

Suppression of damping-off of cucumber caused by *Pythium ultimum* with live cells and extracts of *Serratia marcescens* N4-5

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Abstract

Environmentally friendly control measures are needed for the soil-borne pathogen, *Pythium ultimum*. This pathogen can cause severe losses to field- and greenhouse-grown cucumber and other cucurbits. Live cells and ethanol extracts of cultures of the bacterium *Serratia marcescens* N4-5 provided significant suppression of damping-off of cucumber caused by *P. ultimum* when applied as a seed treatment. Live cells of this bacterium also suppressed damping-off caused by *P. ultimum* on cantaloupe, muskmelon, and pumpkin. Culture filtrates from strain N4-5 contained chitinase and protease activities while ethanol extracts contained the antibiotic prodigiosin, the surfactant serrawettin W1, and possibly other unidentified surfactants. Production of prodigiosin and serrawettin W1 was temperature-dependent, both compounds being detected in extracts from N4-5 grown at 28 °C but not in extracts from N4-5 grown at 37 °C. Ethanol extracts from strain N4-5 grown at 28 °C inhibited germination of sporangia and mycelial growth by *P. ultimum* in *in vitro* experiments. There was no *in vitro* inhibition of *P. ultimum* associated with ethanol extracts of strain N4-5 grown at 37 °C. Prodigiosin, purified from two consecutive thin-layer chromatography runs using different solvent systems, inhibited germination of sporangia and mycelial growth of *P. ultimum*. Another unidentified compound(s) also inhibited germination of sporangia but did not inhibit mycelial growth. There was no *in vitro* inhibition associated with serrawettin W1. These results demonstrate that live cells and cell-free extracts of *S. marcescens* N4-5 are effective for suppression of damping-off of cucumber caused by *P. ultimum* possibly due in part to the production of the antibiotic prodigiosin.

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1. Introduction

Soil-borne pathogens such as the Oomycete, *Pythium ultimum* and the plant-parasitic nematode *Meloidogyne*

incognita can cause significant losses to field- and greenhouse-grown cucumbers and other cucurbits (Zitter et al., 1996; Koenig et al., 1999). Diseases caused by these soil-borne pathogens are typically controlled with strategies employing cultural methods and chemical pesticides (Zitter et al., 1996). There is considerable interest in finding alternatives to chemical pesticides for suppression of soil-borne pathogens due to environmental and health concerns associated with their use (Larkin et al., 1998; Raupach and Kloepper, 1998; Whipps, 2001). Some pesticides such as

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the broad-spectrum chemical fumigant methyl bromide are being phased out from agricultural use (Martin, 2003). A strategy currently being investigated as a means to reduce the use of chemical pesticides is biological control, applied alone or as a component of an integrated pest management strategy (Larkin et al., 1998; Raupach and Kloepper, 1998; Whipps, 2001). Toward this end, we screened a number of fungi and bacteria for suppression of damping-off on cucumber caused by *P. ultimum* and for suppression of *M. incognita* (Roberts et al., 2005). Isolates of the Gram-negative bacterium *Serratia marcescens* were found to be particularly effective in suppression of damping-off of cucumber caused by *P. ultimum* in our disease suppression assays.

S. marcescens is ubiquitous in the environment and has been detected in association with mammals, water, and plants (Grimont and Grimont, 1992) and strains of *S. marcescens* have been reported to have biological control potential (e.g. Kobayashi and El-Barrad, 1996; Press et al., 1997; Someya et al., 2000; Roberts et al., 2005). Unfortunately, certain isolates of *S. marcescens* are considered opportunistic human pathogens, being frequently encountered with a number of debilitating diseases (Grimont and Grimont, 1992; Hejazi and Falkiner, 1997). These isolates can be problematic in hospital settings because of multidrug resistance (Traub, 2000; Stock et al., 2003). It is unlikely that live isolates of *S. marcescens* will be registered for use in the near future as biological control agents for plant diseases due to the perceived risks to human health associated with this bacterium.

A number of inhibitory molecules are produced by *Serratia* spp. including pyrrolnitrin, oocycin A, carbapenem, prodigiosin, and the surfactant serrawettin as well as chitinase and other cell-wall and cell-membrane degrading enzymes (Lindum et al., 1998; Asano et al., 1999; McGowan et al., 1999; Strobel et al., 1999; Kamensky et al., 2003). It is interesting to speculate that cell-free extracts of *S. marcescens*, containing some of these inhibitory molecules, could suppress disease caused by pathogens of cucumber. The benefit of this strategy would be suppression of disease without the perceived risks to human health associated with live cells of *S. marcescens*. The objective of this study was to test the feasibility of this strategy by (1) demonstrating suppression of damping-off of cucumber and other cucurbits caused by *P. ultimum* with live cells of *S. marcescens* N4-5 and (2) by demonstrating effective suppression of damping-off of cucumber caused by *P. ultimum* with seed treatments containing cell-free extracts of *S. marcescens* N4-5.

2. Materials and methods

2.1. Bacteria and oomycete isolates

Strain N4-5 was isolated by baiting and identified as *S. marcescens* (Kobayashi and El-Barrad, 1996).

A spontaneous, rifampicin-resistant mutant of N4-5 was isolated by standard methods (Miller, 1972). This rifampicin-resistant mutant, designated N4-5R2, was similar in growth rate in Nutrient Broth (NB) and in colony morphology with N4-5. *S. marcescens* isolates ATCC 274 and ATCC 8100 were obtained from the American Type Culture Collection (Manassas, VA) for use as controls. *S. marcescens* ATCC 274 produces the compounds prodigiosin and serrawettin W1 while *S. marcescens* ATCC 8100 does not produce either compound (Burger and Bennett, 1985; Matsuyama et al., 1986; Sunaga et al., 2004). *Pseudomonas fluorescens* PF-5 and *Burkholderia ambifaria* BC-F from the Sustainable Agricultural Systems Laboratory culture collection (SASL; USDA-ARS, Beltsville, MD) produce pyrrolnitrin (Roberts and McKenna, unpublished) and were used as positive controls. *P. ultimum* was from the SASL culture collection.

2.2. Confirmation of identity of *Serratia marcescens* N4-5

The identity of strain N4-5 was confirmed from gas chromatographic profiles of cellular fatty acids using the MIDI system (Microbial ID, Inc., Newark, DE) and by sequencing a portion of the 16S rDNA gene. For DNA sequencing analysis, genomic DNA was extracted as described by de Souza et al. (2003) and the 16S rDNA gene amplified by PCR as described by Roberts et al. (2005). Primers for PCR amplification were 8fn (5'-AGA GTT TGA TCT GGC TCA G-3') and 1429r (5'-ACG GCT ACC DTT GTT ACG ACT T-3') (Esikova et al., 2002). PCR products were purified and sequenced as described (Roberts et al., 2005). The LASERGENE (DNASar Inc., Madison, WI) sequence analysis software package and BLAST software package (Altschul et al., 1997) were used for DNA sequence analysis. Nucleotide sequence of 1425 bp of the 16S rDNA gene has been deposited in GenBank under accession number EF035134.

2.3. Preparation of cell-free extracts and culture filtrates

Unless indicated otherwise, cell extracts were prepared from strains grown on Peptone Glycerol (PG) agar plates for 3 d at 28 °C or at 37 °C, the cell mass on PG agar plates extracted with ethanol (10 ml per plate) (Matsuyama et al., 1985), the extract centrifuged at 8000g for 10 min, and the supernatant evaporated to dryness under nitrogen. Strains grown on PG agar plates for 3 d at 28 °C were also extracted individually with ethyl acetate, hexane, or chloroform + methanol (2:1; vol:vol). For this, cell mass from PG agar plates was suspended in sterile distilled water (SDW; 10 ml per plate) and extracted with an equal volume of solvent. Controls were SDW extracted with the organic solvent. All extracts were dried under nitrogen and the residue resuspended as indicated prior to use. Pilot experiments demonstrated that there were no live cells of strain N4-5 in these extracts.

For preparation of culture filtrates, strain N4-5 was grown for 2 d at 22 °C with shaking at 250 rev min⁻¹ in NB or in M56 basal salts (M56) broth (Carlton and Brown, 1981) plus 0.2% glycerol, 0.2% carboxymethylcellulose (CMC), chitin, or laminarin, or M56 basal salts broth plus 0.2% glycerol plus 0.2% CMC, chitin, or laminarin. Cultures were centrifuged at 6000g for 10 min and passed through a 0.2 µm filter. Culture filtrates were stored at -20 °C until used.

2.4. Suppression of damping-off of cucurbits caused by *P. ultimum*

S. marcescens N4-5 was grown overnight in NB, washed and resuspended in SDW, and incorporated into treatments of cantaloupe (*Cucumis melo* cv. Ambrosia), cucumber (*Cucumis sativum* cv. Marketmore 76), muskmelon (*Cucumis melo* cv. Hales Best Jumbo), and pumpkin (*Cucurbita maxima* cv. Baby Bear) seeds in a gelatin formulation as described (Roberts et al., 2005). Seeds coated with gelatin and SDW, but no bacteria, were used as controls. Bacterial cell densities in seed treatments were determined by dilution-plating. Seeds treated with strain N4-5 contained approximately 8.0 log₁₀ CFU N4-5 seed⁻¹. Dried ethanol extracts of strain N4-5, prepared from PG agar plates incubated at 28 °C, were resuspended in 8 ml ethanol, ethyl acetate, acetone, methanol, or SDW and these suspensions incubated with cucumber seeds for 30 s (8 ml [6.4 g cucumber seeds]⁻¹). Cucumber seeds were also incubated in these solvents without cell extract as controls. Treated cucumber seeds were dried under a laminar flow hood.

To produce sporangia, *P. ultimum* was grown at 25 °C for 3 d, flooded with soil extract (Ayers and Lumsden, 1975), and incubated at 25 °C for 7–28 d. Sporangia from these plates were washed and incorporated into soil-less mix (Pro-Mix PGX, Premier Horticulture, Inc., Quakertown, PA). Soil-less mix, soil-less mix amended with sporangia of *P. ultimum* or with SDW, treated seeds, and soil-less mix amended with sporangia of *P. ultimum* or SDW, were added as sequential layers to 6-cm-diameter cups as described by Roberts et al. (1997). For each treatment, eight replicate cups were sown with five seeds each and incubated in a growth chamber at 22 °C for 14 d with a 12 h photoperiod. Treatments were arranged in a completely randomized design. Mean plant stand per cup was determined, analysis of variance (ANOVA) carried out, and differences among means estimated using a protected least-significant difference test (SAS, Cary NC). Experiments were performed at least two times with each seed type and analyzed independently.

2.5. Biochemical characterization of culture filtrates and cell extracts from *Serratia marcescens*

2.5.1. Enzyme assays

Culture filtrate (100 µl) was assayed for carboxymethylcellulase (CMCase), chitinase, laminarinase, and protease

activities. For CMCase activity, 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 5.0, was mixed with 0.2% CMC and incubated at 37 °C. For laminarinase activity, 50 mM MES buffer, pH 5.0, was mixed with 0.2% laminarin and incubated at 37 °C. Reducing sugars liberated due to CMCase or laminarinase activities were determined by the method of Nelson (1944) with a glucose standard. One unit of CMCase or laminarinase activity was defined as the amount of enzyme that released 1 µM glucose reducing equivalent min⁻¹ (ml culture filtrate)⁻¹. Chitinase activity was determined by incubation of culture filtrate with 50 mM citrate buffer, pH 5.0, and 0.2% chitin at 37 °C. Liberated *N*-acetylglucosamine equivalents were determined by the method of Reissig et al. (1955). One unit of chitinase activity was the amount of enzyme that released 1 µM *N*-acetylglucosamine equivalent min⁻¹ (ml culture filtrate)⁻¹. Protease activity was determined by incubating culture filtrate in 100 mM potassium phosphate buffer, pH 7.0, plus 0.4% azocoll at 37 °C (Chavira et al., 1984). One unit of protease activity was the amount of enzyme that increased absorbance at 520 nm 1 unit h⁻¹ (ml culture filtrate)⁻¹. Lower limits of detection were 2.3 U for CMCase and laminarinase activities, 0.3 U for chitinase activity, and 0.001 U for protease activity.

2.5.2. Detection of prodigiosin

Cell extracts from *S. marcescens* strains were grown on PG agar, extracted with ethanol, and dried under nitrogen as above. The residue was dissolved in chloroform:methanol (2:1; vol:vol), applied to Silica gel G thin-layer chromatography (TLC) plates, and developed with freshly prepared chloroform:methanol:7 M ammonia (80:25:4; vol:vol) (Matsuyama et al., 1985) or with chloroform:methanol:5 M ammonia (92:12:4; vol:vol). In some experiments, prodigiosin-like spots from preparations from strains N4-5 and ATCC 274 were scraped from TLC plates (developed with chloroform:methanol:5 M ammonia [80:25:4; vol:vol]), the silica gel fines extracted with methanol, acidified methanol, or alkaline methanol, and subjected to a spectral scan with a Beckman DU650 spectrophotometer (Beckman Coulter Inc., Fullerton, CA).

2.5.3. Detection of surfactant

S. marcescens strains were grown on PG agar, extracted with ethanol, and dried as described above. Residue was resuspended in chloroform:methanol (2:1; vol:vol), and applied to Silica gel G TLC plates as described for prodigiosin detection. TLC plates were developed with chloroform:methanol:7 M ammonia (80:25:4; vol:vol) (Matsuyama et al., 1985) or with chloroform:methanol:acetone:acetic acid (90:10:6:1; vol:vol) made fresh prior to each run, and sequentially sprayed with ninhydrin (0.2% in ethanol), molybdenum blue, and charred by heating at 95 °C after spraying with concentrated sulfuric acid. In some experiments, a second Silica gel G TLC plate spotted with the same extracts was developed concurrently within the same TLC tank. This second plate was segmented and

scraped for analysis of surfactant activity but not sprayed with any detection reagent. Purified serrawettin W1 was used in some experiments as a standard. For detection of surfactant activity, silica gel fines from scraped TLC plates were extracted with SDW and tested using the drop-collapse method (Jain et al., 1991). Cell material from cultures grown on PG agar was also tested for surfactant activity. Cell material was suspended in SDW to an OD 540 nm of approximately 0.20 and the drop-collapse method used to determine surfactant activity. CFU in these suspensions were determined by dilution-plating.

2.5.4. Detection of pyrrolnitrin

Strains were grown in NB 3 or 5 d at 30 °C or 37 °C, in Potato Dextrose Broth 2 or 5 d at 22 °C, in M56 basal salts broth 5 d at 22 °C, and on Potato Dextrose Agar at 22 °C for 5 d (Chernin et al., 1996; de Souza and Raaijmakers, 2003). Broth cultures were centrifuged and the pellet resuspended and extracted with sonication (Vibra Cell, Sonics and Materials, Danbury, CT) in ethyl acetate (de Souza and Raaijmakers, 2003). After centrifugation the supernatant was dried under nitrogen and resuspended in ethyl acetate. Cultures grown on Potato Dextrose Agar were extracted with chloroform (Chernin et al., 1996), dried under nitrogen, and resuspended in ethyl acetate. Strains were also grown on PG agar at 28 °C for 3 d, extracted with ethyl acetate, dried under nitrogen, and resuspended in ethyl acetate. Sample extracts and pyrrolnitrin standard (Cat. No. P8861; Sigma Chemical Co., St. Louis, MO) were spotted on Whatman C18 reverse phase TLC plates (VWR Scientific, Inc., West Chester, PA), developed with methanol:acetonitrile:water (1:1:1; vol:vol), dried, and sprayed with diazotized sulfonilic acid (Mahoney and Roitman, 1990).

2.6. Detection of antibiotic biosynthesis genes

For detection of *prnABC* and *prnD*, which function in biosynthesis of pyrrolnitrin, genomic DNA was isolated by standard protocols (Sambrook and Russell, 2001). PCR primers designed for *prnABC* were PRNA-F1 (5' GCT CAA CCA GAT CAA GTT CC) and PRNC-R1 (5' ATT CGC CGA TCG GAA CC) and *prnD* were PRND-F2 (5' CAG TGC CCG TTT CAC CAC T) and PRND-R2 (5' TGC AGG TTC ATC TGC GAC AT). PCR was conducted in 50 µl elongase polymerase enzyme mixture (Invitrogen Corp., Carlsbad, CA) containing 100 ng genomic DNA; 12 mM Tris, pH 9.1; 3.6 mM (NH₄)₂SO₄; 0.3 mM MgSO₄; 200 µM each dNTP; 0.4 µM each primer; and 1 µl elongase enzyme. PCR was performed in a Perkin Elmer GeneAmp PCR System 9600 (PerkinElmer, Wellesley, MA) with denaturation at 94 °C for 1 min for the first cycle followed by 35 cycles of denaturation (94 °C for 30 s), annealing (30 s at 65 °C), extension (3 min at 68 °C) and a final extension (10 min at 68 °C). PCR products were detected on 0.7% agarose run in 1 × TAE and stained with ethidium bromide. For detection of serrawettin W1 and

prodigiosin biosynthesis genes, total genomic DNA was extracted with the DNeasy Tissue Extraction kit (Qiagen Inc., Valencia, CA) from *S. marcescens* isolates grown to stationary phase in NB. PCR primers were designed for *swrW* which encodes serrawettin W1 synthetase (SW2-F3 5'-GCG ACA AAA GCA ATG ACA AA and SW2-R3 5'-GTC GGC GTA TTG TTC CAA CT) and *pigC* which encodes a condensing enzyme (Williamson et al., 2005) functioning in the prodigiosin biosynthetic pathway (PIG-F1 5'-ATT GCA AAA TCG CAT CAA GG and PIG-R1 5'-AGA ACC AGG TTT CGG TGA CG) using GenBank accessions AB193098.2 and AJ833002.1, respectively. PCR was conducted with PuRe Taq Ready-To-Go PCR beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) in 25 µl reactions in a MJ PTC 200 thermal cycler (MJ Research, Inc., Waltham MA). Final concentrations of components in the reaction mix were 50–100 ng genomic DNA, 10 pMole primer set, 200 mM each dNTP, 10 mM Tris (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, and ~2.5 U PuRe Taq DNA polymerase. Cycling conditions for PCR with PIG primers were initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s), extension (72 °C for 30 s) and a final extension (72 °C for 10 s). Conditions for PCR with SW2 primers were similar to prodigiosin PCR except that the extension time for 35 cycles was 50 s. PCR products were resolved by electrophoresis in 0.8% agarose gels using 0.5 × TBE buffer (1 × Buffer is 89 mM Tris, 89 mM boric acid, 2 mM EDTA). Expected PCR products were eluted from ethidium bromide-stained gels using the QIAquick gel-extraction kit (Qiagen, Inc.). Sequencing of PCR DNA was done by the Auburn University Genomics and Sequencing facility (Auburn, AL). Nucleotide sequences were edited with CHROMAS software (<http://www.technelysium.com.au/chromas.html>) and aligned using BCM Search Launcher utilities (<http://searchlauncher.bcm.tmc.edu/>). PCR sequences were identified using the BLAST search utility and GenBank nucleotide data bank from the National Center for Biotechnology Information, Bethesda, MD (<http://www.ncbi.nlm.nih.gov/>). Accession numbers obtained from GenBank for deposited partial nucleotide sequences are EF122074 for *S. marcescens* N4-5 *swrW*, EF122077 for *S. marcescens* ATCC 274 *swrW*, EF122075 for *S. marcescens* N4-5 *pigC*, and EF122076 for *S. marcescens* ATCC 274 *pigC*.

2.7. In vitro inhibition of *P. ultimum*

For *in vitro* inhibition of germination of sporangia of *P. ultimum*, cell extracts (prepared as described above) were resuspended in SDW; combined with a suspension of sporangia of *P. ultimum* in 25 mM MES, pH 5.0, and NB; incubated at room temperature; and germination of sporangia determined as described (Roberts and Lumsden, 1990). For *in vitro* inhibition of mycelial growth of *P. ultimum*, 75 µl culture extract in ethanol or 75 µl culture filtrate was spotted onto 10-mm-diam. sterile filter disks (Whatman #1) located

at the periphery of NB agar plates, and a single 13-mm-diam. plug of *P. ultimum* added to the center of the Petri dish. Petri dishes were incubated 72 h at room temperature and zones of inhibition around the filter disks measured.

Cell extracts were also fractionated by TLC on Silica gel G plates prior to *in vitro* inhibition experiments. Two plates were run concurrently in a single tank and one plate charred with sulfuric acid for visualization of organic compounds. The second plate was segmented into zones using the charred plate as a guide and silica gel fines scraped into microcentrifuge tubes. Silica gel fines were extracted with SDW for experiments determining *in vitro* inhibition of germination of sporangia or ethanol for experiments determining *in vitro* inhibition of mycelial growth. Controls were silica gel fines from regions of the TLC plates not exposed to sample. Experiments were conducted at least twice for all sporangial germination inhibition and mycelial growth inhibition experiments. Treatments were replicated at least twice. Experiments were analyzed independently.

2.8. Root colonization assays

Cucumber seeds, treated with *S. marcescens* N4-5R2 as described for disease suppression assays (approximately $8.0 \log_{10}$ CFU seed⁻¹), were sown in a natural Hatborough loamy sand soil (pH 5.9), in the soil-less mix used for *P. ultimum* disease suppression assays, and in a pasteurized soil (1:7, compost:sand mixture) in 6.5-cm-diam. × 25 cm-deep pots and incubated in a growth chamber at 22 °C with a 12 h photoperiod. Plants were watered from above as needed. At sampling time for experiments determining total populations per root system (32 and 52 d), plants were removed and sampled by cutting the root just below the seed coat. The root and attached planting medium were placed in SDW, sonicated (Model 8210, Branson Ultrasonics Corp., Danbury, CT), and CFU (root system)⁻¹ determined as described by Roberts et al. (1997). Root system fresh weight was determined for each sample and mean \log_{10} CFU (g fresh weight root tissue)⁻¹ with standard deviation determined. The experiment was performed twice with each potting medium with six replicates per treatment. For experiments to determine the distribution of populations of strain N4-5R2 along roots, a section 7-cm long extending from 14 to 21 cm beneath the soil line was excised after 60 d, placed in SDW, sonicated, and populations of N4-5R2 determined by dilution-plating. The experiment was performed two times with six replicates per treatment. Mean \log_{10} CFU (7-cm root section)⁻¹ with standard deviation was determined. All experiments were analyzed independently.

3. Results

3.1. Identity of strain N4-5

Strain N4-5 was isolated and identified as *S. marcescens* by Kobayashi and El-Barrad (1996). Strain N4-5 was

confirmed to be *S. marcescens* after analysis of cellular fatty acids and sequencing approximately 1425 bp of the 16S rDNA gene. Strain N4-5 had a 40.6% match with *S. marcescens* in the MIDI database. A match of 50% or higher is considered valid at the species level while a match of 20% or higher is considered valid at the genus level. There was no match with any other species. Sequence from the 16S rDNA gene of strain N4-5 most closely matched that from *S. marcescens* isolates N1-14, N1-6, AU736, and N4-1 having 99.5%, 99.4%, 99.2%, and 99.1% DNA sequence identity when compared with sequence from the 16S rDNA gene from these strains, respectively (accession numbers AY514433, AY514431, AY043386, and AY514435).

3.2. Suppression of damping-off of cucurbits caused by *P. ultimum*

S. marcescens N4-5, when applied as a seed treatment in a gelatin formulation, provided effective suppression of damping-off on cucumber, cantaloupe, muskmelon, and pumpkin caused by *P. ultimum* (Table 1). In treatments containing strain N4-5 and *P. ultimum*, plant stands for all crop species tested were similar to the healthy check (gelatin-only treatment in the absence of *P. ultimum*) and significantly greater than the pathogen check (gelatin-only treatment in the presence of *P. ultimum*). Similar results were obtained in two repeated experiments for cucumber, cantaloupe, muskmelon, and pumpkin seed types (data not shown). There was no evidence of phytotoxicity due to treatment of seeds with strain N4-5 in any experiment.

Ethanol extracts of cell mass of strain N4-5 grown at 28 °C resuspended in ethanol, ethyl acetate, acetone, or methanol effectively suppressed damping-off of cucumber caused by *P. ultimum* (Table 2). Plant stands with these seed treatments were all significantly greater than the pathogen check (non-treated cucumber seed in the presence of *P. ultimum*) at both levels of pathogen applied and were similar, or in one case significantly greater than, the live N4-5 seed treatment in the presence of *P. ultimum*. Treatments consisting of ethanol extracts resuspended in ethanol, ethyl acetate, or acetone were also similar to the healthy check (non-treated cucumber seed in the absence of *P. ultimum*). Ethanol extracts of strain N4-5 resuspended in SDW provided minimal disease suppression and only at the higher level of pathogen inoculum. Cucumber seeds treated solely with SDW, ethanol, ethyl acetate, acetone, or methanol as controls did not suppress damping-off of cucumber caused by *P. ultimum*. Similar results were obtained in a second experiment. There was no evidence of phytotoxicity with any treatment in either experiment.

3.3. Biochemical characterization of culture filtrates and cell extracts from strain N4-5

Culture filtrates and cell extracts from strain N4-5 were characterized to identify compounds potentially inhibitory to *P. ultimum*.

Table 1
Suppression of damping-off of cantaloupe, cucumber, muskmelon, and pumpkin caused by *Pythium ultimum* with *Serratia marcescens* N4-5^a

Crop	Treatment ^b	Mean stand pot ^{-1d}	
		Presence of <i>P. ultimum</i> ^c	
		No	Yes
Cantaloupe	Gelatin only	4.63A	0.50B
	N4-5	4.75A	5.00A
Cucumber	Gelatin only	5.00A	0.38B
	N4-5	5.00A	4.40A
Muskmelon	Gelatin only	4.63A	1.75B
	N4-5	4.50A	3.88A
Pumpkin	Gelatin only	4.13A	1.00B
	N4-5	4.88A	4.13A

^aResults from a single experiment for each crop species are presented.

^b*Serratia marcescens* N4-5 was applied in a 10% gelatin formulation at approximately 10^8 CFU seed⁻¹ (Roberts et al., 2005). Gelatin only, gelatin formulation only (no bacteria).

^cNumber of sporangia of *P. ultimum* cm⁻³ applied in the soil-less mix inoculum. For cantaloupe, cucumber, muskmelon, and pumpkin 20, 50, 30, and 30 sporangia cm⁻³ were applied, respectively.

^dNumbers followed by the same letter are not significantly different ($P \leq 0.05$) for that crop species as determined by a protected Least-Significant Difference Test. Least-significant difference ($P \leq 0.05$) for cantaloupe, cucumber, muskmelon, and pumpkin experiments were 1.19, 0.82, 1.29, and 1.00, respectively.

3.3.1. Production of enzymes

Culture filtrates from strain N4-5 contained 0.31 ± 0.04 U chitinase in M56 basal salts broth amended with 0.5% glycerol and 0.5% chitin. Chitinase activity was below detectable limits in culture filtrates from strain N4-5 grown on NB or on M56 basal salts broth amended with 0.5% glycerol. Culture filtrates from strain N4-5 grown on NB, M56 basal salts broth amended with 0.5% glycerol, and M56 basal salts broth amended with 0.5% gelatin contained 1.55 ± 0.09 , 0.24 ± 0.11 , and 0.47 ± 0.05 U protease activity, respectively. No laminarinase or carboxymethylcellulase activity was detected in any culture filtrate tested.

3.3.2. Detection of prodigiosin

Culture extracts from strain N4-5 contained the pigmented compound prodigiosin and production of this compound was temperature-sensitive. The characteristically magenta prodigiosin compound, evident without staining, with approximately the expected R_f (0.96; Dr. T. Matsuyama, personal communication) was detected on Silica gel G plates developed with chloroform:methanol:7 M ammonia (80:25:4; vol:vol) in extracts from strain N4-5 grown on PG agar at 28 °C ($R_f = 0.90$) but not from extracts from N4-5 grown at 37 °C (Fig. 1A). Prodigiosin is not produced by strains of *S. marcescens* grown at 37 °C (Sunaga et al., 2004). This compound in extracts from

Table 2
Suppression of damping-off of cucumber caused by *Pythium ultimum* with extracts of *Serratia marcescens* N4-5

Treatment ^b	Mean plant stand pot ^{-1a}		
	Level <i>Pythium ultimum</i> ^c		
	0	30	100
<i>S. marcescens</i> N4-5 extract in sterile distilled water	4.63ABC	0.13H	2.50EF
<i>S. marcescens</i> N4-5 extract in ethanol	4.50ABC	4.50ABC	4.25ABCD
<i>S. marcescens</i> N4-5 extract in ethyl acetate	4.63ABC	4.25ABCD	3.63BCDE
<i>S. marcescens</i> N4-5 extract in acetone	4.88AB	4.25ABCD	5.00A
<i>S. marcescens</i> N4-5 extract in methanol	4.63ABC	3.50CDE	3.13DE
Sterile distilled water only	4.88AB	0.00H	0.38H
Ethanol only	5.00A	0.00H	0.50GH
Ethyl acetate only	4.63ABC	0.00H	0.13H
Acetone only	4.50ABC	0.63GH	0.13H
Methanol only	4.63ABC	0.63GH	0.88GH
Live <i>S. marcescens</i> N4-5	4.38ABCD	3.50CDE	3.38CDE
No treatment	4.88AB	1.75FG	0.25H

^aNumbers followed by the same letter are not significantly different ($P \leq 0.05$) as determined by a protected Least-Significant Difference Test. Least-significant difference = 1.26.

^bCucumber seeds were incubated with solvents containing ethanol extracts of *S. marcescens* N4-5 or with solvents only and dried prior to sowing. Live *S. marcescens* N4-5 indicates live cells of this bacterium were applied to cucumber seeds at approximately 10^8 CFU seed⁻¹ in a gelatin formulation (Roberts et al., 2005). No treatment indicates that cucumber seeds were not treated prior to being sown.

^cQuantity of sporangia of *P. ultimum* cm⁻³ of soil-less mix inoculum.

strain N4-5 comigrated with a magenta compound from extracts from the prodigiosin-positive-strain ATCC 274 grown on PG agar at 28 °C. There was no corresponding compound in extracts from the prodigiosin-negative-strain ATCC 8100 grown at 28 or 37 °C or in extracts from ATCC 274 grown at 37 °C (Fig. 1A). Similar results (except R_f value) were obtained with culture extracts run on Silica gel G plates developed with chloroform:methanol:5 M ammonia (92:12:4; vol:vol) (data not shown). Prodigiosin-like compounds extracted from Silica gel G plates run with preparations from strains N4-5 and ATCC 274 had identical spectral scans (data not shown) and the expected absorbance maxima for prodigiosin in acidified methanol ($\lambda_{\max} = 535$ nm), methanol ($\lambda_{\max} = 535$ nm), and alkaline methanol ($\lambda_{\max} = 470$ nm) (Cang et al., 2000). The above experiments were performed at least twice with each of two separate ethanol extracts from the above *S. marcescens* strains with similar results.

PCR and subsequent DNA sequence analysis confirmed the presence of *pigC*, a gene involved in the biosynthesis of prodigiosin (Williamson et al., 2005), in strains N4-5 and ATCC 274. Primers designed for *pigC* were used to amplify a segment of *pigC* by PCR from DNA from strains N4-5

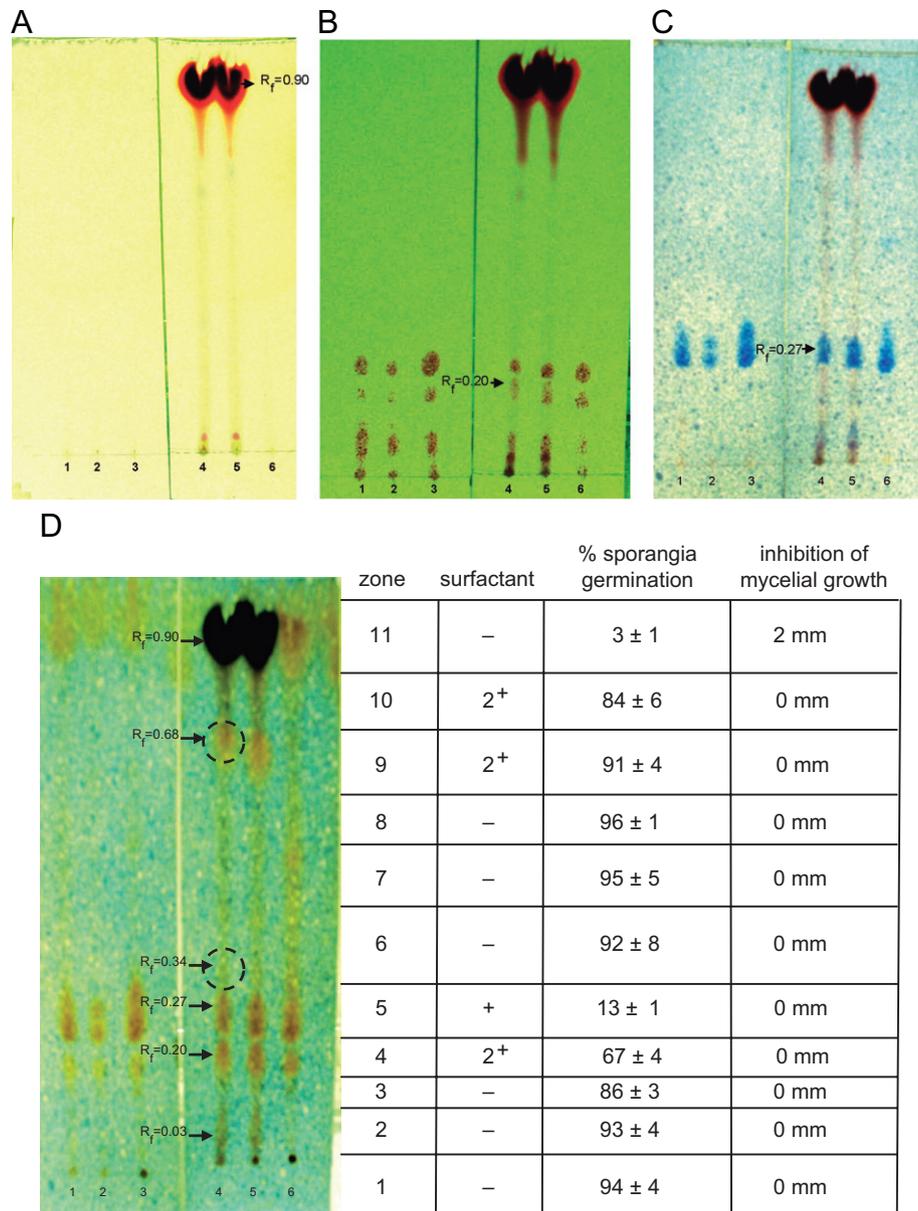


Fig. 1. Thin-layer chromatography of ethanol extracts of *Serratia marcescens* strains on Silica gel G developed with chloroform:methanol:7 M ammonia (80:25:4; vol:vol). In (A)–(D) ethanol extracts of cultures of N4-5, ATCC 274, and ATCC 8100 grown at 37 °C were spotted in lanes 1, 2, and 3, respectively, and ethanol extracts of cultures of N4-5, ATCC 274, and ATCC 8100 grown at 28 °C were spotted in lanes 4, 5, and 6, respectively. (A) no detection reagent applied; (B) ninhydrin reagent applied for detection of amino groups; (C) molybdenum blue reagent applied for detection of phospholipid; (D) charred with concentrated sulfuric acid and heat for general detection of organic compounds. Zone, a TLC plate run concurrently within the same TLC tank was segmented into the indicated zones. Surfactant, surfactant activity associated with sterile distilled water extracts of TLC fines from this zone as determined by the method of Jain et al. (1991). See Table 3 footnote for surfactant activity rating system. % sporangia germination, percent germination of sporangia of *P. ultimum* after incubation with SDW extracts of TLC fines from this zone. Inhibition of mycelial growth, zone of inhibition of growth of mycelia of *P. ultimum* on NA plates around ethanol extracts of TLC fines.

and ATCC 274. The expected-sized fragment, 494-bp, was detected in agarose gels (Fig. 2). Analysis of 384-bp of DNA sequence from the PCR fragment from strain N4-5 showed 99% identity with *S. marcescens pigC* in the databank (Genbank accession number AJ833002.1). Similarly, analysis of 437-bp of DNA sequence from the PCR fragment from ATCC 274 showed 100% identity with Genbank accession number AJ833002.1.

3.3.3. Detection of surfactants

Cultures from strain N4-5 contained at least one surfactant and production of this compound(s) was temperature sensitive. Standardized suspensions in SDW of cell material from strains N4-5 and ATCC 274 (positive control for surfactant activity and the surfactant serrawettin W1) grown at 28 °C had surfactant activity as determined by the drop-collapse method (Table 3). Cell

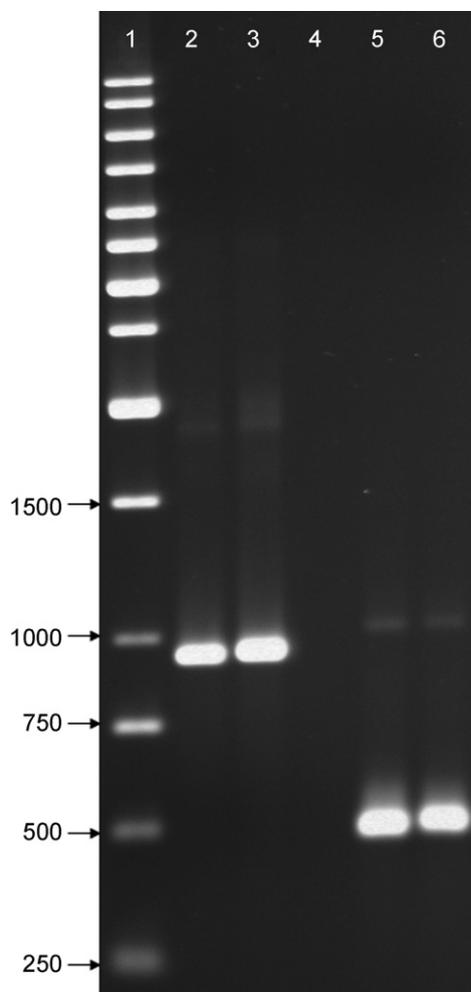


Fig. 2. Agarose gel-electrophoresis of PCR products for a prodigiosin biosynthesis gene (*pigC*) and serrawettin W1 synthetase (*swrW*) from *Serratia marcescens* strains N4-5 and ATCC 274. PCR were conducted with *swrW*-specific primers (lanes 2, 3) and *pigC*-specific (lanes 4, 5) on genomic DNA from isolate N4-5 (lanes 3, 6) and isolate ATCC 274 (lanes 2, 5). The estimated sizes of PCR products from *pigC* and *swrW* are 494 and 932 bps, respectively. Lane 1 has a 1-kb ladder molecular weight marker (GeneRuler™, Fermentas Life Sciences).

material from strains N4-5 and ATCC 274 grown at 37 °C had limited surfactant activity while cell material from strain ATCC 8100 (negative control for surfactant activity) grown at 28 or 37 °C did not have surfactant activity. Similar results were obtained in a second experiment (data not shown).

A TLC plate that was run concurrently with a second TLC plate, that had been sequentially sprayed with the ninhydrin reagent (for detection of amino groups), molybdenum blue (for detection of phospholipid), charred with sulfuric acid (for general detection of organic compounds), was segmented into zones, and the TLC fines extracted with SDW and tested for surfactant activity (Fig. 1). Surfactant activity was detected in zones 4, 5, 9, and 10 from ethanol extracts of strain N4-5 grown at 28 °C (Fig. 1D). Zone 4 contained a compound with $R_f = 0.20$ that was ninhydrin-positive, zone 5 also contained a

ninhydrin-positive compound, while there was no clearly resolving compound in zone 10. Zone 9 contained a compound ($R_f = 0.68$) with approximately the expected R_f for the surfactant serrawettin W1 (0.55–0.63; Matsuyama et al., 1985) on Silica gel G plates developed with chloroform:methanol:7 M ammonia (80:25:4; vol:vol). This compound comigrated with a compound from extracts from the serrawettin W1-positive-strain ATCC 274 grown on PG agar at 28 °C. This compound in zone 9 ($R_f = 0.68$) was not detected in extracts from strain N4-5 grown on PG agar at 37 °C. As expected for serrawettin W1, these compounds were not produced at 37 °C and did not react with the ninhydrin reagent or molybdenum blue reagent but were charred with sulfuric acid (Sunaga et al., 2004). The darkened region trailing the prodigiosin spot was magenta in color, not the expected purple color of a ninhydrin-positive compound. There was no corresponding compound in extracts from the surfactant-negative-strain ATCC 8100 (Matsuyama et al., 1986) grown at 28 or 37 °C or in extracts from ATCC 274 grown at 37 °C. The above experiments were performed at least twice with each of two separate ethanol extracts from the above *S. marcescens* strains with similar results.

The presence of serrawettin W1 in ethanol extracts of strain N4-5 grown on PG agar at 28 °C was confirmed by TLC using purified serrawettin W1 as standard. A compound that did not react with the ninhydrin reagent or molybdenum blue but comigrated with the serrawettin W1 standard on Silica gel developed with chloroform:methanol:acetone:acetic acid (90:10:6:1; vol:vol) a compound present in N4-5 cell extracts ($R_f = 0.35$) comigrated with the serrawettin W1 standard ($R_f = 0.35$). No compounds in cell extracts from strain N4-5 grown at 37 °C comigrated with the serrawettin W1 standard in TLC experiments developed with either solvent system (data not shown).

PCR and subsequent DNA sequence analysis confirmed the presence of *swrW*, which encodes serrawettin W1 synthetase (Li et al., 2005), in strains N4-5 and ATCC 274. Primers designed for *swrW* were used to amplify a segment of *swrW* by PCR from DNA from strains N4-5 and ATCC 274. The expected-sized fragment, 932-bp, was detected in agarose gels (Fig. 2). Analysis of 748-bp of DNA sequence from the PCR fragment from strain N4-5 showed 99% identity with *swrW* in the database (GenBank accession number AB193098.2). Similarly, 258 bp of DNA sequence of the 5'-terminus and 241 bp of DNA sequence of the 3'-terminus of the SWS-specific PCR product from ATCC 274, showed 99% and 100% identity, respectively, with

Table 3
Surfactant activity associated with suspended cell material from *Serratia marcescens* strains grown at 28 and 37 °C^a

Treatment	Drop collapse ^b						
	Dilution:	none	1:2	1:5	1:25	1:125	1:250
<i>S. marcescens</i> N4-5 grown at 37 °C		+	–	–	–	–	–
<i>S. marcescens</i> N4-5 grown at 28 °C		++++	+++	++	+	–	–
<i>S. marcescens</i> ATCC 274 grown at 37 °C		+	–	–	–	–	–
<i>S. marcescens</i> ATCC 274 grown at 28 °C		++++	+++	++	+	–	–
<i>S. marcescens</i> ATCC 8100 grown at 37 °C		–	–	–	–	–	–
<i>S. marcescens</i> ATCC 8100 grown at 28 °C		–	–	–	–	–	–
Sterile distilled water		–	–	–	–	–	–

^aSurfactant activity was determined by the method of Jain et al. (1991). All non-diluted treatments contained approximately 10¹⁰ CFU ml⁻¹ bacteria.

^bDrop collapse was determined with non-diluted cell material or cell material diluted with sterile distilled water. + + + +, —; + + +, —; + +, —; +, —; —, —.

corresponding sequences in the database (GenBank Accession number AB193098.2).

3.3.4. Pyrrolnitrin production

Certain isolates of *S. marcescens* produce the antibiotic pyrrolnitrin (Kamensky et al., 2003), however no compounds from samples from *S. marcescens* N4-5 prepared a variety of ways (see Section 2) comigrated with the pyrrolnitrin standard after TLC (data not shown). Pyrrolnitrin was detected by TLC in preparations from cultures of *P. fluorescens* PF-5 and *B. ambifaria* BC-F grown and extracted under the same conditions (Roberts and McKenna, unpublished). PCR confirmed the absence of pyrrolnitrin biosynthetic genes in strain N4-5. PCR amplifications containing primer pairs designed for *prnD*, a highly conserved gene in pyrrolnitrin-producing strains (de Souza and Raaijmakers, 2003), or *prnABC* failed to amplify PCR product in reactions containing N4-5 genomic DNA (data not shown). Primer pairs for *prnD* and *prnABC* amplified DNA fragments of the expected size, 503 and 1982 bp, respectively, in reactions containing *P. fluorescens* PF-5 genomic DNA (data not shown).

3.4. In vitro inhibition of *Pythium ultimum* with compounds in extracts from strain N4-5

Non-fractionated ethanol extracts of cell mass of strain N4-5 prepared as for the damping-off suppression assay (Table 2) contained a compound(s) that inhibited germination of sporangia of *P. ultimum* and growth of mycelia of *P. ultimum*. Production of this inhibitory compound(s) was temperature sensitive. When cell mass of strain N4-5 from four PG agar plates incubated at 28 or at 37 °C was extracted with ethanol, dried, and the residue resuspended in SDW, sporangial germination was inhibited by a 1:125 dilution (with SDW) of the 28 °C preparation while there was only slight inhibition of germination of these sporangia associated with the non-diluted 37 °C preparation. Incubation of sporangia with the 1:125 dilution of the 28 °C preparation resulted in 0.7 ± 0.7% germination, incubation with the non-diluted 37 °C preparation resulted in

55.0 ± 2.0% germination, and incubation with the 1:5 dilution (with SDW) of the 37 °C preparation resulted in 82.0 ± 4.0% germination. There was 95.0 ± 3.0% germination of sporangia in the SDW-only control. Similar results were obtained in a second experiment (data not shown). Experiments could not be run with extract residue resuspended in ethanol or other organic solvents as these solvents inhibited germination of sporangia. When residue from these ethanol extracts of strain N4-5 was resuspended in ethanol and tested for inhibition of mycelial growth by this Oomycete, inhibition of growth was only detected with the 28 °C preparations. Zones of inhibition of mycelial growth on NA agar were 15.0 ± 2.0 mm for the non-diluted 28 °C preparation and 0.0 ± 0.0 mm for the non-diluted 37 °C preparation. There was no zone of inhibition with the ethanol-only control. Similar results were obtained in a second experiment (data not shown). Ethanol extracts of cell mass of strain N4-5 grown on PG agar at 28 or at 37 °C that were resuspended in SDW did not inhibit mycelial growth. No zones of inhibition were detected on NA agar with these preparations or with the SDW-only control in two experiments.

Ethanol extracts of strain N4-5 grown on PG agar at 28 and 37 °C were fractionated by TLC and these fractions tested for inhibition of germination of sporangia of *P. ultimum* (Fig. 1D). The strongest inhibitory activity was associated with the compound prodigiosin or a compound that comigrated with prodigiosin under our TLC conditions. This inhibition was only associated with the 28 °C preparations (data not shown). Germination of sporangia was always less than 5% when incubated with TLC fines scraped from the prodigiosin-region of the TLC plate. Hexane, ethyl acetate, and chloroform:methanol (2:1; vol:vol) extracts of strain N4-5 grown on PG agar also all contained strong sporangial germination inhibitory activity that was associated with prodigiosin. As with ethanol extracts, the inhibitory compound(s) almost completely inhibited sporangial germination (data not shown). Ethanol extracts of strain N4-5 grown on PG agar at 28 and 37 °C that were fractionated by TLC were also tested for inhibition of mycelial growth (Fig. 1D).

Only the zone of the TLC plate containing prodigiosin from the 28 °C preparation inhibited mycelial growth. No fractions from the 37 °C preparation inhibited mycelial growth (data not shown). Similar results were obtained in repeated experiments (data not shown).

Prodigiosin was purified from ethanol extracts of strain N4-5 grown on PG agar by (1) TLC on a Silica gel G plate developed with chloroform:methanol:5 M ammonia (80:25:4; vol:vol), (2) extraction of the magenta-colored prodigiosin spot with chloroform:methanol (2:1; vol:vol), (3) TLC on a second Silica gel G plate developed with chloroform:methanol:5 M ammonia (92:12:4; vol:vol), and (4) extraction of the magenta-colored prodigiosin spot from the second TLC plate with SDW. This prodigiosin preparation resulted in $0.0 \pm 0.0\%$ sporangial germination in two experiments while controls (TLC fines not exposed to the sample but developed with the same two solvent systems) resulted in $89.0 \pm 6.0\%$ and $95.0 \pm 1.0\%$ sporangial germination in two experiments. When the magenta-colored prodigiosin spot was extracted from the second TLC plate with chloroform:methanol (2:1; vol:vol) and tested for inhibition of growth of mycelia of *P. ultimum*, zones of inhibition on NA agar were 3.5 ± 0.5 mm and 3.5 ± 0.5 mm in two experiments. There were no zones of inhibition of mycelial growth associated with control preparations in either experiment.

A second inhibitory zone (zone 5) was associated with a phospholipid compound with R_f of 0.27 on Silica gel G developed with chloroform:methanol:7 M ammonia (80:25:4; vol:vol) in one experiment (Fig. 1). Compounds in this zone inhibited sporangial germination only (Fig. 1D). In a second experiment, inhibition of sporangial germination was associated with a compound that was slightly more mobile when using this solvent system (R_f approximately 0.34). There was no inhibition of germination with phospholipid compounds of R_f 0.27. No compounds other than prodigiosin inhibited mycelial growth in either experiment.

3.5. Cucumber root colonization

S. marcescens N4-5R2 effectively colonized the cucumber root system in a natural soil, the soil-less mix used in damping-off assays, and a pasteurized soil. Populations per root system detected at 32 and 52 d were 5.07 ± 1.22 and $5.66 \pm 0.66 \log_{10}$ CFU (g fresh weight root)⁻¹ in the natural soil, 6.39 ± 0.97 and $6.62 \pm 0.23 \log_{10}$ CFU (g fresh weight root)⁻¹ in the soil-less mix, and 5.87 ± 0.40 and $5.73 \pm 0.49 \log_{10}$ CFU (g fresh weight root)⁻¹ in the pasteurized soil, respectively. Results of a second experiment were similar (data not shown). *S. marcescens* N4-5R2 was also detected on root segments at substantial distances down the cucumber root system from the soil line after 60 d in all three planting media. \log_{10} CFU of N4-5R2 on 7-cm sections of root from 14 to 21 cm below the soil line were 4.10 ± 2.15 , 4.16 ± 0.52 , and 3.27 ± 0.38 , respectively, from cucumber grown in the natural soil, the soil-less mix, and

the pasteurized soil. Results from a second experiment were similar (data not shown). Initial populations of strain N4-5R2 applied to cucumber seed in these experiments were approximately $8.0 \log_{10}$ CFU seed⁻¹.

4. Discussion

S. marcescens has versatile plant-beneficial properties with strains of this species suppressing diverse fungal, bacterial, and viral pathogens on several crops (Kobayashi and El-Barrad, 1996; Press et al., 1997; Someya et al., 2000). Pigmented and non-pigmented strains of *S. marcescens* were also shown to suppress damping-off caused by the Oomycete *P. ultimum* (Roberts et al., 2005). We show here that *S. marcescens* N4-5 effectively suppressed damping-off of cucumber and other cucurbits caused by *P. ultimum* in growth chamber experiments (Table 1). This strain was not previously characterized for suppression of damping-off caused by *P. ultimum*. Strain N4-5, like other strains of *S. marcescens* we have studied (Roberts et al., 2005), produced compounds that had broad spectrum antibiotic activity. Cell extracts from N4-5 were inhibitory to *P. ultimum* while culture filtrates from this bacterium were inhibitory to the nematode *Meloidogyne incognita in vitro* (Meyer, Crowley, and Roberts, unpublished).

It is unlikely that strain N4-5 or other live isolates of *S. marcescens* will be granted registration for agricultural use by the US Environmental Protection Agency in the near future. Parallels exist between isolates of *S. marcescens* and isolates of the *B. cepacia* complex regarding perceived risks to human health; despite studies suggesting that prodigiosin production by *S. marcescens* is a marker of the environmental origin of strains of this species (Ding and Williams, 1983; Aucken and Pitt, 1998). Isolates from the *Burkholderia cepacia* complex are ubiquitous in nature and have beneficial properties such as suppression of plant diseases (Parke et al., 1991; Hebbar et al., 1992; Li et al., 2002) and bioremediation (Kilbane et al., 1982; Vega et al., 1988; Shields et al., 1991; Shields and Reagin, 1992). As with *S. marcescens*, certain isolates of the *B. cepacia* complex are considered opportunistic human pathogens having been associated with clinical infections of immunocompromised patients (Chiarini et al., 2006). Isolates of the *B. cepacia* complex were commercially available for control of plant pathogenic fungi and nematodes (DenyTM, Stine Microbial Products, USA) but the US Environmental Protection Agency cancelled the registration of all biopesticide products containing *B. cepacia* complex isolates in 2003 in response to the medical community and existing scientific data at the time (Chiarini et al., 2006).

Nevertheless, it may be possible to exploit the biotechnological potential of isolates of *S. marcescens* for use in certain agricultural applications without releasing live cells of this bacterium into the environment. Certain disease interactions do not require live cells of biocontrol agents for delivery of inhibitory biochemicals or other traits for suppression of disease. For example, resting structures of

P. ultimum rapidly germinate in response to nutrients released from germinating seeds and can colonize these seeds within as little as 4 h (Nelson et al., 1986; Osburn et al., 1989). Certain species, such as cucumber, quickly lose susceptibility to damping-off caused by *P. ultimum*. Cucumber seeds germinated for 4–8 h prior to exposure to infective propagules of *P. ultimum* escaped damping-off (Hadar et al., 1983). Since the window of vulnerability is short in duration, the infection court is limited spatially to the seed and emerging radicle. This allows direct application of inhibitory biochemical compounds to the infection court at the time of seed treatment. Colonization of the rhizosphere by bacterial biocontrol agents is not required for suppression of damping-off of cucumber caused by *P. ultimum* (Roberts et al., 1997). We show here that suppression of damping-off of cucumber caused by *P. ultimum* with seed treatments containing cell-free extracts of *S. marcescens* N4-5 was effective and similar to that obtained with live cells of N4-5 (Table 2). It remains to be seen if seed treatment with cell-free extracts from N4-5 will effectively suppress *P. ultimum* on crops with longer windows of vulnerability as it may not be possible to deliver the inhibitory compounds to the entire infection court at the time of seed treatment. The inhibitory compound(s) in these cell-free extracts may also be unstable in the soil environment over longer periods of time.

Seed treatment with chemical pesticides has been used routinely for suppression of damping-off diseases. However, the use of more environmentally benign natural products with low human toxicity from microbes such as *S. marcescens* may provide control of certain plant diseases without the risks that are associated with many chemical pesticides. It should be noted that commercialization of natural products, such as inhibitory compounds produced by *S. marcescens* N4-5, is subject to registration and economic evaluation.

Cell-free extracts of strain N4-5 and other isolates from *S. marcescens* may have use in preparations containing multiple biocontrol agents. Combining multiple biocontrol agents within a single preparation is an approach that has been tested for enhancing biological control (Larkin et al., 1998; Raupach and Kloepper, 1998; Meyer and Roberts, 2002). It is thought that a combination of biocontrol agents is more likely to have a greater variety of traits responsible for suppression of one or more pathogens and also is likely to have these traits expressed over a wide range of environmental conditions (Lemanceau and Alabouvette, 1991; Lemanceau et al., 1993; Pierson and Weller, 1994; Crump, 1998). This approach has had documented successes (Raupach and Kloepper, 1998; Meyer and Roberts, 2002). However, there are reports indicating decreased performance of preparations containing combined biocontrol agents relative to individual application of these biocontrol agents (Meyer and Roberts, 2002). It is thought that strains combined in biocontrol preparations must be compatible for increased disease suppression to

occur (Baker, 1990; Janisiewicz and Bors, 1995; Janisiewicz, 1996; Raupach and Kloepper, 1998; Roberts et al., 2005). Strains of *S. marcescens* and strains from the *B. cepacia* complex were combined with isolates of *Trichoderma virens* in assays targeting suppression of damping-off of cucumber caused by *P. ultimum* and *R. solani* (Roberts et al., 2005). Biological control performance was improved with *T. virens* combined with certain of these bacteria. However, populations of *T. virens* were suppressed in cucumber rhizosphere by isolates of *S. marcescens* and *B. cepacia*. It may be possible to improve biological control performance by improving compatibility among biocontrol agents in these preparations by applying cell-free extracts of microbes such as *S. marcescens* N4-5 in combination with *T. virens*. As shown in the current study, strain N4-5 colonizes cucumber rhizosphere effectively. Cell-free extracts of N4-5 would contribute toward suppression of damping-off of cucumber caused by *P. ultimum* without live cells of N4-5 competing with and inhibiting *T. virens* in cucumber rhizosphere.

Ethanol extracts of strain N4-5 grown at 28 °C resuspended in a number of different organic solvents suppressed damping-off of cucumber caused by *P. ultimum* when applied as a seed treatment (Table 2). The pigmented antibiotic prodigiosin appeared to play a large role in this disease suppression based on *in vitro* inhibition experiments. The majority of inhibitory activity with regard to germination of sporangia and mycelial growth of *P. ultimum* was associated with fractions of these ethanol extracts containing prodigiosin (Fig. 1D). Similar results were obtained with fractions created using the solvents hexane, ethyl acetate, and chloroform:methanol (2:1; vol:vol). There was some inhibition of sporangial germination associated with other TLC fractions but this inhibition was low and did not consistently associate with any compounds detected by charring (Fig. 1D). Only the TLC fraction associated with prodigiosin inhibited growth of mycelia of *P. ultimum*. Additionally, other inhibitory compounds, reported to be produced by other strains of *S. marcescens*, were not inhibitory in *in vitro* assays or were not produced by strain N4-5. The surfactant serrawettin W1 was produced by strain N4-5 but there was no inhibition of *P. ultimum* by fractions that contained this compound (Fig. 1D). The broad-spectrum antibiotic pyrrolnitrin and the genes involved in its biosynthesis were not detected with N4-5. We are currently constructing prodigiosin-deficient knock-out mutants of strain N4-5 to confirm the role of prodigiosin in suppression of damping-off of cucumber.

Prodigiosin is a secondary metabolite consisting of three pyrrole rings linked in a linear mode. This compound is produced by *S. marcescens* and other microbes as an exolipid. There is no defined role for prodigiosin in the metabolism of producing strains but it has been suggested that this compound functions in metabolic overflow from primary metabolism (Bu'Lock, 1961; Hood et al., 1992; Williamson et al., 2005). Prodigiosin has been of research

interest because of reports of its anti-fungal, anti-bacterial, anti-protozoal; and anti-insectal activities (Williams and Quadri, 1980; Tsuji et al., 1990; 1992; Demain, 1995; Someya et al., 2001). It is also reported to be immunosuppressive in humans and have anti-cancer activities (Mandarville, 2001). If prodigiosin proves to be a major factor in inhibition of damping-off of cucumber caused by *P. ultimum* it may be possible to improve its effectiveness through chemical modification for enhanced stability or toxicity. Prodigiosin has been shown to be amenable to chemical modification (Gale et al., 2005), allowing further pharmaceutical and other industrial applications to be pursued. Pyrrolnitrin is an example of a natural product that served as a lead structure in the development of the more stable compounds fenpiclonil and fludioxonil, two agricultural commercial fungicides (Ligon et al., 2000).

In summary, we present evidence based on TLC, PCR and sequence analysis, spectral scans, and biological tests that strongly indicate the involvement of prodigiosin produced by *S. marcescens* N4-5 in the activity of this strain in the biological control of *P. ultimum*-induced damping-off of cucumber.

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