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Application of 16S rDNA-DGGE and Plate Culture to Characterization of Bacterial Communities Associated with the Sawfly, *Acantholyda erythrocephala* (Hymenoptera, Pamphiliidae)

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Abstract Culture-based analysis was employed in parallel with PCR amplification of 16S rDNA, coupled with denaturing gradient gel electrophoresis (DGGE), to profile bacterial species associated with different developmental stages of the pine false webworm (PFW), *Acantholyda erythrocephala*, a sawfly pest responsible for incidents of severe defoliation in commercially important tree plantations in North America. Culture-based analysis revealed that *Pseudomonas* spp. along with *Bacillus sphaericus* and *Arthrobacter* sp. were the predominant components of the microflora of the internal organs and identified life-stage-specific associations including *Photorhabdus temperata* with egg and larval samples and a *Janthinobacterium* sp. with eonymphs. PCR-DGGE confirmed the predominance of *Pseudomonas* spp. and *B. sphaericus* in the majority of samples but did not detect *Arthrobacter* sp., *P. temperate*, or *Janthinobacterium* sp. In contrast, DGGE revealed the presence of a *Chryseobacterium* sp. as the predominant component of the PFW micoflora at all life stages, with the exception of adults. This species had been infrequently cultured, at low levels, from a limited number of samples and the existence of a possible relationship between this bacterium and the PFW had gone unnoticed using the culture-based approach. Our findings highlight the advantages of applying a dual approach to the study of microbe-insect associations and demonstrate that the benefits of one system can be used to overcome some of the limitations of the other.

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Introduction

Acantholyda erythrocephala, the pine false webworm (PFW), was introduced into eastern North America from Europe around 1925. Since 1981, the PFW has caused moderate to severe defoliation in saw-timber pine stands in New York state, USA, and in Ontario, Canada [2]. The PFW has six life stages: eggs, larvae, eonymph, pronymph, pupae, and adult. In spring, insects emerge from the soil and mate. Eggs remain in intimate contact with the vascular system of the plant and continue development (spring to summer). After emergence, larvae crawl down to the base of the needle and start to feed on old needle foliage and construct silken tubes (webs). Following their arboreal development, larvae drop to the ground and burrow into the soil where they develop into eonymhs. Transformation into pronymphs occurs during late summer or early autumn. Pronymphs remain underground for at least one winter (diapause), with transformation into pupae occurring at the beginning of spring [12].

Studies of the interactions between invertebrate hosts and their microflora have revealed direct bacterial involvement in nitrogen fixation, methanogenesis, cycling of organic compounds, production of pheromones, and biocide degradation [5]. Some endosymbiotic bacteria supply their hosts with nutrients, while others generate reproductive defects or

protect against the development of disease [5, 6]. Traditionally, studies of insect-microbe interactions have employed culture-based methods with identification based on phenotypic, genetic, and biochemical characteristics. The limitations of such an approach are well established [1, 15] and have served to stimulate the application of culture independent methods to the study of insect-bacterial interactions [10, 17, 18].

In this study, as part of a broader investigation into sawfly population dynamics and identification of potential biological control agents, we employed both culture-based methods and polymerase chain reaction (PCR) amplification of the total 16S rRNA gene fragments, in combination with denaturing gradient gel electrophoresis (DGGE), to examine the microbial communities associated with different life stages of PFW.

Materials and Methods

Field Sampling and Rearing From spring 2003 through summer 2004, PFW pronymphs, eonymphs, pupae and adults were collected in Bruce, Grey, and Simcoe Counties in Ontario and transported live to the Atlantic Forestry Centre, Fredericton, New Brunswick. Eonymphs, pronymphs, and pupae were maintained in clean sand at 4°C and were processed within 48 h of arrival. Field-caught adults were provided with potted, 3-year-old red pine trees (*Pinus resinosa*) for oviposition and larvae were allowed to feed on the same trees. PFW rearing was performed in the laboratory at 20°C under a 16-h light:8-h dark cycle.

Specimen Preparation All life stages were surface cleansed with quick rinses (<1 min) in 0.25% sodium hypochlorite, then 70% ethanol, followed by a rinse in sterile distilled water, then dissected to excise internal tissues. Internal tissues from different PFW life stages—larvae ($n = 25$), pronymphs ($n = 24$), eonymphs ($n = 20$), pupae ($n = 30$), and adults ($n = 4$)—as well as frass and eggs in groups of 20 ($n = 20$), were placed in FastRNA Tubes (Savant Bio101 Inc.) containing 1 mL of phosphate-buffered saline (PBS), pH 7.2. Homogenization was conducted in a Fast Prep 120 (Bio101 Inc.) at a setting of 4 for 30 s. For bacteriological analysis duplicate 100-µL volumes were removed, serially diluted in PBS, and used as inocula for nutrient agar (NA) plates with incubation aerobically at 25°C. Plates were examined daily over 14 days, with samples of the dominant colony types subcultured to generate pure cultures.

PCR-DGGE PCR assays were performed as previously reported [13], using DNA extracted from duplicate 200-µL aliquots of the tissue homogenates which had also been examined for culturable bacteria. The broadly conserved bacterial 16S rDNA gene primers were p515f (gccagcagccgcggtaa; *E. coli* positions 515–533) and p806r (ggactaccagggtatctaat; *E. coli* positions 806–787). For DGGE, the p515 forward primer was modified at the 5' end with a 40-bp GC-rich clamp sequence that terminated gel migration of products within a concentration gradient of urea/formamide. Amplicons were examined by electrophoresis using 1.5% agarose gels and ethidium bromide staining. DGGE analysis of the 16S rRNA gene amplicons was undertaken as previously described [13]. Briefly, amplicons were separated in a 40–70% gradient using the DCode system (Bio-Rad) for 14 h at 80 V in 1 × Tris/Borate/EDTA (TBE) buffer at a constant temperature of 60°C. Relative mobility standards consisted of p515 (no GC clamp)/p806r PCR amplicons produced from laboratory subcultures of the dominant bacterial species cultured from the insect samples (Table 1) and identified by nucleotide sequence similarity to known 16S rDNA sequences using BLAST (Basic Local Alignment Search Tool). Gels were stained with ethidium bromide and documented with an Imagemaster digital camera and associated annotation software (Amersham Pharmacia Biotech). DGGE bands of interest were extracted and sequenced as previously described [13].

Results and Discussion

In designing this study we sought to sample multiple samples over a number of months, for the specific purposes of identifying the predominant species, possible trends in terms of microbial succession during insect development, and the presence of species known to be involved in pathogenesis or symbiosis in other insects.

The levels of bacterial growth recovered from insect samples were in the range of 1.7×10^3 to 3.6×10^6 cfu mL^{-1}. Eggs and pupae yielded the lowest bacterial counts, with the highest numbers recorded in the frass samples. We observed that many colonies recovered on nutrient agar plates showed highly similar morphologies. Yet upon subculture, as pure cultures on the same medium, it was possible to perceive a variety of distinct colony types which displayed a range of unique textural and olfactory characteristics: features that went undetected when growing on the original mixed culture plates. Given that it would have been impractical to have produced pure cultures of each individual colony, we believe it is possible that our culture-based assessment may have underestimated species diversity. Bacterial identification was performed by sequence analysis of approximately 240 nucleotides of the V5 region of the 16S rRNA gene, with a total of 17 different culturable bacterial species being identified (Table 1). Predominant species included

Table 1 Culturable bacterial species, identified based on partial 16S rDNA sequencing with comparison to sequences deposited in Genbank and used to produce reference ladders for DGGE gels

Ladder A			Ladder B			Ladder C		
Band	Identification[a]	GenBank accession no.	Band	Identification[a]	GenBank accession no.	Band	Identification[a]	GenBank accession no.
A	*Chryseobacterium* sp. (99%)	AY427792	A	*Pseudomonas* sp. (99%)	AY439233	A	*Agrobacterium tumefaciens* (99%)	AF501343
B	Rhizobiaceae bacterium (97%)	AY178080	B	*Pseudomonas fluorescens* (100%)	AJ581980	B	*Pseudomonas* sp. (100%)	AY308043
C	*Serratia fonticola* (98%)	AY236502	C	*Stenotrophomonas maltophilia* (98%)	AY472115	C	*Pseudomonas* sp. (100%)	AY336121
D	*Arthrobacter* sp. (100%)	AY383043	D	*Paenibacillus xyanilyticum* (100%)	AY427832	D	*Janthinobacterium* sp. (97%)	AJ551147
E	*Rhodococcus* sp. (99%)	AF420421	E	*Bacillus sphaericus* (100%)	AY304997	E	*Photorhabdus temperata* (99%)	AY296252
			F	Microbacteriaceae (97%)	AF408987	F	*Pantoea agglomerans* (99%)	AY315453

Note: DGGE, denaturing gradient gel electrophoresis

[a] The bacteria are represented by 16S rDNA sequences covering ~240 nucleotides. Values in parentheses represent the percentage nucleotide sequence homology to the closest relative identified in GenBank

Arthrobacter sp., *Pseudomonas* spp., and *Bacillus sphaericus*, with *Stenotrophomonas maltophilia*, *Paenibacillus* spp., and *Pantoea agglomerans* also isolated frequently (Table 3). *Photorhabdus temperata* was recovered from the majority (16/20) of egg and (18/25) larval samples but was absent from all other developmental stages, with the exception of a single adult specimen. An additional, apparently stage-specific association was noted for a purple pigmented *Janthinobacterium* sp., which was detected only in eonymph (14/20) samples. Indeed, eonymph samples consistently yielded the greatest diversity (14 of 17) of colony types (Table 3). Prior to the eonymphal molt, fully fed European pine sawfly (*Neodiprion sertifer*) larvae evacuate all solid matter from their gut and thereafter the insect is entirely dependent on internal mechanisms to regulate the gut environment [16]. The accumulation of solid gut material at the eonymphal molt stage has been recorded in other sawfly species of the family Diprionidae and was associated with increased growth of *Enterobacter cloacae* [16]. In the case of the PFW, an accumulation of stagnant material in the gut at the eonymph life stage may have provided a suitable environment for the diverse bacterial growth recorded in this study.

The banding patterns generated by a representative subset of the 123 samples analyzed by PCR-DGGE are shown in Fig. 1a and b, and the identities of the major bands are provided in Table 2. This approach confirmed the predominance of some of the species identified via culture, and identified others which were either undetected or underestimated. A minority (8/17) of the species detected by culture were also detected by PCR-DGGE (Table 3). It is pertinent to note that many of the undetected culturable species, including *Serratia fonticola*, *Rhodococcus* sp., and A*grobacterium tumefaciens*, represented minor components of the culturable microflora. In contrast, *Arthrobacter* sp., *Janthinobacterium* sp., and *P. temperata* were recorded as major colony types in a variety of samples and their absence highlighted some of the possible limitations of PCR-DGGE community profiling [9].

Regarding egg and larval samples, major bands were identified as a *Chryseobacterium* species (Fig. 1a), which had been cultured at low numbers from a single larval and two pooled egg samples, and a *Pseudomonas* sp. (GenBank accession number AY30843*)*, which had been frequently cultured from such samples (Table 3). In addition, *Photorhabdus luminescens* was detected as a strong band in most egg and larval samples (Fig. 1a), despite not having been detected by plating. Examination of later life stages revealed the continued predominance of the *Chryseobacterium* species (band 5; Fig. 1b) which had been isolated sporadically from pronymph, eonymph, and pupal samples at low numbers (Table 3). Yet again, *Pseudomonas* species

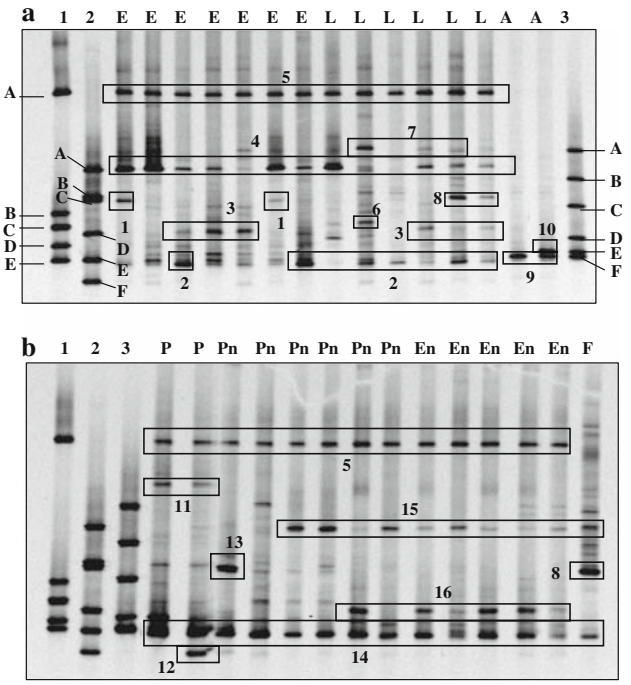

Fig. 1 Analysis of 16S rDNA fragments recovered by PCR-DGGE analysis using DNA extracted from pine false webworm tissue homogenates. 1, 2, and 3 refer to the species ladders based on culturable bacteria (see Table 1 for details). For additional lanes E, egg; L, larva; A, adult; P, pupa; Pn, pronymph; En, eonymph; F, frass. The relationships of the isolated fragments to other sequences in GenBank are provided in Table 2

were frequently detected as strong bands in these sample types (Fig. 1b). In support of the culture-based data, *B. sphaericus* emerged as a dominant band in the later developmental stages (band 14; Fig. 1b), while it had not been detected in either egg or larval samples (Fig. 1a and Table 3).

The majority of the species identified in this study by PCR-DGGE and culture have previously been reported in association with insects and/or plant material, an observation which supported their isolation from PFWs. In contrast, the identification of a *Leptotrichia* sp. and putative *Eubacterium* sp. in 4 of the 25 larval samples, which had been collected and processed at different times, was somewhat intriguing. These genera are most commonly associated with warm-blooded animals, although *Eubacterium* spp. have been isolated less frequently from plant products and soil [8, 14]. Both require anaerobic atmospheres for growth, and as such neither would have been detected using the culture method employed in our study.

The observation that a *Chryseobacterium* sp. was found as a dominant band in all samples, with the exception of those produced from adult insects or from frass, suggested that this bacterium was somehow benefical to the PFW. *Chryseobacterium* strains have been found in a variety of ecological niches including aquatic animals, soil, sewage, fresh water, marine sediments, clinical samples, and various food sources and associated with plants [3]. A feature of some *Chryseobacteria* spp. is their ability to resist both subzero environmental temperatures and alternating, natural freeze-thaw cycles [19]. These abilities are linked to the possession of both ice recrystallization inhibition and ice shaping activities, which promote autosurvival and may be exploited by other bacteria in the same environment to enhance thermal stress tolerance [19]. In Ontario PFW

Table 2 Relationship of excised DGGE band sequences, amplified from PFW samples, to other sequences in GenBank

Band no.	Identification[a]	GeneBank acession no.	Homology (%)
1	*Nocardia ignorata*	AY191254	100
2	*Photorhabdus luminescens*	AY444555	99
3	*Paenibacillus* sp.	AY337581	100
4	*Pseudomonas* sp.	AY439233	99
5	*Chryseobacterium* sp.	AY427792	99
6	*Eubacterium* sp.	AY230774	94
7	*Leptotrichia* sp.	AF189244	99
8	*Pseudomonas* sp.	AY315457	100
9	*Pantoea agglomerans*	AY315453	99
10	*Photorhabdus temperata*	AY296252	99
11	*Flavobacterium* sp.	AY162137	92
12	Microbacteriaceae	AF408987	97
13	*Stenotrophomonas maltophilia*	AY472115	98
14	*Bacillus sphaericus*	AY304997	100
15	*Pseudomonas* sp.	AY308054	99
16	*Paenibacillus xyanilyticum*	AY427832	100

Note: DGGE, denaturing gradient gel electrophoresis; PFW, pine false webworm.
[a] The bacteria are represented by 16S rDNA sequences covering ~240 nucleotides. Values in the last column represent the percentage nucleotide sequence homology to the closest relative identified in GenBank. Band numbers refer to those provided in Fig. 1a and b

Table 3 Correlation between bacterial species and insect instar

Culturable bacteria							Identifed by DGGE						
Bacterial species	E	L	Pn	En	P	A	Bacterial species	E	L	Pn	En	P	A
B. sphaericus	—	—	X	X	X	X*	*B. sphaericus*	—	—	X	X	X	—
Chryseobacterium sp.	X*	X*	X*	X*	X*	—	*Chryseobacterium* sp.	X	X	X	X	X	—
P. agglomerans	—	X*	X*	X	X*	X	*P. agglomerans*	—	—	—	—	—	X
Microbacteriaceae	—	—	X	—	X	—	Microbacteriaceae	—	—	X*	—	X	—
P. temperate	X	X	—	—	—	—	*P. temperate*	—	—	—	—	—	X*
S. maltophilia	—	X	X	X*	X	X*	*S. maltophilia*	—	—	X	—	—	—
P. xyanilyticum	—	X*	X	X*			*P. xyanilyticum*	—	—	X*	X	—	—
Pseudomonas sp. AY439233	X	X	—	X*	—	—	*Pseudomonas* sp. AY439233	X	X	—	—	—	—
A. tumefaciens	—	—	—	X*	—	—	*P. luminescens*	X	X	—	—	—	—
Arthrobacter sp.	X	X	X	X	X*	X	*Paenibacillus* sp.	X	X	—	—	—	—
Janthinobacterium sp.	—	—	—	X	—	—	*Pseudomonas* sp. AY308054l	—	—	X*	X	—	—
P. fluorescens	X	X*	X	X	X*	X	*N. ignorata*	X*	—	—	—	—	—
Rhizobiaceae bacterium	—	—	—	X	—	—	*Eubacterium* sp.	—	X*	—	—	—	—
Rhodococcus sp.	—	—	X*	X*	—	—	*Leptotrichia* sp.	—	X*	—	—	—	—
S. fonticola	—	—	X*	X*	X*	—	*Flavobacterium* sp.	—	—	—	—	X*	—
Pseudomonas sp. AY308043	X	X	X*	X*	X	X	*Pseudomonas* sp. AY315457	—	X*	—	—	—	—
Pseudomonas sp. AY336121	X	X*	X	X	—	X*							

Note: DGGE, denaturing gradient gel electrophoresis; E, egg; L, larvae; Pn, pronymph; En, eonymph; P, pupae; A, adult. X, recovered from >50% of specimens; X*, recovered from <50% of specimens; —, not recovered

eonymphs are commonly encountered just below the soil surface, to a maximum depth of 9 cm, at times when soil temperatures are near zero [11]. Similarly, pupation occurs during periods of low soil temperatures, when ice crystals are still present in the soil [11]. Numerous insect species have developed mechanisms, e.g., production of antifreeze proteins, cryoprotectants, and extracellular ice nucleators, to tolerate subzero temperatures [4], and bacteria are involved in some of these adaptive processes [7]. Against this background, we would speculate that *Chryseobacterium* may play a role in enhancing thermotolerance in the PFW.

The combined results of our diphasic analysis indicated that the microflora of this insect was predominated by a limited number of species and demonstrated that some of the apparent life-stage-specific associations identified would have gone unnoticed had only culture or PCR/DGGE been used. The increasing popularity of PCR-based, culture-independent methods to the study of microbial communities is based mainly on the premise that they will help to reveal the nature of the 99% of the bacterial community which cannot be cultured on bacteriological media. Yet it is relevant to observe that, in common with culture-based methods, culture-independent techniques also suffer from a number of limitations which must be considered when interpreting the results obtained using these approaches [9].

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