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Analysis of Bacterial Communities Associated with Insect Biological Control Agents using Molecular Techniques

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Abstract

Investigations of the diversity of bacterial communities associated with field-collected specimens of two insect biological control agents of spotted knapweed*, Agapeta zoegana* and *Cyphocleonus achates*, were made using molecular methods. The objective was to assess the bacterial communities of each insect to evaluate the potential compatibility of plant pathogenic fungi as a supplement to insect diets for mass rearing of the two species. Synergistic interactions of plant pathogenic fungi with root-attacking insects have been extensively correlated with reductions in stand density of rangeland weeds. This synergism has been noted both in foreign and domestic stands of leafy spurge (*Euphorbia esula*) and spotted knapweed (*Centaurea maculosa*). The bacterial flora of larvae of the biological control agent *Aphthona nigriscutis* of *Euphorbia esula* was also investigated as an indication of possible factors that may affect their establishment, survival and capacity to impact the weed. After DNA extraction of whole larvae, the V6 to V8 region of 16S rDNA was PCR-amplified. Community profiles were obtained using denaturing gradient gel electrophoresis (DGGE) and isolated bands were excised, reamplified and sequenced for phylogenetic comparisons. Phylogenetic analysis indicated that some bands represented microorganisms causing ice nucleation, antagonism toward soilborne plant pathogens and symbionts associated with mating incompatibility. The results suggest that these insect biological control agents maintain diverse microbial communities, an important consideration in formulating artificial diets that may contain antibiotics or other preservatives. Furthermore, the findings imply that microbial communities associated with insects influence the establishment, and impact of these insects.

Keywords: *Agapeta zoegana, Cyphocleonus achates, Aphthona nigriscutis*, DGGE, microbial communities

In the practice of biological control of weeds, the failure of agents to establish has been noted in numerous instances. For example, in the landmark work conducted for the control of *Opuntia* spp., approximately 55 species of insects were imported into Australia for release and fewer than 12 were noted to have established (Dodd, 1940). Thirty-seven insect species were available as possible biocontrol agents of St. John's Wort and of 5 species imported from Europe, only 2 established in Australia (Wilson, 1943). Such a failure rate raises questions about factors that should be considered in addition to or instead of climate matching as criteria to consider when assessing limitations on survival and reproduction of insects introduced for biological weed control. The capacity of microbes to affect insect reproduction, survival and fitness is well-established (Buchenr, 1965; Douglass, 1989; Lee *et al*. 1992). The reproductive consequences of bacterial endosym-

bionts as an issue in biological control of weeds has previously been emphasized (Campbell *et al.* 1992).

The gut microflora of many insects includes a high diversity of microflora (Bignell 1984). In many instances, the association between microorganisms and insects is casual and transitory, where microorganisms are probably derived from the diet upon which the insect feeds. In other cases, the relationship between certain microorganisms and their insect hosts is more intimate and specific. Many insects, across a broad taxonomic range, possess obligate microbial endosymbionts that benefit the insect in some way (Douglas 1989). Generally, insects with a restricted or nutrient poor diet are likely to have endosymbionts. To date, none of these bacterial endosymbionts have been cultured.

Other microorganisms associated with insects have been implicated in altering the population ecology of their hosts. The most studied of these are the *Wolbachia*, a common and widespread group of bacteria found in insect reproductive tissue. These bacteria are cytoplasmically inherited rickettsia that cause a number of reproductive alterations in their host, including cytoplasmic incompatibility between strains, parthenogenesis induction, and feminization of genetic males (reviewed in Werren, 1997). *Wolbachia* have additionally been shown to increase fecundity of insects (Vavre *et al.* 1999), and the nature of their effects vary with strain, arthropod species, and host genetic system (Johanowicz and Hoy 1998). These rickettsiae have not been successfully cultured outside of host cells. Ice-nucleating bacteria have been shown to decrease the overwintering capacity of the Colorado Potato Beetle (Lee *et al.* 1994). Such bacteria, even in a killed form, have been shown to raise the lowest temperature at which the insect will survive in soil. In addition, there may be as yet undiscovered associations of individual microbes or microbial communities that can affect the establishment of insects or their ability to overwinter. Components of the microbial flora of insects may also affect the ability through antagonism or competition of plant pathogen partners in synergisms with insects. Such synergisms are correlated with the dramatic stand reductions observed sometimes among the hundreds of instances where insects have been released for control of leafy spurge and knapweed (Caesar 2000).

Microscopy and cultivation have long been considered of limited usefulness in describing microbial diversity in environmental samples. Microscopic techniques generally cannot distinguish between morphologically similar but metabolically different bacteria, and cultivation selects for only those organisms able to grow in the culture media. The introduction of denaturing gradient gel electrophoresis (DGGE) analysis of small subunit (SSU) rDNA has allowed the study of microbial diversity in complex environments (Muyzer, *et al*.1993). In brief, a region of the rDNA gene is PCR-amplified from mixed population DNA, and the resulting mixed PCR products are separated on a polyacrylamide gel with a linear, increasing denaturing gradient. PCR products with different sequences are separated into discrete bands during electrophoresis. The bands can be excised and sequenced, then compared with the sequences of known organisms to determine the phylogenetic positions of the organisms represented by the bands.

Understanding microbial communities of insects may be the first step to greatly increasing the success rate of insect releases against exotic and invasive perennial weeds. The object of this study was a preliminary description of the bacterial flora of three insect species that are important components of biological control programs of leafy spurge and knapweed. In the present study, we examined the bacterial communities associated with *Agapeta zoegana* (Lepidoptera: Cochylidae) and *Cyphocleonus achates* (Coleoptera:

Curuliondae) two insects released for the biological control of knapweeds (*Centurea* spp.), and *Aphthona nigriscutis* (Coleoptera: Chrysomelidae), released for the control of leafy spurge (*Euphorbia escula*).

Materials and Methods

DNA extraction

A. zoegana larvae were collected from spotted knapweed roots at sites in Corvallis, Montana and Palisade, Colorado in June 1998. *C. achates* larvae were collected from the Palisade site. *A. nigriscutis* larvae were collected from a field release site in northeastern Montana. Larvae were randomly assigned to two groups. One group was surface-sterilized with 10% bleach for 20 minutes, followed by 3 rinses with sterile MilliQ water. The other group was rinsed 3x with sterile MilliQ water. Individual larva were frozen in liquid nitrogen, ground with a mortar and pestle, and transferred to a 1.5 ml centrifuge tube while still frozen. DNA was extracted using a QIAamp tissue extraction kit (QIAGEN Inc., Valencia, CA) according to manufacturer's directions, except the final eluate volume was reduced to 200 µl. DNA size and yield were determined by analyzing 5 µl on a 1 % agarose gel.

For diet studies, neonate *A. zoegana* larvae from eggs of adults collected in Corvallis were transferred to an artificial diet developed at the USDA/ARS Insect Biological Control Laboratory (IBCL) in Columbia, MO. This diet contained per 3000ml water: agar, 95 g; casein, 126 g; sucrose, 135 g; raw wheat germ, 175 g; Wesson salt mix, 36 g; Alphacel, 25 g; potassium sorbate, 4 g; methyl paraben, 5.4 g; raw linseed oil, 26 ml; vitamin mix, 36 g; aureomycin, 4g; Tenox, 0.8 g; 45 % KOH, 9 ml; and 40 % formalin, 3 ml. The diet was supplemented with 2 % w/v ground and autoclaved spotted knapweed root. Larvae were collected from the diet after 6 weeks and treated in the same manner as field collected specimens.

Insect PCR and DGGE

Primers U984-GC (5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GCC GAA CCT TAC) and L1401 (5' GCG TGT GTA CAA GAC CC) (Zoetendahl, *et al*., 1998; Nubel, *et al*., 1996) were used to amplify the V6 to V8 regions of 16S rDNA. PCR mixtures of 100 µl contained 100 pmol of each of the primers, 3 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase, 200 µM dNTPs, 500 mM KCl, and 100 mM Tris-HCl (pH 8.3). Two-microliter samples were amplified in a Thermal Cycler 480 (Perkin-Elmer) using the following temperature cycles: initial cycle of 94 C for 3 min; followed by 35 cycles of 94 C for 30 s, 52 C for 30 s, and 68 C for 1 min; and a final cycle of 68 C for 7 min.

PCR products were separated by DGGE using the DCode system (BioRad, Inc., Hercules CA) according to manufacturer's instructions. Gels consisted of 1 mm-thick 6% polyacrylamide with a denaturing gradient of 30 to 60 % (100% denaturant corresponds to 7 M urea and 40% [vol/vol] deionized formamide) and 0.5X TAE buffer. DGGE was performed at 60 C and 160V with 0.5X TAE buffer for 6 H. Gels were stained with Sybr green I nucleic acid stain (Molecular Probes, Eugene, OR) for 60 min and images were captured using a Fluor-S MultiImager (BioRad, Inc., Hercules, CA).

DNA bands from DGGE gels were excised with a razor blade and placed in 2 ml screw-capped vials containing 0.2 ml sterile water and approximately 0.25 g of 1 mm acid-washed glass beads (Sigma, St. Louis, MO). Vials were vortexed for 2 min to dis-

perse the acrylamide and incubated overnight at 4 C to elute the DNA. Ten microliters of the supernate were used as a template to reamplify the DNA, using primers U984-GC and L1401. The PCR conditions were the same as described for DGGE amplifications.

PCR products were purified using QIAprep spin columns (QIAGEN, Inc., Valencia, CA), and sequenced in both directions with the same primers as used in PCRs. Template sequences were labeled with BigDye™ Terminator cycle sequencing (PE Applied Biosystems) and analyzed on an ABI 377 sequencer. Sequences were compared to sequences from identified organisms in a ribosomal database (RDP, Maidak, *et al*. 1999).

Results and Discussion

DGGE and temperature gradient gel electrophoresis (TGGE) are now routinely used as molecular tools to compare the diversity of microbial communities from a variety of environments (Muyzer, 1999). We were able to amplify fragments of SSU rRNA genes from most field-collected *A. zoegana*, *C. achates* and *A. nigriscutis* (Figs.1 to 3). Surface sterilization had no consistent effect on microbial community patterns, suggesting that the organisms detected were probably internal insect inhabitants.

Agapeta zoegana larvae from both sites had similar bacterial communities (Fig. 1). Relatives of *Acinetobacter* spp., *Duganella zoogloeodes*, *E. coli*, *Pseudomonas* spp., and *Propionibacterium acnes* were routinely detected in larvae at both Montana and Colorado sites. *Acinetobacter* spp., *Pseudomonas* spp., and *Propionibacterium* spp. are common soil inhabitants with different metabolic characteristics that may be advantageous to their insect hosts. Some species of *Propionibacterium* are lipolytic (Jarvis *et al.*, 1998) and *Acinetobacter* spp. have been implicated in phosphate storage. Pseudomonads are well known for their metabolic diversity in catabolizing aromatic compounds and could poten-

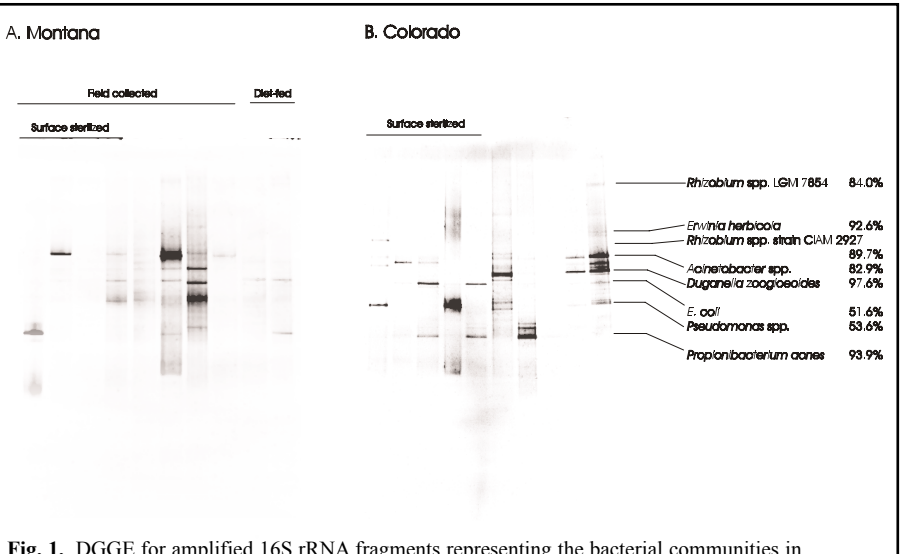

Fig. 1. DGGE for amplified 16S rRNA fragments representing the bacterial communities in field-collected *Agapeta zoegana* larvae residing in spotted knapweed (*C. maculosa*) roots collected from sites in A) Montana and B) Colorado. Listed to the right of the gels are closest relatives of organisms represented by the bands, with corresponding percent identity.

tially be associated with metabolism of recalcitrant or allelopathic plant residues. Diet-fed larvae had less diverse bacterial community patterns than field collected specimens (Fig. 1). Other research has also suggested that field-collected insects differed in culturable bacterial populations when compared with diet-reared insects (Smalley and Ourth, 1979). In these results, antimicrobials in the diet may have adversely affected the bacterial populations.

Compared with *A. zoegana* bacterial communities, *C. achates* larvae had little diversity (Fig. 2). Our small sample size limited our ability to draw many conclusions. However, from these few individuals it was evident that the bacterial communities of *C. achates* were more conserved than those of *A. zoegana*.

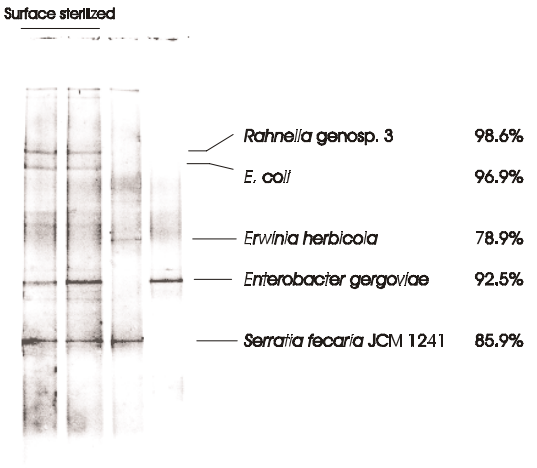

Fig. 2. DGGE for amplified 16S rRNA fragments representing the bacterial communities in field-collected *Cyphoceonus achates* larvae residing in spotted knapweed (*C. maculosa*) roots. Listed to the right of the gels are closest relatives of organisms represented by the bands, with corresponding percent identity.

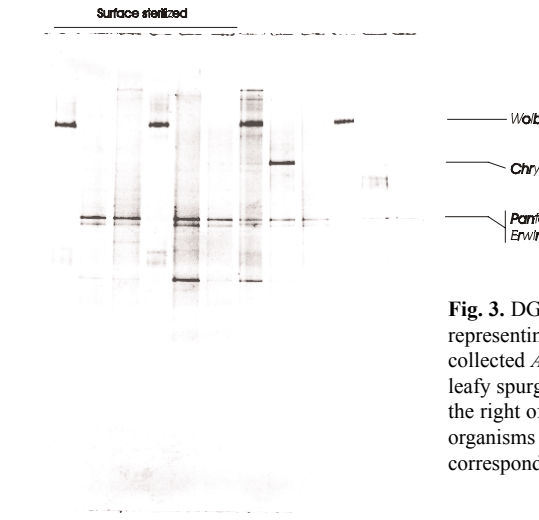

Fig. 3. DGGE for amplified 16S rRNA fragments representing the bacterial communities in field-collected *Aphthona nigriscuitis* larvae residing in leafy spurge (*Euphorbia esula*) roots. Listed to the right of the gels are closest relatives of organisms represented by the bands, with corresponding percent identity.

As with *C. achates*, *A. nigriscutis* bacterial community patterns were simple and fairly conserved between individuals (Fig. 3). Of particular interest was the detection of *Wolbachia*-like sequences in 4 of the 10 larvae we analyzed. We confirmed the presence of this endosymbiont in these specimens using *Wolbachia*-specific primers (data not shown). Infection by *Wolbachia* may offer an explanation for the female-biased sex ratios observed at *A. nigriscutis* release sites, and the involvement of *Wolbachia* in the skewed ratio of *A. nigriscutis* requires further study. We also routinely found sequences related to the *Pantoea agglomerans/Erwinia herbicola* complex. *Erwinia herbicola* is a common epiphyte and found in the rhizosphere of weed species (Kremer, *et al*., 1990). *Erw. herbicola* produces a variety of antimicrobial substances including bacteriocins and the antifungal toxin herbicolin (Ishimura, *et al*. 1988), and has been used as a biological control agent against a host of plant pathogens. The presence of *Erw. herbicola* in these insect biological agents has important implications in the establishment of pathogens directed against the target weeds. Pathogens act synergistically with root-attacking insects in reducing the stand density of rangeland weeds (Caesar 2000), and *Erw. herbicola* is a potential antagonist of these pathogens. In addition, many *Erw. herbicola* strains have been shown to be ice-nucleating (Turner, *et al*., 1990), and ingestion of these bacteria by insects may decrease the cold tolerance of the insect (Strong-Gunderson, *et al*., 1990), thus adversely affecting winter survival.

Separation of SSU rRNA sequences from mixed microbial communities, followed by cloning and sequencing, is a powerful technique in elucidating relationships between those communities and their environment. This study has shown that larvae of the insect biological control agents *A. zoegana*, *C. achates*, and *A. nigriscutis* harbor a diversity of bacteria. Some of these bacteria may contribute to nutritional requirements of the insects, while others may be associated with preventing the establishment of exogenous microorganisms, or the ecology of the insect population.

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