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Enteric bacteria of field-collected Colorado potato beetle larvae inhibit growth of the entomopathogens *Photorhabdus temperata* and *Beauveria bassiana*

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ABSTRACT

The nematode *Heterorhabditis marelatus* fails to reproduce in the Colorado potato beetle, *Leptinotarsa decemlineata*, possibly due to interference from the enteric bacteria of the beetle. Specifically, the enteric bacteria inhibit the growth of *Photorhabdus temperata*, the enteric symbiont of the nematode, in vitro. However, previous work was based on a laboratory culture of *L. decemlineata*, and we wished to determine if similar bacteria were present in the field. Therefore, we cultured the enteric bacteria of fourth-instar larvae collected from the field at two locations in Maryland and Virginia. Representatives of the genera *Pantoea, Enterobacter, Pseudomonas, Acinetobacter, Serratia, Stenotrophomonas, Curtobacterium, Bacillus, Lactococcus* and *Enterococcus* were identified by sequencing of their 16S rDNA. Isolates belonging to the genera *Pantoea, Enterobacter, Pseudomonas, Serratia* and *Bacillus* inhibited the growth of *P. temperata*. A number of these isolates also inhibited the entomopathogenic fungus *Beauveria bassiana* in vitro.
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1. Introduction

The entomopathogenic nematode *Heterorhabditis marelatus* Liu and Berry and its bacterial symbiont, *Photorhabdus temperata* Fischer-Le Saux et al. are capable of killing the Colorado potato beetle, *Leptinotarsa decemlineata* (Say); however, the nematode is incapable of completing its reproductive cycle in the beetle (Armer et al., 2004). We recently examined both nematode vectored and artificially induced infection of *L. decemlineata* by *P. temperata* and found that *P. temperata* could not prevent spread and growth of the enteric bacteria within the host cadaver. In many cases the level of hemocoel contamination by other bacteria was substantial and could easily interfere with either the growth of *P. temperata* or its host nematode. In vitro growth-inhibition experiments demonstrated that many enteric isolates inhibited the growth of *P. temperata* but were not significantly inhibited by *P. temperata* (Blackburn et al., 2007).

The enteric bacteria of insects play a large and complex role in the biology of their hosts. Aside from the more obvious contribution to digestion and nutrition, the indigenous bacteria can displace or inhibit pathogenic species in a phenomenon termed "colonization resistance" (Dillon and Dillon, 2004). Although the competition *P. temperata* appears to encounter when colonizing *L. decemlineata* is distinct from colonization resistance in that it does not prevent disease in an individual, it does represent a plausible mechanism for inhibiting the establishment of a pathogen in a population.

Our previous experiments were conducted on a laboratory colony of *L. decemlineata* whose gut microbiota might not reflect that of field populations. Thus, we wished to sample field-collected *L. decemlineata* to determine how their enteric bacteria differed from our laboratory colony, and to determine if these bacteria inhibit *P. temperata* or the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin.

2. Materials and methods

2.1. Bacterial isolation and culture

All bacteria described in this study were isolated and grown on solid medium consisting of 2% Bacto® Proteose Peptone #3 (PP3) in 1.6% Bacto® agar (both obtained from Becton Dickinson and Co., Sparks, MD, USA). Liquid cultures were grown in 2% PP3. The choice of media obviously affects which bacteria can be isolated from a given source. Since our primary objective was to assess the effect of enteric species on the growth of *P. temperata*, we chose PP3 because we are familiar with the behavior of *P. temperata* on this medium.

Bacteria were isolated from apparently healthy fourth-instar *L. decemlineata* collected from potato at the Beltsville Agricultural Research Center (Beltsville, MD, USA) and from the Virginia Tech Eastern Shore Agricultural Research and Extension Center (Painter, VA, USA). Collections were made on two separate occasions at both sites in May and June 2007.

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Larvae were placed in sterile 50 ml plastic centrifuge tubes that were placed in a horizontal position. When larvae defecated, 5 μl of fluid was pipetted from the frass and used for inoculations. In cases where 5 μl could not be recovered, 10 μl of sterile PP3 medium was pipetted onto the frass, mixed, and 5 μl of the excess fluid recovered for inoculation. In the first collections from each site, the fluid was used to inoculate 100 ml of PP3 in a 500-ml flask, which was then shaken at 250 rpm for 24 h at 25° C. At 24 h, serial dilutions of the cultures were prepared with sterile PP3, and 10 μl aliquots of selected dilutions spread on 100-mm plates of PP3 agar. Apparent single colony isolates were selected from these plates after 48–72 h for sub-culturing. For some samples from the first Beltsville collection and all samples from the second collections at each site, serial dilutions of the fluid recovered from frass were spread directly onto plates.

Bacterial isolates from individual larvae were chosen for further analysis based on colony morphology and appearance. We were less concerned with the relative abundance of different bacteria than we were with generating a reasonably accurate picture of the spectrum of bacterial species that could be cultured, albeit on PP3 alone, from the beetle larvae. All colonies that appeared unique or occurred at low frequency were retained for further study. When many colonies appeared to be of the same type, a number of these apparently identical isolates were retained. All isolates were maintained in culture on PP3 agar and were sub-cultured bi-weekly.

2.2. Identification of bacteria

Individual enteric bacterial isolates were identified by PCR amplification and sequencing of conserved 16S ribosomal DNAs. For each isolate, DNA was purified from 2 ml of liquid culture. DNA was isolated using the Quantum Prep miniprep kit (BioRad, Hercules, CA, USA) as specified by the manufacturer for use as template in polymerase chain reaction (PCR). Nearly full length 16S rDNA was amplified for each isolate using primers universal to prokaryotes, R16F0 and R16R0 (Lee et al., 1993). Thirty-five PCR cycles were conducted in a model 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) using 30 s denaturation at 94 °C, 1.5-min annealing at 55 °C and 2-min primer extension (10 min in final cycle) at 72 °C. Bacterial 16S rDNA amplicons were sequenced directly. Products were separated on 1.5% NuSieve agarose gel (FMC, Rockland, ME, USA) in modified-1X TAE (0.04 M Tris–acetate and 0.1 mM EDTA), and excised for sequencing using ABI BigDye V1.1 (Applied Biosystems with both amplification primers and a nested universal primer 533F: 5′-GTGCCA GCMGCCGCGGTAA-3′). Cycle sequencing conditions were 35 cycles at 96 °C, 10 s; 50 °C, 5 s; 60 °C for 4 min. Automatic sequencing was carried out on an ABI Prism Model 3100 (Applied Biosystems). Sequences of 16S rDNAs were edited and assembled (DNASTAR, SeqMan component) and compared with those of known bacteria using BLAST searches (Altschul et al., 1990).

2.3. Phylogenetic analysis

Preliminary assignment of bacterial isolate 16S rDNA sequences to phylogenetic groups was performed by comparing them with sequences of bacterial type strains using the Seqmatch tool from the Ribosomal Database Project-II (Cole et al., 2005). Phylogenetic analyses were undertaken to examine relationships among new isolates preliminarily assigned to (1) Enterobacteriaceae, and to (2) *Pseudomonas* groups. 16S rDNA sequences from the new bacterial isolates and type strain sequences from closely related bacteria were aligned separately using the CLUSTAL W algorithm of the MegAlign component of the Lasergene suite (DNASTAR, Inc., Madison, WI, USA). Phylogenetic analyses were performed using PAUP version 4.10b (Sinauer Associates, Sunderland, MA, USA). Uninformative characters were excluded from analyses. Phylogenetic trees were constructed by heuristic search via random stepwise addition and TBR branch swapping to find the optimal phylogenetic tree(s). *Xanthemonas campestris* (Pammel) Dowson and *Yersinia enterocolitica* (Schliefstein and Coleman) Frederiksen were designated outgroups to root the *Pseudomonas* and Enterobacteriaceae trees, respectively.

2.4. Bacterial growth experiments

The growth of 12 selected enteric isolates in the presence of the primary phase of *P. temperata*, and their effect on the growth of *P. temperata*, were examined as described by Martin (2002). Briefly, interactive effects on the growth of two isolates of bacteria were evaluated by measuring the widths of two parallel lines of bacteria streaked on a single agar plate. Primary phase *P. temperata* isolated from *H. marelatus* and maintained on PP3 agar (Blackburn et al., 2007) was used. Two sets of 100 mm plates of PP3 agar were included. On the first set, one streak of an enteric bacterium was paired with one streak of *P. temperata* 1 cm apart. The second set of plates served as controls, with two streaks of the same bacterium on the same plate. Plates were incubated at 25° C for 72 h. A stereomicroscope equipped with a calibrated ocular micrometer was used to measure the width of each streak in the first set of plates at five pre-determined points. These five measurements were then averaged to obtain a single value for each streak. One of the two streaks on each control plate was measured similarly. The test was replicated four times.

The widths of the streaks of enteric isolates paired with *P. temperata* were compared to the widths of their respective control streaks by factorial analysis of variance (ANOVA; PROG GLM, SAS Institute, 2006) with isolate and pairing (with *P. temperata* or with itself) as main effects. The widths of each enteric isolate were also compared individually with their respective controls. The widths of streaks of *P. temperata* were analyzed similarly. The width of each enteric isolate was also expressed as a proportion of the width of its control streak. These data were analyzed by ANOVA for effects of enteric isolate, and means were separated by the least significant difference (LSD) test. Widths of streaks of *P. temperata* were also analyzed in this manner. Lastly, a test for correlation between the absolute widths of the streaks of the enteric isolates with the widths of their respective *P. temperata* streaks was performed (PROC CORR, SAS Institute, 2006). In all analyses, each plate was treated as a replicate.

2.5. Fungal growth experiments

Starter cultures of 15 selected enteric bacteria were prepared by streaking 60-mm plates (48 mm inside diameter) of PP3 and incubating them at 24° C for 24 h. Clean plates of PP3 were then inoculated with *B. bassiana* (ARSEF 6721) by pipetting 10^4 conidia in 10 μl of 10% glycerol in water onto each plate. A sterile plastic spreader was used to evenly distribute conidia across the plate. To inoculate plates with bacteria, a sterile 6-mm paper disk (Becton, Dickinson and Co.) was pressed onto the starter plate of each isolate. The disk was then pressed onto the center of a plate with *B. bassiana* conidia, then removed and discarded. Plates without *B. bassiana* were similarly inoculated with each bacterial isolate. In addition, three plates inoculated with conidia only and one uninoculated plate were included. Plates were incubated at 24° C. The test was replicated four times.

Seven days after inoculation, the density of fungal growth was rated visually. A scale of 0–4 was used, where zero was no fungal growth visible to the unaided eye and four was fungal growth similar to that on plates inoculated with *B. bassiana* only. At this time,

the diameter of the zone of bacterial growth was also measured. Density data were $\sqrt{x} + 0.5$ transformed prior to statistical analysis, but means presented are of untransformed data. Density data were analyzed by ANOVA for effects of bacterial isolate. Ratings of uninoculated plates and plates inoculated with *B. bassiana* only were included as controls. Means were separated by LSD. Diameters of the bacterial zones were analyzed by ANOVA for effects of isolate, fungus treatment (i.e., with versus without *B. bassiana*) and the interaction thereof. A test for correlation between fungal density and bacterial zone diameter was also performed. Treatments of *B. bassiana* only and uninoculated plates were not included in analyses of bacterial zone diameter.

3. Results

3.1. Identification of bacteria

In all, 76 bacterial isolates were identified to genus level from a total of 20 larvae collected from the two sites (Table 1). The most prevalent types of bacteria isolated from both sites were from the closely related genera *Enterobacter* (29 isolates) and *Pantoea* (15 isolates), with *Acinetobacter* (12 isolates) and *Pseudomonas* (10 isolates) also occurring frequently. Other types of bacteria whose occurrence appeared more incidental belonged to the genera *Bacillus* (3 isolates), *Lactococcus* (2 isolates), *Curtobacterium* (2 isolates), *Serratia* (1 isolate), *Stenotrophomonas* (1 isolate) and *Enterococcus* (1 isolate). The 16S rDNA sequences of representative isolates have been deposited in GenBank (Accession Nos. EU693534–EU693575).

Phylogenetic relationships among new isolates and type strain Enterobacteriaceae (*Pantoea*, *Enterobacter* and *Serratia)* based on 16S rDNA sequences are shown in Fig. 1, while analysis of *Pseudomonas* isolates is presented in Fig. 2. Phylogenetic analysis of 16S rDNA sequences revealed that the new *Enterobacter* isolates were of two lineages. New *Enterobacter* isolates 2B1C, 2B1E, 2B2A 2B2B, 2B2C, 2B2D, 2B5C, 2B5D, 2B5E, 2B8D and 2V4A formed a monophyletic clade with closely related characterized *Enterobacter asburiae* Brenner et al. and *Enterobacter cancerogenus* (Urosevic) Dickey and Zumoff; new *Enterobacter* isolates 2B7A, 2B7B, 2B8C, 2V2A and 2V5B grouped with the characterized species *E. ludwigii* Hoffman et al. (Fig. 1). All *Pantoea*-related isolates (2V1E1A, 2V2D2, 2B8A, 2B4D, 2B7C, 2B7D and 2B7E) formed a monophyletic clade with the two type species strains of *P. agglomerans* (Ewing and Fife) Gavini (Fig. 1). The sole *Serratia* isolate 2V1A was most closely related to characterized species *Serratia marcescens* Bizio (Fig. 1). Phylogenetic analysis of 16S rDNA sequences revealed that the new *Pseudomonas* isolates were also of two lineages. New *Pseudomonas* isolates B3G and 2V1D were closely related to characterized species *Pseudomonas koreensis* Kwon et al., while isolates 2B5A, 2B5B, BD3E, 2V1C, 2V2E and 2V2F all grouped within the large clade containing characterized species *Pseudomonas putida* (Trevisan) Migula, *Pseudomonas fulva* Iizuki and Komagata, *Pseudomonas oryzihabitans* Kodama et al., *Pseudomonas entomophila*, *Pseudomonas plecoglossicida* Nishimori and other characterized *Pseudomonas* species (Fig. 2).

3.2. Bacterial growth experiments

Results of bacterial growth experiments scored at 72 h are shown in Fig. 3. As a group, the growth of the enteric isolates next to *P. temperata* appeared to be stimulated ($F = 16.28$; df = 1, 68; $P = 0.0001$); however, on an individual basis, none of the isolates were significantly stimulated ($P > 0.05$). Growth of *P. temperata* next to the enteric isolates was significantly suppressed, both as a group, and in most individual comparisons. The only isolates that did not significantly inhibit *P. temperata* were *Pantoea* 2B7D, *Stenotrophomonas* 2V2H and *Acinetobacter* 2V3A; the latter significantly stimulated growth of *P. temperata*. There were substantial differences in absolute growth among the enteric isolates, and there was a weak but significant negative correlation between the width of the *P. temperata* streaks and the width of the enteric isolate streaks ($r = -0.3130$, $P = 0.0342$). Width of the enteric isolate streaks as a proportion of their controls did not vary among isolates ($F = 1.77$; df = 11, 31; $P = 0.1036$). In *P. temperata*, this proportion was affected by the enteric isolate with which it was paired ($F = 8.77$; df = 11, 31; $P = 0.0001$). In the latter case, *Acinetobacter* isolate 2V3A differed significantly ($P < 0.05$) from the other isolates by LSD.

3.3. Fungal growth experiments

Fungal density was significantly affected by bacterial isolate ($F = 47.16$; df = 16, 56; $P = 0.0001$) (Fig. 4). *Pseudomonas* isolates B3G and 2V1D completely inhibited fungal growth, with no visible growth on any plate. A third isolate, *Enterobacter* 2B1C, consistently reduced fungal growth to low levels. The LSD indicated that all the isolates inhibited fungal growth except *Pantoea* isolates 2B7D, 2B4D and 2B7C, which did not differ from the treatment of

Table 1
Phylogenetic grouping of 16S rDNA sequences from representative *Leptinotarsa decemlineata* isolates

Nearest type strain	Type strain accession nos.	*L. decemlineata* isolate (% identity)
Bacilli		
Bacillus cereus ATCC 14579	AF290547	B2A (99.0)
Lactococcus lactis subsp. cremoris ATCC 19257	M58836	2V3B (99.5), 2V2C (98.8)
Enterococcus casseliflavus NCIMB 11449	Y18161	2V5C (99.7)
γ-Proteobacteria		
Pantoea agglomerans NCTC9381T	AJ251466	2V1E1A (100), 2V2D2 (100)
Pantoea agglomerans ATCC 27155	AF130953	2B7D (99.8), 2B4D (98.8)
Enterobacter asburiae JCM6051	AB004744	2B5D (99.7), 2B1C (100)
Enterobacter cancerogenus LMG2693	Z96078	2B2B (99.9), 2B2C (99.7), 2V4A (99.8)
Enterobacter ludwigii DSMZ 16688	AJ853891	2V5B (99.8), 2V2A (99.7), 2B7B (99.6)
Pseudomonas koreensis Ps 9–14	F468452	B3G (99.8), 2V1D (99.6)
Pseudomonas parafulva AJ 2129T	AB060132	B3C (99.9), 2B5B (99.8)
Pseudomonas oryzihabitans IAM1568	D84004	2V1C (99.3)
Pseudomonas plecoglossicida FPC951	AB009457	BD3E (99.6), 2V2F (99.7)
Acinetobacter calcoaceticus NCCB 22016	AJ888983	2V3A (99.8), 2V2D1 (100)
Serratia marcescens subsp. *marcescens* ATCC 13880	M59160	2V1A (99.9)
Stenotrophomonas maltophilia ATCC 13637T	AB008509	2V2H (99.0)
Actinobacteria		
Curtobacterium flaccumfaciens pv. *flaccumfaciens* LMG 3645	AJ312209	2B4A (99.6)

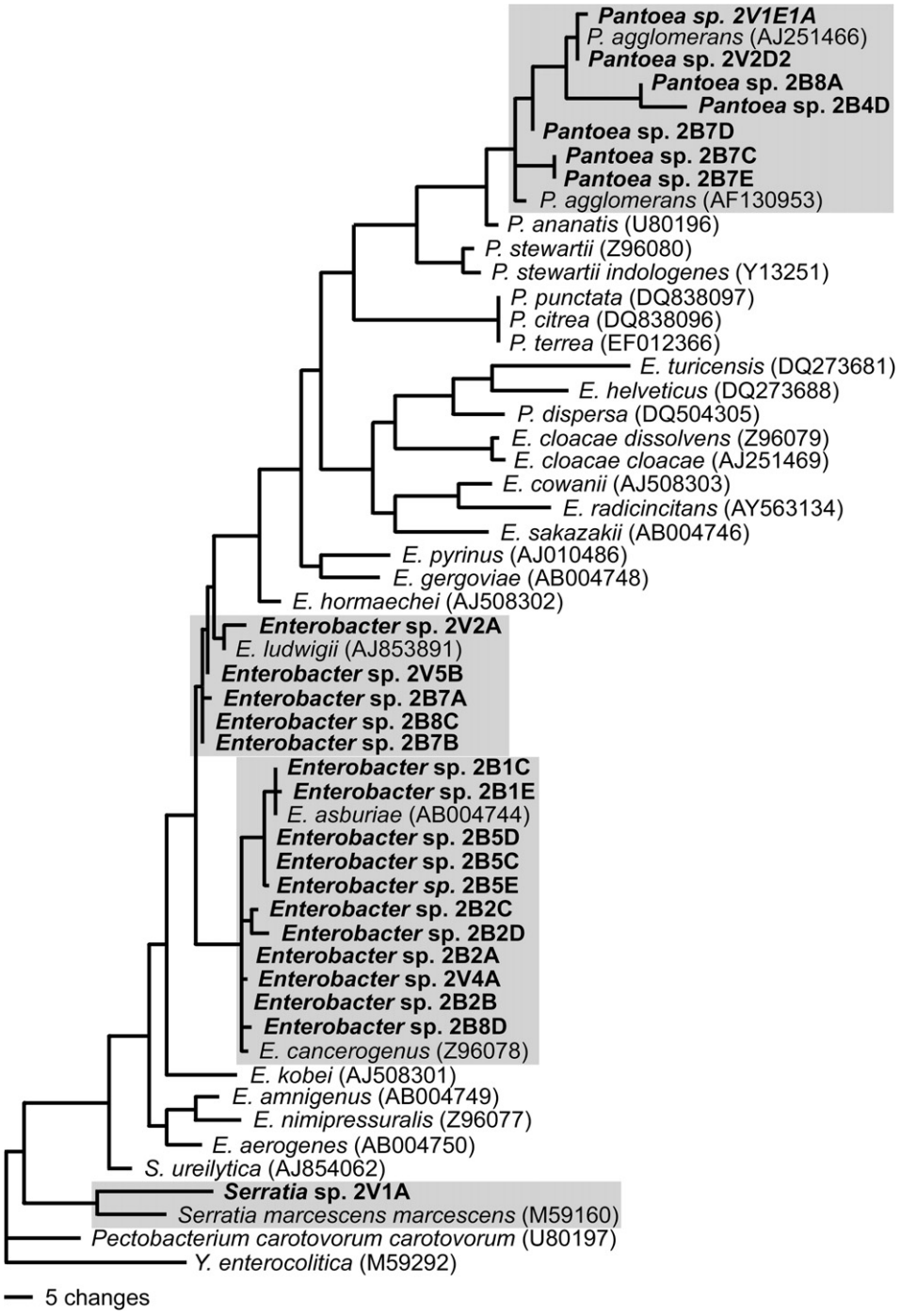

Fig. 1. Phylogenetic relationships among Enterobacteriaceae (*Enterobacter*, *Pantoea* and *Serratia*) isolates from *Leptinotarsa decemlineata* (indicated in boldface), and accepted type strains from these genera.

B. bassiana alone. The diameter of the bacterial zone varied significantly among isolates ($F = 27.94$; df = 14, 87; $P = 0.0001$) but was not affected by the presence or absence of *B. bassiana* ($F = 0.12$; df = 1, 87; $P = 0.7286$) or by the interaction of isolate and fungus treatment ($F = 0.26$; df = 1, 87; $P = 0.9964$). There was a significant negative correlation between diameter of the bacterial zone and fungal density ($r = -0.8710$, $P = 0.0001$).

4. Discussion

Blackburn et al. (2007) found that infection of laboratory-reared *L. decemlineata* larvae by *P. temperata* typically resulted in a mixed culture of bacteria within the insect cadaver, regardless of whether the infection was vectored by the nematode *H. marelatus* or by injection. Enteric species isolated from these larvae belonging to the genera *Pantoea*, *Pseudomonas*, *Acinetobacter*, *Serratia* and *Klebsiella* were able to inhibit the growth of *P. temperata* to a greater extent than *P. temperata* was able to inhibit their growth. In the current study we have cultured bacteria from field-collected *L. decemlineata* and found representatives of the genera *Pantoea*, *Enterobacter*, *Pseudomonas*, *Acinetobacter*, *Serratia*, *Stenotrophomonas*, *Curtobacterium*, *Bacillus*, *Lactococcus* and *Enterococcus*. Results of growth-inhibition experiments conducted on *P. temperata* in the current study are similar to those of the previous study; many

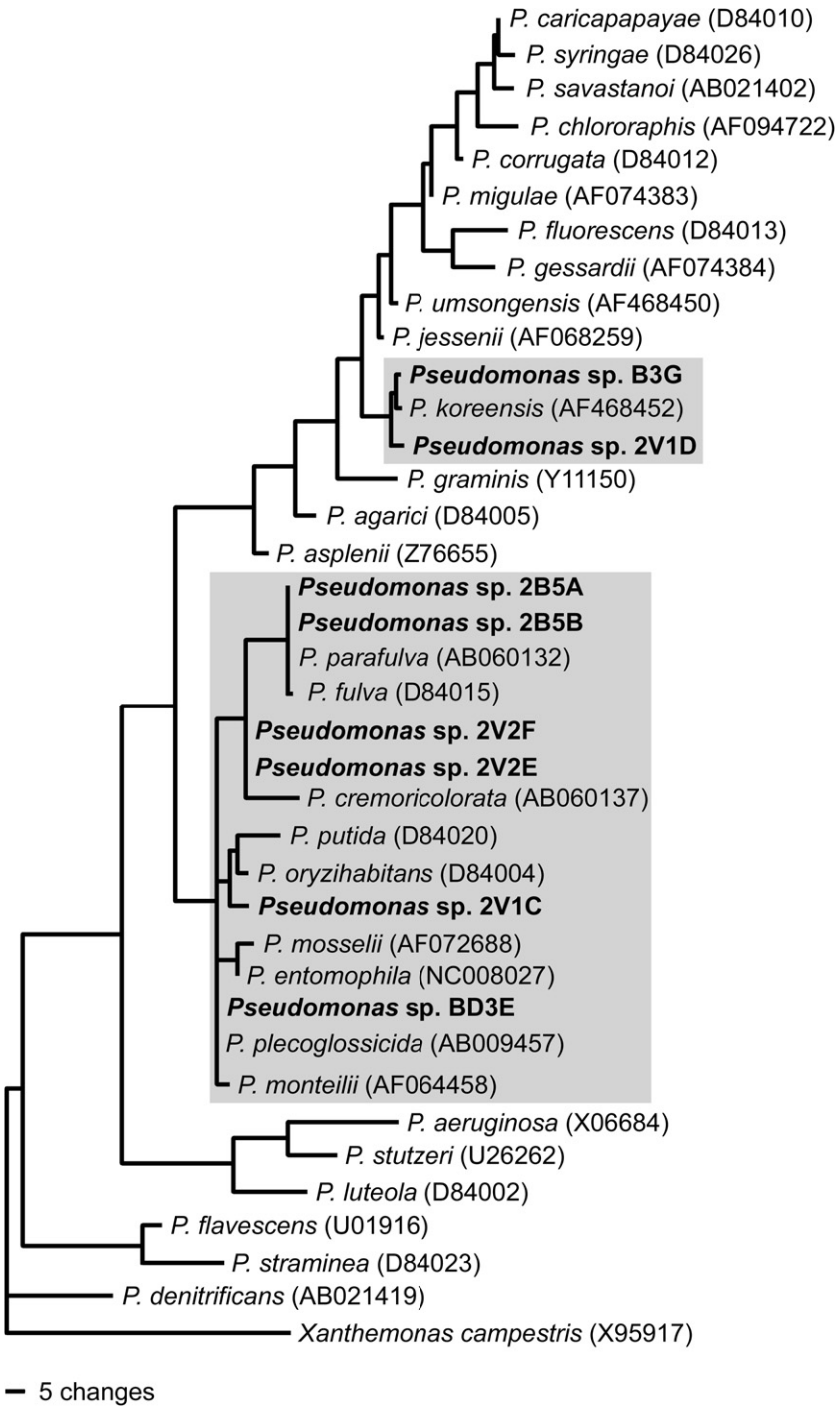

Fig. 2. Phylogenetic relationship of *Pseudomonas* isolates from *Leptinotarsa decemlineata* (indicated in boldface) and representative type strains from the *Pseudomonas*. Type strains were chosen to represent the major phylogenetic groups of the *Pseudomonas* as described by Anzai et al. (2000).

enteric isolates inhibited *P. temperata* to a greater extent than *P. temperata* inhibited them. In fact, as a group, the enteric isolates that we tested were stimulated by *P. temperata*. Only three of 12 isolates tested did not significantly inhibit *P. temperata*: *Acinetobacter* 2V3A, *Stenotrophomonas* 2V2H and *Pantoea* 2B7D. Curiously, *Acinetobacter* 2V3A actually stimulated *P. temperata*.

Isolates representing the genera *Pseudomonas*, *Enterobacter* and *Serratia* also inhibited the growth of the entomopathogenic fungus *B. bassiana* in vitro. In particular, *Pseudomonas* isolates B3G and 2V1D were found to completely inhibit the growth of *B. bassiana*. In contrast, the *Pantoea* isolates that we tested had little effect on the fungus.

With the exception of *Lactococcus* and *Enterococcus*, representatives of all the genera found in the frass of *L. decemlineata* have been demonstrated to be endophytes of potato (Sturz and Matheson, 1996; Sturz et al., 1998; Garbeva et al., 2001; Reiter et al., 2002; Sessitsch et al., 2004; Berg et al., 2005), suggesting that the culturable gut flora of *L. decemlineata* larvae may be largely determined by the endophytic community of the host plant.

Among those bacteria that we could culture from larval frass, the most commonly isolated were species of the closely related genera *Enterobacter* and *Pantoea*. Based on 16S rDNA sequence comparisons with accepted type strains, the closest matches were for *P. agglomerans*, *E. asburiae*, *E. cancerogenus* and *E. ludwigii*. Both

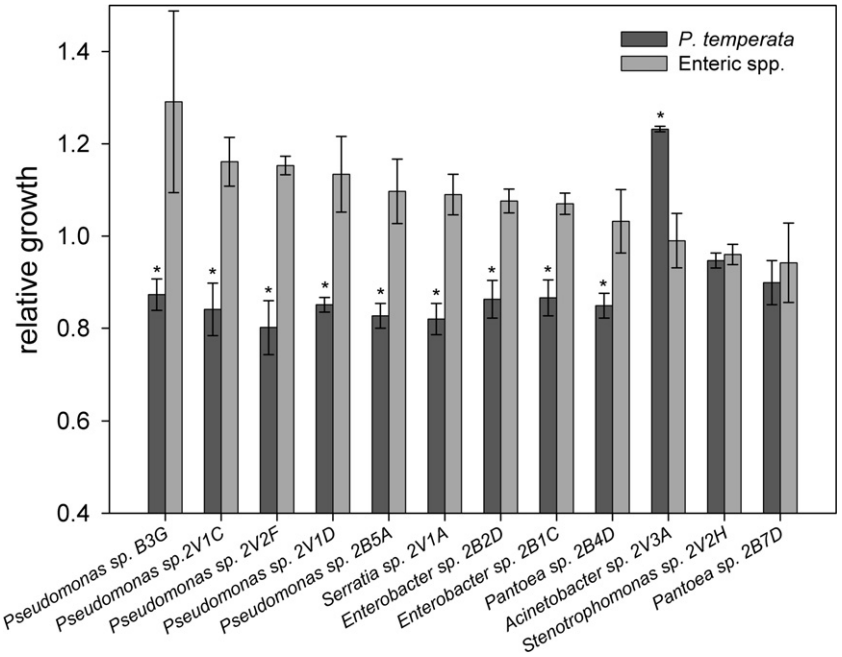

Fig. 3. Growth of *Photorhabdus temperata* and 12 enteric isolates grown next to each other relative to the growth of each bacterium next to itself at 72-h post-inoculation. Asterisks indicate growth significantly different ($P < 0.05$) from that of controls.

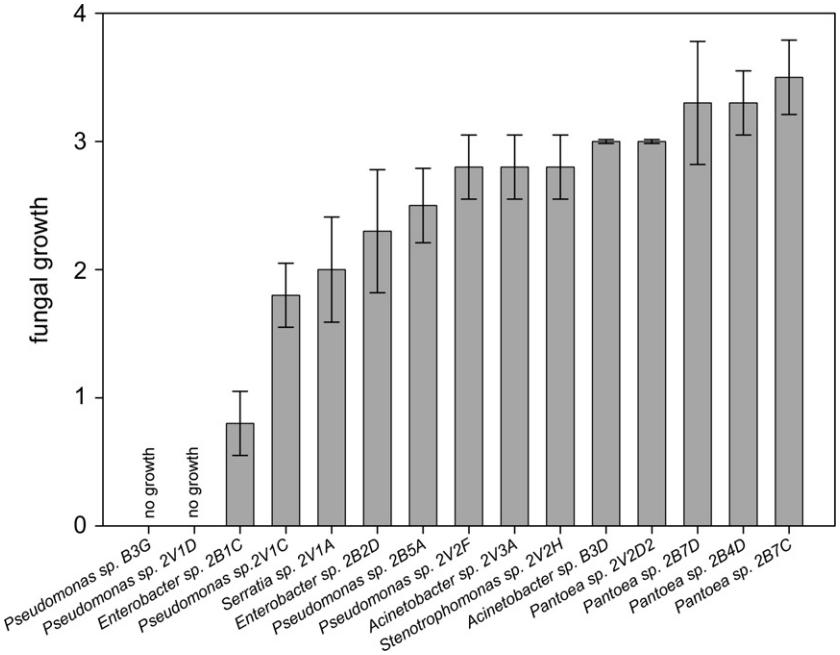

Fig. 4. Growth of *Beauveria bassiana* in the presence of 15 enteric isolates 7 days post-inoculation. The scale on the Y axis is from 0 to 4 where zero was no fungal growth visible to the unaided eye and four was fungal growth similar to that on plates inoculated with *B. bassiana* only.

P. agglomerans and *E. cancerogenus* have specifically been cited as potato endophytes (Sturz and Matheson, 1996; Garbeva et al., 2001; Sessitsch et al., 2004), while *E. asburiae* has been shown to be associated with a variety of other plants (Asis and Adachi, 2003). *Pantoea agglomerans* and *Enterobacter* spp. have also been isolated or identified from diverse insect species (Dillon and Charnley, 1995; Broderick et al., 2004; Vasanthakumar et al., 2006; Dunn and Stabb, 2005; Nishiwaki et al., 2007; Blackburn et al., 2007). In the only prior study of the enteric bacteria of *L. decemlineata*, Steinhaus (1941) apparently reported members of the genera *Pantoea* and *Enterobacter* as *Flavobacterium acidificum* sp. nov. and *Aerobacter aerogenes*, respectively. *Aerobacter aerogenes* is synonymous with the current *Enterobacter aerogenes* Hormaeche and Edwards, and *F. acidificum* was considered by Holmes et al. (1984) to be identical to *Erwinia ananas* Serrano, which is now classified as *Pantoea ananatis* (Serrano) Mergaert et al.

In both plants and insects, *P. agglomerans* appears to offer its host some protection from colonization by both fungal and bacterial pathogens. It has been shown to inhibit *Erwinia amylovora* (Burrill) Winslow, the causative agent of fire blight (Wright et al.,

2001) and has been shown to inhibit growth of several fungal pathogens on tomato (Enya et al., 2007). In the desert locust, *Schistocerca gregaria* (Forskal), *P. agglomerans* has been shown to be involved in the production of anti-fungal phenols that inhibit *Metarhizium anisopliae* (Metschnikov) Sorokin (Dillon and Charnley, 1995) and, with other gut bacteria, to resist colonization by *S. marcescens* (Dillon et al., 2005). We found that one of two *Pantoea* isolates and both of the *Enterobacter* isolates we tested significantly inhibited *P. temperata*. These same *Pantoea* isolates had little or no effect on the growth of *B. bassiana*; however, *Enterobacter* isolate 2B1C strongly inhibited the fungus. The failure of *Pantoea* isolates to inhibit *B. bassiana* in the present study may be due to the absence of plant material in our assays that might be necessary for the production of anti-microbial phenolics (Dillon and Charnley, 1995).

Phylogenetic analysis of 16S rDNA sequences from our *Pseudomonas* isolates, together with sequences from type strains representing major groups of the *Pseudomonas sensu stricto* (Anzai et al., 2000) suggests two groups. Most of the isolates are clustered within the *P. putida* group, with 16S rDNA sequences that most closely resembled either *Pseudomonas parafulva* Uchino, *P. oryzihabitans* or *P. plecoglossicida*. *Pseudomonas putida* and closely related members of this group have been repeatedly reported as endophytes of potato (Sturz et al., 1998; Garbeva et al., 2001; Reiter et al., 2002; Sessitsch et al., 2004; Berg et al., 2005), and Steinhaus (1941) reported "*Pseudomonas ovalis* Chester" from *L. decemlineata*, a bacterium presumed by Palleroni (1984) to be *P. putida*. A second group, isolated from both sampling sites, most closely resembles *P. koreensis* Kwon et al., a recently described species isolated from agricultural soils in Korea (Kwon et al., 2003). Bacteria with 16S rDNA sequences highly similar to *P. koreensis* have been isolated from both ginseng and tomato, and shown to inhibit phytopathogenic fungi (Cho et al., 2007; Enya et al., 2007). Interestingly, both of the *Pseudomonas* isolates from this group (B3G and 2V1D) were the most potent inhibitors of *B. bassiana* that we found, and both inhibited *P. temperata* as well. As a group, the *Pseudomonas* isolates displayed the greatest inhibitory activity against both entomopathogens.

In our prior study, a strain of *S. marcescens* associated with our laboratory colony of *L. decemlineata* was found to strongly inhibit *P. temperata*. We were only able to obtain a single putative isolate of *S. marcescens* from field-collected larvae, suggesting that this species occurs only sporadically in agricultural settings. However, this isolate did inhibit both *P. temperata* and *B. bassiana*.

The term colonization resistance refers to the concept that the normal gut flora is capable of inhibiting the growth of pathogens and affords the individual host a degree of protection from disease, but in a broader sense the idea can be extended to populations. Blackburn et al. (2007) described a possible example of colonization resistance operating at the level a population. Although the nematode *H. marelatus* and its symbiont *P. temperata* were capable of killing *L. decemlineata*, it appeared that *P. temperata* was incapable of excluding the gut bacteria from propagating within the host cadaver to the extent that *Photorhabdus* spp. can in other insects, leading to reproductive failure of the nematode. When the effectiveness or persistence of a control agent is at least partially dependent on reproduction within the host, if reproduction is inhibited, the host population will benefit even though individual hosts may die. In the present study, we have examined field-collected *L. decemlineata* and found that their enteric flora includes many species that inhibit *P. temperata* in vitro. Some of these same isolates also have inhibitory effects on the growth of the entomopathogenic fungus *B. bassiana*. Although replication of *B. bassiana* in the target insect may be less crucial for persistence in the field, the enteric bacteria may still offer the beetles some resistance to infection. In addition to inhibiting the growth of ingested conidia, foliage surfaces of plants infested with *L. decemlineata*, and the insects themselves, are generally contaminated by frass. Surface contamination by the enteric bacteria, or their metabolites, may inhibit the germination and growth of *B. bassiana* across the cuticle or trachea. Regarding this last point, it should be noted that certain chrysomelid beetles, such as *Lema trilinea* White, form shields of fecal matter on their backs. While this has been correctly interpreted as a defense against predation (Morton and Vencl, 1998), it is also possible that the bacteria or bacterial metabolites contained in these fecal masses protect the larvae from pathogens, and it would be worthwhile to investigate the relative susceptibility of species forming such shields to pathogens.

The concept of biological control is based on principles of population dynamics. In the best case, a pest population is regulated by the density-dependent action of predators, parasitoids or pathogens. Although current understanding of the population dynamics of plant endophytes and the enteric bacteria of insects is very limited, it is clear that these bacterial communities constitute potentially potent antagonists to bacterial or fungal biological control agents.

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