PRELIMINARY EXAMINATION OF GUT BACTERIA FROM *NEODIPRION ABIETIS* (HYMENOPTERA: DIPRIONIDAE) LARVAE

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Abstract *J. ent. Soc. Ont.* 138: 49–63

The gut microbiotas of insects are important for many processes, including digestion, nitrogen fixation, and nutrient recycling. Bacterial 16S ribosomal DNA (rDNA) extracted from excised *Neodiprion abietis* larval guts was amplified using PCR. Two combinations of primers produced six fragments that were separated using Denaturing Gradient Gel Electrophoresis (DGGE). The DNA fragments were sequenced directly. BLAST-n analysis and comparison-rank searches, using the Ribosomal Database Project II, revealed four predicted bacterial species, one that had similarity to Alphaproteobacteria and three that aligned with Gammaproteobacteria. Phylogenetic analysis by maximum parsimony and neighbour joining confirmed these findings and suggest that *Rahnella, Yersinia*, *Enterobacter*, and a *Caulobacter*-like species inhabit the *N. abietis* larval gut.

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Introduction

The balsam fir sawfly, *Neodiprion abietis* (Hymenoptera: Symphyta: Diprionidae), is an indigenous phytophagous insect in North America. The larvae feed predominantly on balsam fir (*Abies balsamea* Mill), but will also consume white spruce (*Picea glauca* Moench) and black spruce (*Picea mariana* Mill.) (Wallace and Cunningham 1995). Outbreak populations typically occur every 5-15 years, lasting 4-5 years in duration (Piene et al. 2001; Moreau et al. 2005). Larvae emerge in early summer after overwintering as eggs sheltered in the needles of the host plant. Male larvae pupate after their fifth instar, whereas female larvae may go through an additional instar before pupation. Adults emerge in late summer and, after mating, females lay eggs in current year foliage, using a saw-

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like ovipositor. The majority of current knowledge regarding diprionid sawflies is based on ecological (Wallace and Cunningham 1995; Li et al. 2005; Moreau et al. 2005) and anatomical studies (Bordas 1895; Maxwell 1955). Current knowledge of the structure and organization of the sawfly digestive tract is limited, and almost nothing is known about the microbiota of sawfly guts.

The insect gut is a complex and highly structured organ. Gut morphology and function are dependent on several factors: the insect taxon, its stage of development, feeding behaviour of each developmental stage, food source, environment that the insect inhabits, and the inhabiting microorganisms (Wigglesworth 1972; Chapman 1985; Nation 2002; Dillon and Dillon 2003). The first comparative review of hymenopteran guts was made, by Bordas (1895), describing the macro-morphology of guts from selected insects of every family in the order Hymenoptera. Sixty years later, Maxwell (1955) compared the internal anatomy of larvae from 132 species, in eleven families of North American and European sawflies, within the suborder Symphyta. Neither of these reviews on sawfly gut morphology mentioned gut microbes.

The natural microbiota of the gut represent microbial-host interactions that range from pathogenic to obligate mutualism. Studies that define the composition of microbial communities in the digestive system have primarily been performed using termites, tsetse flies, aphids, and cockroaches (Dillon and Dillon 2003). Recent interest in insect endosymbionts, such as bacteria in the genera *Wolbachia*, *Buchnera*, or *Wigglesworthia* bacteria, has increased our knowledge about relationships of microbes with their insect hosts. Termite microbiota are best characterized, primarily because the functional roles of gut microbes in other insects have not been investigated (Brune 1998; Bignell 2000; Breznak 2000). In termites, microbes are mainly located within specialized regions and structures of the gut. The majority of the termite microbiota are found in pouches of the hindgut, where bacterial densities can reach 10^{11} cells per ml of gastric fluid (Breznak and Pankratz 1977). In the midgut, microbial communities are typically sparse and tend to localize between the microvilli of the epithelial cells (Breznak and Pankratz 1977). Microorganisms may colonize the gut wall, attach to surfaces such as spines, or course freely in the lumen (Bignell 2000). Depending on the termite species and its food source, the functional role of the microbes may range from fermentation and hydrogen production to nitrogen recycling and carbon elimination (Breznak and Pankratz 1977; Brune 1998; Bauer et al. 2000; Bignell 2000; Brauman et al. 2001).

Recent studies of gut microbiota in the ant genera *Camponotus, Solenopsis*, and *Tetraponera* (Hymenoptera: Formicidae), have shown that bacteria localize to bacteriocytes within the midgut and the pouch of the hindgut (Shannon et al. 2001; Sauer et al. 2002; van Borm et al. 2002a; Li et al. 2005). These symbiotic microbiota are members of Alpha, Beta, and Gamma divisions of Proteobacteria, as well as Flavobacteria (van Borm et al. 2002a), and including a novel candidate genus, *Blochmannia* (Sauer et al. 2000; Sauer et al. 2002). Classification of these microbes was accomplished by culture-independent methods as these bacteria often cannot be cultured outside of their hosts (Schroder et al. 1996). In addition, media for culturing has typically been developed for medical studies and the growth conditions for fastidious microorganisms are often lacking, leading to misrepresentative sampling of the gut microbiota (Dillon and Dillon 2003). To surmount these difficulties, sequence analysis of 16S ribosomal DNA (rDNA) has become widely accepted as a tool

for investigating unculturable microbes in these often complex communities (Hongoh et al. 2003a, b).

This manuscript represents the first preliminarily analysis of the gut microbiota of a diprionid species. PCR was used to amplify bacterial 16S ribosomal DNA (rDNA), extracted from excised larval guts, and along with Denaturing Gradient Gel Electrophoresis (DGGE) revealed 4 distinct DNA products. BLAST-n analysis and comparison rank searches of the Ribosomal Database Project II (RDP II) database showed similarity to Alphaproteobacteria and Gammaproteobacteria. Maximum parsimony and neighbour joining analyses confirmed these observations.

Materials and Methods

Larval collection

Balsam fir branches, containing *Neodiprion abietis* larvae, were collected from forest stands near Old Man's Pond (near Corner Brook), Newfoundland, Canada (N 49º 05"59' W 57º 56"05'). Larvae were maintained on balsam fir in paper bags at 4ºC. Head capsule widths of healthy larvae were measured using a dissecting microscope with a calibrated objective. Larvae with head-capsule widths between 0.68-1.4 mm, corresponding to 2nd to 4th instar larvae, were harvested for histological preparation and extraction of total DNA from the excised gut.

PCR amplification, DGGE, and sequencing of bacterial *16S* gene

Larvae harvested for molecular characterization of sawfly-gut bacteria were surface sterilized with a 60 second wash in 5% bleach, followed by a 60 second rinse in DEPC-treated water (0.1% diethyl pyrocarbonate). Larvae were submerged in sterile phosphate buffered saline (PBS, pH 7.4), and anterior and posterior segments were excised just posterior of the head capsule and immediately anterior to the eighth proleg, respectively. The cuticle was secured and the gut was pulled from the body cavity. The excised gut was transferred to fresh PBS and the peritrophic membrane, containing the food bolus, was pulled from the gut lumen using forceps. The gut tissue was immediately placed into RNAlater (Ambion Inc., Austin, Texas) and stored at -20ºC.

DNA was purified using TRIzol (Invitrogen Co., Burlington, Ontario), following the manufacturer's protocol. Two primer sets were used to ensure amplification of the targeted bacterial 16S rDNA. Primers p984f-GC (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGG GGGCACGGGGGGAACGCGCCGAACCTTAC-3') and p1401r (5'-GCGTGTGT ACAAGACCC-3') were used to amplify the V6 to V8 regions of 16S ribosomal DNA (Nòbel et al. 1996; Frederick and Caesar 2000). Primers p515f-GC (5'-CGCCC GGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGCCAGCAGCCGCGGTAA -3') and p806r (5'-GGACTACCAGGGTATCTAAT-3') were used to amplify the variable V4 region of 16S rDNA (Relman 1993). PCR mixtures of 50 µl volume contained reaction buffer (10 mM Tris-HCl pH 8.3 at 25°C, 50 mM KCl, 1.5 mM MgCl, 0.001% gelatin), 10 µM each of dATP, dTTP, dCTP, and dGTP, 0.1 µM of each primer, 1 unit *Taq* polymerase (Qiagen, Mississauga, Ontario) and approximately 10 ng insect genomic DNA template. PCR was conducted using a Mastercycler EP thermal cycler (Eppendorf, Mississauga,

Ontario), with the following settings: (i) 94°C for 5 min, 1 cycle; (ii) 94°C for 30 sec, 52°C for 30 sec, 72°C for 45 sec, 40 cycles; (iii) 72°C for 5 min, 1 cycle. On completion of thermal cycling, 10% of the reaction was loaded on a 1% agarose gel and electrophoresed in 1X TBE buffer (90 mM Tris Borate, pH 8.3, 2 mM EDTA) for 2 hrs at 60 V. The gel was stained with ethidium bromide and visualised using UV illumination.

Subsequently, the PCR products (50% of the reaction) were separated by DGGE using the DCode system (BioRad) according to the manufacturer's instructions. Gels consisted of 1 mm thick 6% polyacrylamide with a denaturing gradient of 30-70 % (100% denaturant corresponds to 7 M urea and 40 % vol/vol deionized formamide) and 1X TAE buffer (90 mM Tris Acetate, pH 8.3, 2 mM EDTA) for 16 hours. Electrophoresis was performed at 60ºC and 80 V in 1X TAE running buffer for 16 hours. Gels were stained with SYBR Gold nucleic acid stain (Invitrogen) for 30 minutes and images captured upon UV illumination. DNA bands were excised with a sterile razor blade and placed in 100 µl of sterile distilled H_2O. The samples were placed at 94ºC for 5 minutes to elute the DNA from the polyacrylamide and were stored at 4ºC overnight. Five µL of the supernatant were used as template to reamplify the individual DNA bands. The PCR conditions were the same as above, but with only 30 cycles of amplification. The PCR products were gel purified using the QIAquick Gel Extraction Kit (Qiagen), and samples stored at -20ºC until ready for sequencing. Sequencing was performed by Ontario Genomics Innovation Centre, using an ABI 3730 DNA Analyzer (BigDye version 3.1).

Sequence data was analysed using BLAST-n (http://www.ncbi.nlm.nih.gov/BLAST) and the Similarity Rank program of the RDP II (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) (Maidak et al. 1999), to determine similarity with known bacterial species (in the database). Closely related species, as well as gut microbiota listed in recent publications (Boursaux-Eude and Gross 2000; Sauer et al. 2000; Shannon et al. 2001; van Borm et al. 2002b; Hongoh et al. 2005), were used to construct phylogenetic trees using neighbour joining and maximum parsimony algorithms, with 1000 bootstrap replicates. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar et al. 2004).

Results

DGGE separated four 16S rDNA fragments when using the p984f-p1401r primer set (Table 1, #1 to 4), and two fragments after amplification from the p515f-p806r primer set (Table 1, #5 and 6). BLAST-n analysis and similarity rank comparisons to the RDP II sequence database predicted four bacteria matches: *Rahnella* sp. (sequence #1, GenBank Accession No. EF140875), *Yersinia* sp. (sequences #2-4, GenBank Accession No. EF140876-EF140878), an Enterobacteriaceae (sequence #5, GenBank Accession No. EF140880), and an Alphaproteobacteria (sequence #6, GenBank Accession No. EF140879).

Phylogenetic analysis confirmed the predicted identities of the first four bacteria and showed their close relationship to other known insect-gut microbes in the Enterobacteriaceae family of Gammaproteobacteria. Maximum parsimony and neighbor joining analyses suggest that sequence #1 was most closely related to *Rahnella aquatilis*. Both analyses weakly supported the clustering of sequences #2-4 with *Yersinia*, with the degree of their

TABLE 1. Highest NCBI BLAST-n[1] results and RDP II comparison values[2] (from GenBank and the RDP II databases) for portions of 16S ribosomal DNA sequences obtained from DNA extracted from the microbiota of *Neodiprion abietis* larval-midguts (after amplification by PCR and separation by DGGE).

DGGE fragment (primer set)	Amplicon size (bp)	Predicted Identity	Blast Match Accession Number (% identity)	RDP (% identity)
#1 (p984f-GC/p1401r)	318	*Rahnella* sp.	U90758 (99%), DQ440548 (97%)	S000438772 (96.5%), S000653581 (96.5%)
#2 (p984f-GC/p1401r)	321	*Yersinia* sp.	AJ627599 (99%), AJ627600 (99%)	S000539482 (94.6%), S000539483 (94.6%)
#3 (p984f-GC/p1401r)	314	*Yersinia* sp.	AJ627599 (99%), AJ627600 (99%)	S000539482 (95.3%), S000539483 (95.3%)
#4 (p984f-GC/p1401r)	308	*Yersinia* sp.	AJ627599 (99%), AJ627600 (99%)	S000539482 (100%), S000539483 (100%)
#5 (p515f-GC/p806r)	180	Enterobacteriaceae sp.	AY859722 (97%) U93263 (97%) DQ440548 (97%)	S000015297 (100%), S000497099 (100%)
#6 (p515f-GC/p806r)	184	Uncultured Alphaproteobacterium	AJ459874 (100%), DQ163946 (100%)	S000093253 (100%), S000600168 (100%)

[1] NCBI BLAST website:http://www.ncbi.nlm.nih.gov/BLAST

[2] RDP II website: http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp

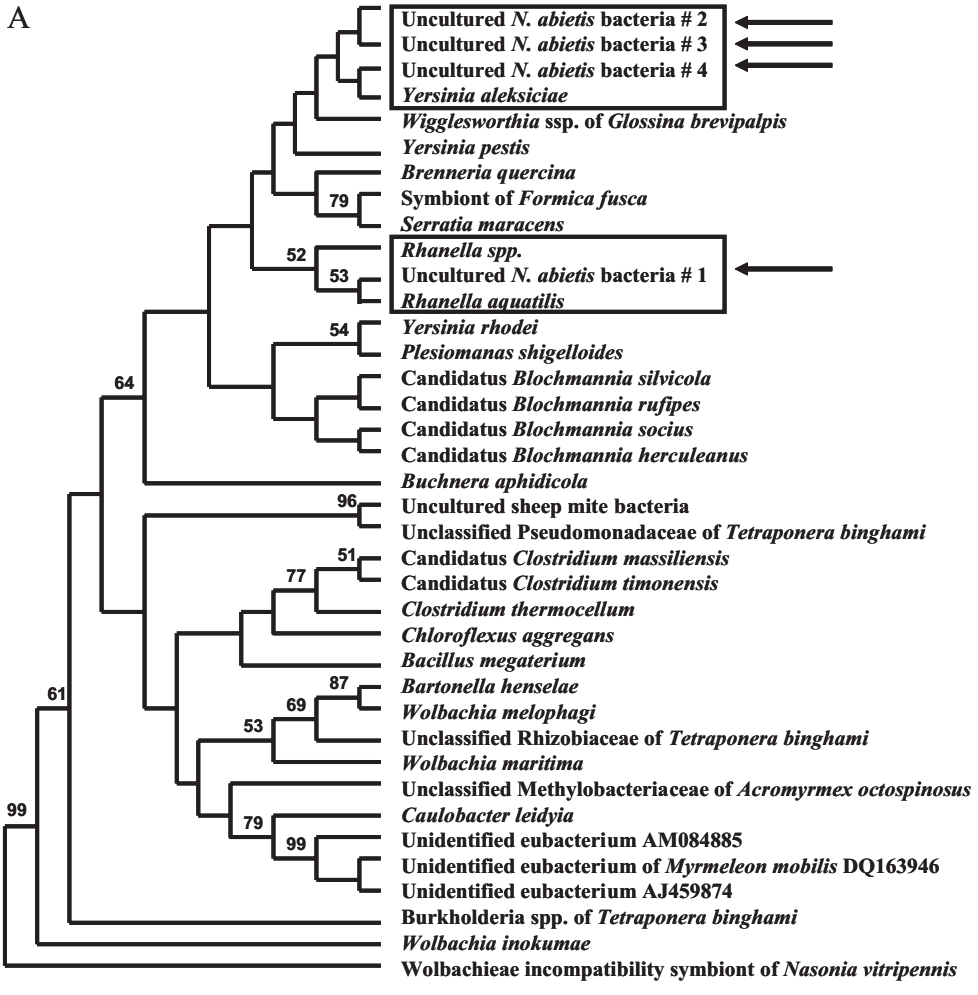

FIGURE 1. Phylogenetic analyses of bacterial 16S ribosomal DNA gene sequences amplified from insect guts. Maximum parsimony (A and C) and neighbour joining trees (B and D) were inferred using the Mega 3.1 program with 1000 bootstrap repetitions. Support values >50% are listed at nodes. Sequences of 16S ribosomal DNA from the microbiota of *N. abietis* larval guts are indicated with arrows. Bacteria identified by sequences #1–4 (from primer set p984f-GC/p1401r) are represented in Trees A and B, while bacteria identified by sequences #5 and #6 (primer set p515f-GC /p806r) are represented in Trees C and D. Boxes indicate groups referred to in Table 1.

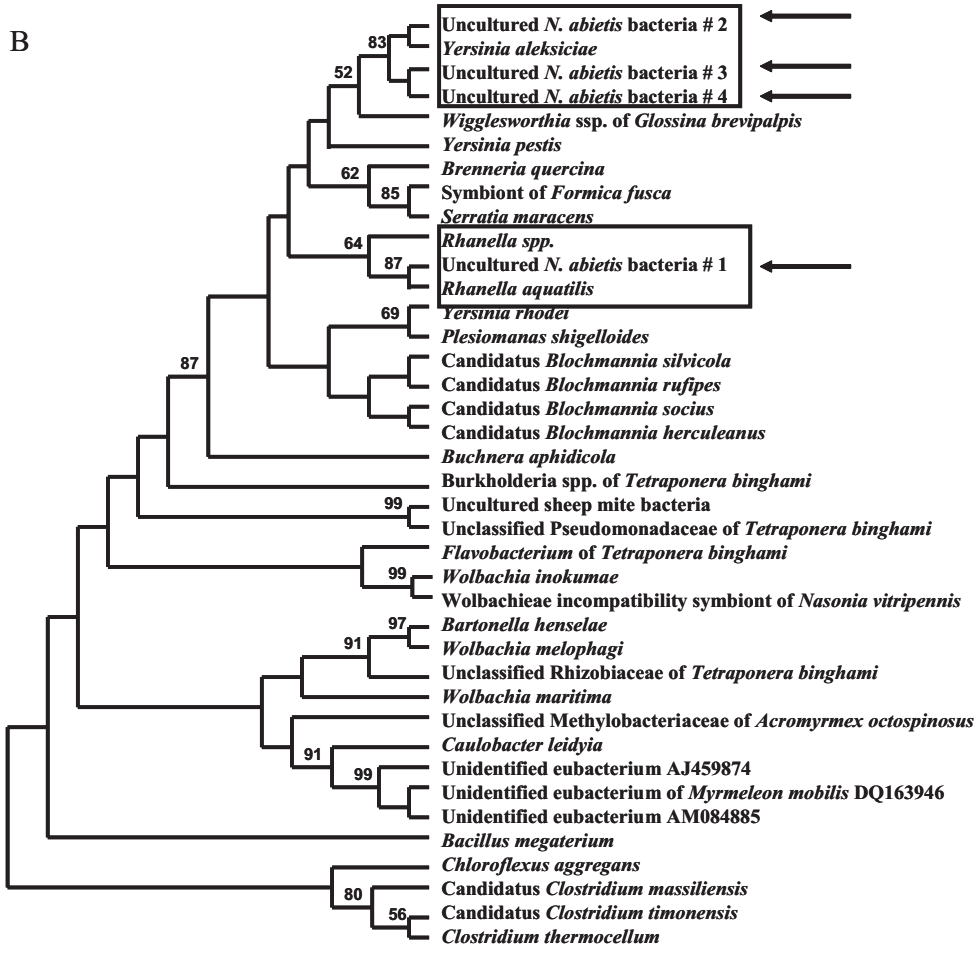

FIGURE 1. Continued

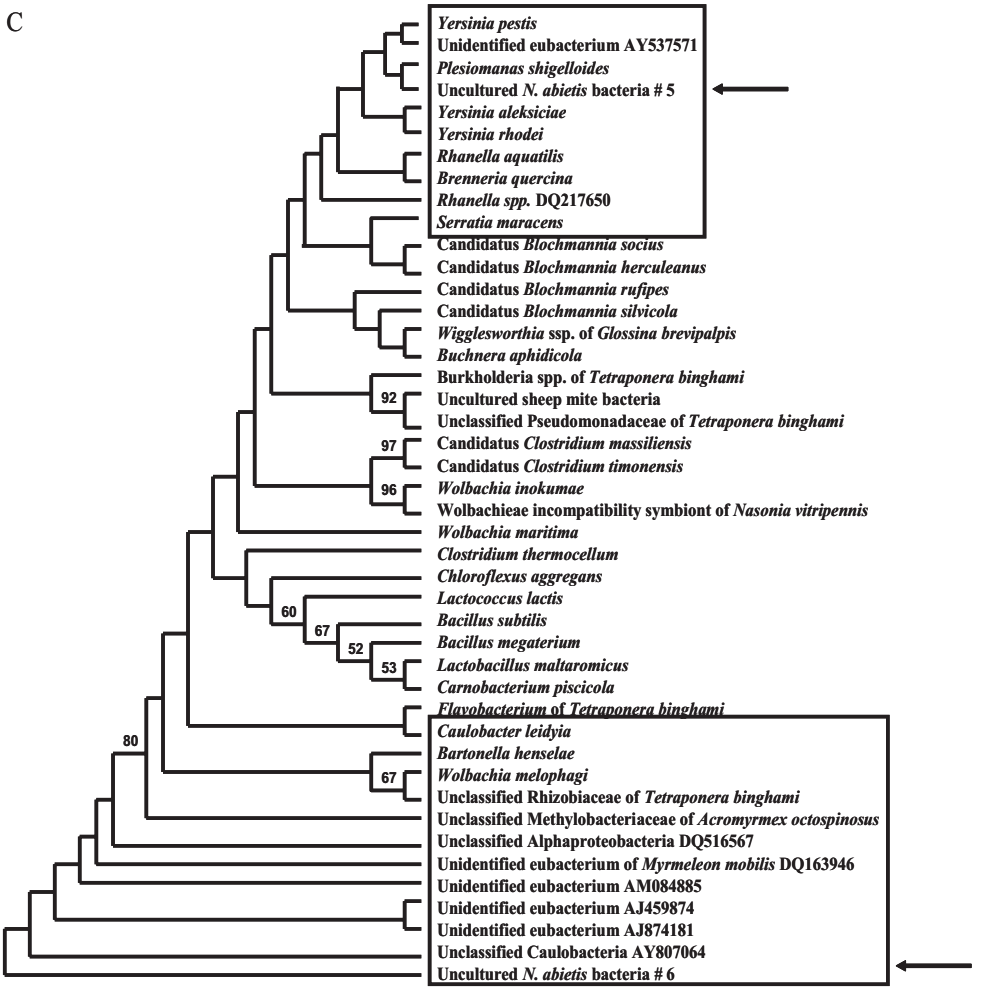

FIGURE 1. Continued

D

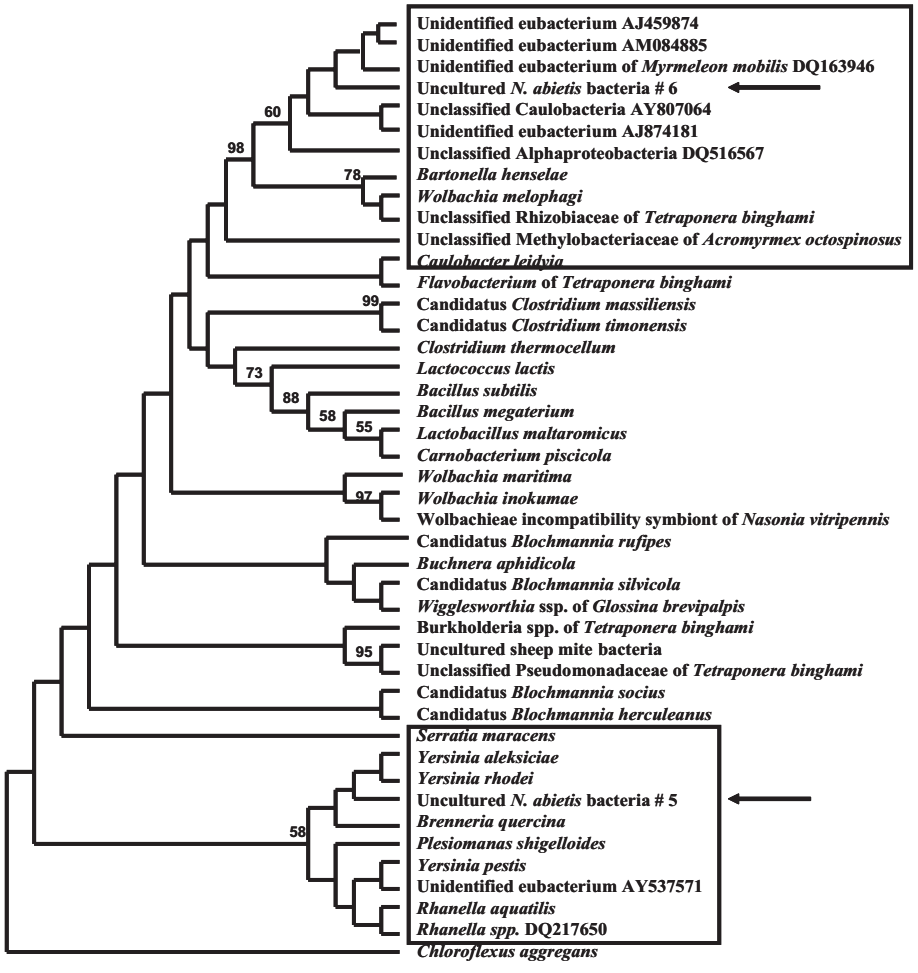

FIGURE 1. Continued

relatedness to *Yersinia aleksiciae* varying (Table 1 and Figure 1). The three 16S rDNA sequences showed 98.7% identity to each other and were approximately 320 bp in length.

The identity of the bacterium from which sequence #5 was derived was not determined beyond Enterobacteriaceae because results from maximum parsimony and neighbor joining analyses were inconsistent. The difficulty in confirming the identity of this bacterium may be a result of the small amplicon size (180 bp). However, sequence #6 (184 bp), which was amplified with the same primer set, clearly clustered with the Alphaproteobacteriaceae. Maximum parsimony analysis suggested that bacteria, from which sequence #6 was derived, belonged to the genus *Caulobacter.*

Discussion

The microbiota identified by 16S rDNA sequences from *N. abietis* gut tissues include those that have been found ubiquitously in the environment and likely originated from the host's diet (Selenska-Pobell et al. 1995; Dillon and Charnley 2002; Sprague and Neubauer 2005). Similarly, other free-living microbial species have been isolated from other sawfly gut tissues, including *Pristiphora geniculata*, *Acantholyda erythrocephala*, and *Pikonema alaskensis* (R. Graham, unpublished data). Fragments #1-5 (based on 16S rDNA sequences) represent bacteria that belong to the Gammaproteobacteria, specifically those in the Enterobacteriaceae family of Gram-negative, anaerobic microbes.

Neither *Rahnella aquatilis* nor *Yersinia aleksiciae* have been published as insect gut microbes, although *R. aquatilis* has been isolated from both chicken ticks (Montasser 2005) and the intestinal contents of snails (Brenner et al. 1998). An uncultured *Rahnella* sp. was reported in GenBank (Accession # U84730) from an isolate of the microbial gut flora from the coleopteran genera *Phaleria* and *Latreille* (Tenebrionidae). *Rahnella* spp. have been isolated from foliage (Hashidoko et al. 2002; Izumi et al. 2006) and ferment several polysaccharides (Brenner et al. 1998). Additionally, *Rahnella* spp. have been recognized as strong nitrogen fixers (Brenner et al. 1998; Izumi et al. 2006). This characteristic would be important for nitrogen recycling in nutrient-poor diets and possibly promote its retention as a symbiont within the gut, perhaps originally acquired through the sawfly's diet.

Species of *Yersinia* have been isolated from other insect guts (Ulrich et al. 1981); therefore it is not surprising that we found related bacteria in the gut of *N. abietis.* No beneficial characteristics have been attributed to *Yersinia*. Their ubiquitous presence in soils and detritus suggest that this bacterium is more likely to be a transient microbe ingested with food matter, rather than part of the permanent flora of the sawfly gut.

16S-sequence analysis of fragment #5 and subsequent phylogenetic comparisons to other Gammaproteobacteria was inconsistent and poorly resolved. Maximum parsimony indicated that the closest relative to the *N. abietis* bacteria was *Plesiomanas shigelloides*, while neighbour joining analysis suggested that *Y. rhodei* was more closely related. BLAST-n searches of the 16S ribosomal sequence commonly aligned *Serratia* spp. with high degrees of identity (97%). *Yersinia, Rahnella,* and *Serratia* spp. have been shown to cluster closely together in a Group B of the enterobacterial genera, with the main signature nucleotides located between positions 590-649 (Sproer et al. 1999). The p515f-p806r primer set amplifies the variable V4 region of 16S rDNA between base pairs 627 and 807. This

region only overlaps the signature nucleotides by 22 bp, making a positive identification difficult. Therefore bacterial sequence #5 can only be classified as an Enterobacteriaceae until further data is collected.

Finally, 16S-sequence analyses indicated that bacterium #6 was a member of the Alphaproteobacteria, showing high similarity with uncultured bacteria of insect larvae and soil (GenBank AJ459874, DQ163946, and AM084885; Table 1; Figure 1 C, D). An uncultured *Caulobacter* (GenBank AY807064) aligned within the unidentified Alphaproteobacteria, supported by a 98% bootstrap value, suggesting that the *N. abietis* bacterium #6 may be Caulobacter-like. Although Caulobacteria have typically been isolated from aquatic environments, a few isolates have been reported from the intestinal contents of a millipede (Abraham et al. 1999) and the mite *Tetranychus urticae* (Hoy and Jeyaprakash 2005). If *N. abietis* bacterium #6 is a *Caulobacter*, this microbe may play a key role in nutrient acquisition since Caulobacteria have been shown to uptake phosphorus from nutrient-poor environments (Gonin et al. 2000). Chemical analyses of current year foliar nutrients have reported phosphorous levels at 900-4000 ppm along the eastern US coastline and in the Laurentide-Onatcheway region of Québec, Canada (Bauce et al. 1994; Richardson 2004). Although foliar chemical data for balsam fir growing in Newfoundland could not be found, it is known that phosphorous levels decline rapidly in trees growing in harsh conditions (Richardson 2004). Due to the often severe climate of Newfoundland, one would predict phosphorous levels at the lower end of the range reported.

The diversity of the gut microbiota of *N.* abietis, using a PCR prospecting approach, is relatively low compared to the variety of microbes observed in termite and cockroach guts (Cruden and Markovetz 1984; Hongoh et al. 2003a). Approximately 270 phylotypes have been detected in the gut of *Reticulitermes speratus* and the bacteria were classified into 9 of the 20 phyla of eubacteria (Hongoh et al. 2003a). In contrast, only 6 phylotypes were detected in *N. abietis* and were classified within a single eubacterial phylum (Proteobacteria).

Although low levels of bacterial diversity within insect guts are not uncommon, the microbiota are generally composed of multiple phyla. The gut of the gypsy moth, *Lymantria dispar* (Order Lepidoptera) has a microbial diversity that ranges from 7 to 15 phylotypes, depending on its diet source (Broderick et al. 2004). A total of 13 genera were identified from larvae feeding on all diet sources and were classified within the Actinobacteria, the Bacteroidetes/Chlorobi group, Firmicutes, and Proteobacteria. Similar results were obtained from cultured isolates and 16S sequence analysis of microbes detected within the midgut of *Culex quinquefasciatus* (Order Diptera), where bacteria from 13 genera were identified (Pidiyar et al. 2004). The majority of mosquito bacteria belonged to the Gammaproteobacteria class (60% of cultured and 46% of culture-independent), while Actinobacteria and Firmicutes constituted the remainder of the bacterial types.

While diet influences the acquisition of bacterial flora observed in insect guts, morphology is often a significant factor affecting the diversity of the gut microbiota. Many termites and cockroaches have evolved complex and convoluted guts (Wigglesworth 1972; Brune and Friedrich 2000) that allow the retention of bacteria in specialized fermentation structures. Insects possessing simple and straight alimentary canals, such as the Diprionidae, Lepidoptera, and many Diptera, generally have a lower diversity of gut microbes (Dillon and Dillon 2003). Due to the selective diet of *N. abietis* and the simple morphology of its gut, the low level of bacterial diversity is not unexpected.

Nonetheless, 16S rDNA sequencing and phylogenetic analyses identified six phylotypes in the larval gut of the balsam fir sawfly; four of the bacteria were clustered with *Rahnella sp*. and *Yersinia sp.*, while the other two bacteria were determined to belong to the Enterobacteriaceae and Caulobacteriaceae. Whether or not they are present as obligate endobionts, they may variously play significant roles as associated microflora in sawfly larvae.

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