of these compounds and provides novel insights into the natural functions, regulation, and evolution of antiprotozoan genes and compounds in environmental bacteria. Understanding the mechanisms underlying soil bacterium-protozoan interactions can also be instrumental in development of new strategies to control human- and animal-pathogenic protozoans.

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