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ORIGINAL ARTICLE

Analysis of cellulolytic and hemicellulolytic enzyme activity within the *Tipula abdominalis* (Diptera: Tipulidae) larval gut and characterization of *Crocebacterium ilecola* gen. nov., sp. nov., isolated from the *Tipula abdominalis* larval hindgut

Theresa E. Rogers and Joy Doran-Peterson

Microbiology Department, University of Georgia, Athens, Georgia, USA

Abstract In forested stream ecosystems of the north and eastern United States, larvae of the aquatic crane fly *Tipula abdominalis* are important shredders of leaf litter detritus. *T. abdominalis* larvae harbor a dense and diverse microbial community in their hindgut that may aide in the degradation of lignocellulose. In this study, the activities of cellulolytic and hemicellulolytic enzymes were demonstrated from hindgut extracts and from bacterial isolates using model sugar substrates. One of the bacterial isolates was further characterized as a member of the family *Microbacteriaceae*. Taxonomic position of the isolate within this family was determined by a polyphasic approach, as is commonly employed for the separation of genera within the family *Microbacteriaceae*. The bacterial isolate is Gram-type positive, motile, non-sporulating, and rod-shaped. The G + C content of the DNA is 64.9 mol%. The cell wall contains B2γ type peptidoglycan, D- and L-diaminobutyric acid as the diamino acid, and rhamnose as the predominant sugar. The predominant fatty acids are 12-methyltetradecanoic acid (ai-$C_{15:0}$) and 14-methylhexadecanoic acid (ai-$C_{17:0}$). The isolate forms a distinct lineage within the family *Microbacteriaceae*, as determined by 16S rRNA sequence analysis. We propose the name *Crocebacterium ilecola* gen. nov., sp. nov., to accommodate this bacterial isolate. The type species is T202^T (ATCC BAA-1359; GenBank Accession DQ826511).

Key words cellulolytic, hemicellulolytic, hindgut

Introduction

Larvae of the aquatic crane fly *Tipula abdominalis* (Say) (Diptera: Tipulidae) are major shredders of leaf litter detritus in small order stream ecosystems of the northern and eastern United States (Martin, 1987). The *T. abdominalis* larval gut is composed of a foregut, gastric ceca, midgut, pylorus, ileum, hindgut and rectum (Fig. 1). The pH of the midgut ranges from 8.5 to 11.6, with the highest alkalinity located in the middle of the midgut, and the hindgut pH range is from 7.1 to 7.5 (Martin, 1987). The midgut and the hindgut harbor a dense microbial community, while other sections of the alimentary tract are not colonized. The midgut lumen consists of a microbial community similar to that found on conditioned leaf detritus with 10^8 cells/mg, and the midgut wall is not colonized. The hindgut lumen and wall are densely colonized, with 10^9 and 10^{10} cells/mg, respectively (Klug & Kotarski, 1980). The hindgut is compartmentalized into anterior and posterior sections, and the anterior hindgut is commonly referred to as the fermentation chamber (Martin, 1987). The morphological diversity of the microbial community residing in the *T. abdominalis*

Correspondence: Joy Doran-Peterson, Microbiology Department and Biofuels, Biopower, and Biomaterials Initiative, University of Georgia, 527 Biological Sciences Building, 1000 Cedar Street, Athens, GA 30602, USA. Tel: 706 542 4115; fax: 706 542 2674; email: jpeterso@uga.edu

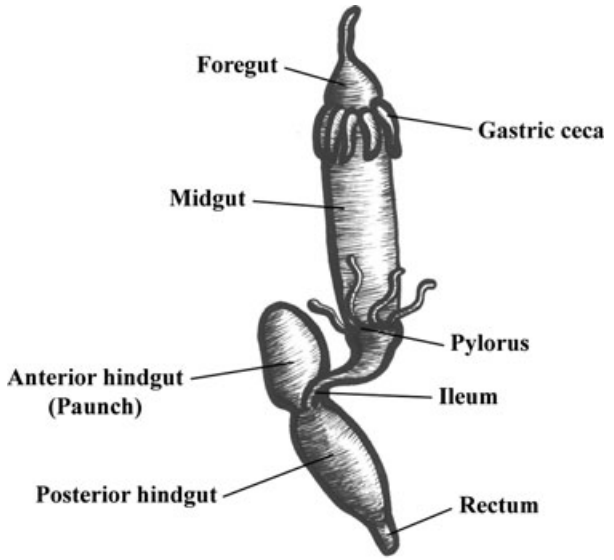

Fig. 1 *Tipula abdominalis* alimentary tract. Figure is redrawn from Klug and Kotarski (1980). Figure is not drawn to scale.

larval alimentary tract was studied via microscopy and direct isolation (Klug & Kotarski, 1980). The microbial communities associated with the anterior and posterior hindgut lumen and wall are different and more morphologically diverse than the microbial community associated with leaf detritus. The posterior hindgut wall harbors the most diverse community overall. Flagellate and ciliate protozoans are not common inhabitants of the *T. abdominalis* larval gut, and were only found in young or unhealthy larvae (Klug & Kotarski, 1980).

The majority of *T. abdominalis* larval nutrition is derived from leaf litter detritus rather than microbial biomass (Cummins & Klug, 1979). It is unlikely that degradation of cellulosic material is due to ingested fungal cellulases since these enzymes would be inactivated by the high pH of the midgut (Martin, 1987; Suberkropp & Klug, 1974). Sinsabaugh and colleagues successfully demonstrated exoglucanase activity within the hindgut and the assimilation of purified cellulose by transport of organic and amino acids across the gut wall (Sinsabaugh *et al.*, 1985). These data lead to the hypothesis that the microbial symbionts are responsible for the degradation of cellulosic material in the *T. abdominalis* larval gut rather than insect tissue-level synthesized cellulases. However, this contribution of the gut microbiota to the degradation of leaf litter detritus within stream ecosystems has not yet been fully established.

This study aimed to demonstrate the activity of cellulolytic and hemicellulolytic enzymes within the *T. abdominalis* larval gut. The midgut and hindgut of *T. abdominalis* were separated in order to assay their activities at physiologically relevant pH values of 11.0 and 7.4, respectively (Martin *et al.*, 1980). Although the high pH in the midgut is generally associated with proteolytic activity (Graca & Barlocher, 1998) and inactivation of fungal cellulases (Barlocher & Porter, 1986; Martin *et al.*, 1980; Sinsabaugh *et al.*, 1985; Suberkropp *et al.*, 1976), alkaline cellulases from bacterial soil isolates have been described previously (Singh *et al.*, 2004). It was therefore necessary to measure cellulolytic and hemicellulolytic activity within the midgut at pH 11.0. Previous studies of the bacterial isolates from *T. abdominalis* hindgut homogenates demonstrated activity on methylumbelliferyl- (MU-) conjugated sugars (J. Doran-Peterson, submitted). MU-conjugated substrates have also been used to demonstrate the activity of extracellular enzymes in marine systems, the digestive tracts of freshwater snails, and of the larvae of two Trichoptera species (Brendelberger, 1997; Hoppe, 1983; Schulte *et al.*, 2003). In this study, MU-conjugated sugars were used to measure cellulolytic and hemicellulolytic enzyme activity from *T. abdominalis* larval gut extracts.

In addition to demonstrating the activity of cellulolytic and hemicellulolytic enzymes within the *T. abdominalis* larval gut, we also report the isolation of strain T202^{T} from the hindgut of a *T. abdominalis* larva. Characterization was performed using a polyphasic approach, which differentiates taxonomic lineages by assessing both phylogenetic and phenotypic characteristics. Based on the phylogenetic and chemotaxonomic data presented within, we propose a novel genus within the family *Microbacteriaceae*, "*Crocebacterium*" gen. nov., and the type species, "*Crocebacterium ilecola*" sp. nov., to accommodate this bacterium.

Materials and methods

Collection site

Fourth instar *T. abdominalis* larvae were collected from Shope Fork Creek, Coweeta, NC. Stream parameters were as follows: pH 5.35; dissolved oxygen, 11.2 mg/L; water temperature, 7.7°C; conductivity, 14.58 μs/cm; turbidity, 0.47 nephelometric turbidity units; alkalinity, 12.0 mg/L; $<$ 1 mg/L NO_3^-; and $<$ 0.1 mg/L PO_4^{3-}. Larvae were stored at 12°C in an aerated 4 L container filled with filter-sterilized stream water and leaves collected from the stream of capture. Water was changed every 3–4 days. Of the 12 larvae, four per day were dissected and their gut homogenates were assayed 14, 23 and 28 days following capture.

Larval dissection

Tipula abdominalis larval midguts (with foregut attached) and hindguts were removed and separated during dissection under : buffered salts solution $(BSS)_{7.4}$ buffer, pH 7.4 (10.8 mmol/L K_2HPO_4, 6.9 mmol/L KH_2PO_4, 21.5 mmol/L KCl, 24.5 mmol/L NaCl, 1.0 mmol/L DTT) (Leadbetter & Breznak, 1996) (Fig. 1). Midguts were placed in 5 mL BSS_{11} buffer, pH 11.0 (25 mmol/L Na_2CO_3, 24.5 mmol/L KCl, 1.0 mmol/L DTT), and hindguts were placed in 5 mL $BSS_{7.4}$ buffer, pH 7.4.

Sample preparation

Protocol was modified from Schulte *et al.* (2003) and Brendelberger (1997). Midguts and hindguts were sonicated in a Branson 1510 ultrasonic bath (Branson Ultrasonics, Danbury, CT, US) for 1 min, then centrifuged at 3 500 *g* and 4°C for 30 min (Schulte *et al.*, 2003). The supernatant was removed and placed into a new tube, and from here on is termed the "enzyme extract." The centrifuged gut pellet was resuspended in buffer of appropriate pH and from here on is termed the "resuspended gut extract." From both the enzyme extract and resuspended gut extract, a 1 mL aliquot was filtered through a 0.2 μm filter to screen for cell-free enzyme activity: "filtered enzyme extract" and "filtered resuspended gut extract." Another 1 mL aliquot was boiled for 30 min to serve as a negative control: "boiled enzyme extract" and "boiled resuspended gut extract" (Schulte *et al.*, 2003). To determine if controls were free of viable micro-organisms, 200 μL aliquots of the filtered and boiled extracts were spread-plated onto tryptic soy agar (TSA, DIFCO, Detroit, MI, US) and compared to plated enzyme extracts and resuspended gut extracts, described below. All gut extracts were assayed on the day of dissection. A previously studied bacterial isolate from the *T. abdominalis* gut (isolate 27, accession number AY504453) known to demonstrate activity on all MU-substrates in this study was used as a positive control. The unfiltered gut extracts represent micro-organisms residing within the gut lumen and those that were easily dislodged from the gut wall. The resuspended gut extracts represent the micro-organisms attached to the gut wall, and the filtered gut extracts represent cell-free enzymes.

Preparation of stock solutions and standards

Stock solutions of the MU-standard and substrates were prepared by dissolving in 5 mL of 70% (v/v) ethanol, then diluting to 50 mL with autoclaved distilled water to a final concentration of 2 mmol/L. Standard concentrations of methylumbelliferone sodium salt (Sigma, St Louis, MO, US) were 100.0, 50.0, 25.0, 20.0, 15.0, 10.0 and 1.0 μmol/L (Schulte *et al.*, 2003). New standards were prepared daily since fluorescence diminished upon storage at −20°C. The five MU-conjugated sugars used as model substrates were 4-methylumbelliferyl-α-L-arabinofuranoside (MUA, Sigma), 4-methylumbelliferyl-β-D-cellobioside (MUC, Sigma), 4-methylumbelliferyl-1 β-D-glucopyranoside (MUG, Sigma), 4-methylumbelliferyl-β-D-mannopyranoside (MUM, Sigma), and 4-methylumbelliferyl-β-D-xyloside (MUX, Sigma). In contrast to the methylumbelliferone sodium salt standard, the MU-conjugated substrate solutions were stable upon storage at −20°C.

Enzyme assay

Enzyme activity was assayed in black 350 μL 96-well plates (NUNC, Rosklide, Denmark) with a spectrofluorometer Spectra MAX® GEMINI EM (Molecular Devices, Sunnyvale, CA, US). Unfiltered, filtered, and boiled enzyme extracts and resuspended gut extracts (40 μL) were mixed with BSS buffer, pH 11.0 for midgut and 7.4 for hindgut (155 μL), and MU-substrate (5 μL). Top read, endpoint fluorescence enzyme assays began with substrate addition and were incubated for 20–30 min at 25°C. Fluorescence was measured at 360 nm emission and 435 nm excitation with no cutoff wavelength, and values were the average of 20 readings (Schulte *et al.*, 2003). Fluorescence values directly relate to liberated methylumbelliferone concentrations.

Isolation and screening of hindgut microbiota

Hindgut and resuspended gut extracts of two larvae were plated onto TSA and Luria-Bertani agar (LBA, DIFCO) media in 50 and 75 μL aliquots and incubated at room temperature until no new colonies appeared (after 9 days). A total of 198 colonies were streaked onto additional TSA or LBA plates for isolation. Colonies were picked according to morphology and incubation time before colony appearance to reduce repetition of isolates, picking only 3–4 colonies with similar morphology if found on different plates or from different gut extracts. Glycerol stocks of 40% (w/v) glycerol were made of each bacterial isolate for storage at −80°C. Isolates were then screened on TSA + 1 g/L MUA, MUC, MUG, MUM or MUX by replica plating. Cleavage of MU-conjugated

sugars, MUA, MUC, MUG, MUM and MUX was determined by the method of Sharrock (1988). Fluorescence was detected by exposure to UV light after incubation for 1 week.

Morphological, physiological and chemotaxonomic characterization of isolate T202^T

Isolate T202^T colony morphology was observed on TSA at 24–25°C. Cell morphology was observed by phase-contrast microscopy and bright-field microscopy. Gram-staining was performed as described (Ayers, 2000). Motility was studied by phase-contrast microscopy and motility test medium (BBL) (2003b). Temperature range and the optimum for growth were determined in TSB, pH 7.0. For temperatures above 5°C, a temperature gradient incubator (Scientific Industries, Bohemia, NY, US) was used with agitation. For temperatures between 0°C and 5°C, cells were grown on TSA, pH 7.0. pH range and the optimum for growth were determined in TSB at 24–25°C. NaCl tolerance was determined on LBA with NaCl concentrations between 0% and 10% (w/v). Growth under anaerobic and microaerobic conditions was examined using BBL GasPak® jar systems (BBL Microbiology Systems, Cockeysville, MD, US). Presence of endospores was determined by staining as previously described (Doetsch, 1981).

Isolate T202^T was grown at 24–25°C for all physiological tests mentioned below. Catalase activity was determined by the addition of 3% (v/v) hydrogen peroxide solution. Oxidase activity was tested using freshly prepared 1% tetramethyl-*p*-phenylenediamine dihydrochloride (Tarrand & Groschel, 1982). Methyl red (MR), Voges-Proskauer (VP), nitrate reduction, formation of H_2S, urease activity, extracellular DNase activity, and hydrolysis of casein and starch were determined following the manufacturer's instructions (DIFCO). Hydrolysis of carboxymethyl cellulose (CMC), xylan, and pectin was determined as previously described (MacFaddin, 1985; Mondou *et al.*, 1986; Vera & Dumoff, 1974; Wood & Kellogg, 1988). Various enzyme activities and acid production from a variety of carbohydrates were investigated using API Staph, Strep, and Coryne systems (BioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions, except incubation was at 25°C for 48 h. Antibiotic tolerance was determined by the Kirby-Bauer disc-diffusion method using BBL™ Sensi-Discs (BBL Microbiology Systems) (2003a). Cell wall analysis was performed by the DSMZ identification service (Braunschweig, Germany). Peptidoglycan structure was analyzed as described by Schleifer and Kandler (1972) and Schleifer (1985). Amino acid quantification was performed by gas chromatography (MacKenzie, 1987). Sugars present in the cell wall were determined as described by Staneck and Roberts (1974). Fatty acid methylesterase (FAME) analysis was performed by MIDI LABS (Newark, DE, US).

DNA extraction and phylogenetic analysis of isolate T202^T

DNA extraction was modified from Harwood and Cutting (1990). Briefly, pelleted cells were resuspended in lysis buffer (50 mol/L Na_2 EDTA, 0.1 mol/L NaCl, pH 7.5) with 3 mg lysozyme/mL and incubated at 37°C for 10 min. Sodium dodecyl sulfate (SDS) was added to 1.5% (w/v) final concentration, and the sample was incubated an additional 5 min at 37°C. Phenol (pH 7.8) extraction, chloroform : isoamyl alcohol (24 : 1) extraction, and ethanol precipitation were then performed. DNA G + C content was determined as described in Mesbah and Whitman (1989) and confirmed by the DSMZ by the methods of Cashion *et al.* (1977), Mesbah and Whitman (1989), Tamaoka and Komagata (1984) and Visuvanathan *et al.* (1989). Sequencing of the full 16S rRNA gene was performed by MIDI LABS (Newark, DE, US) using primers corresponding to *Escherichia coli* 16S rRNA positions 005 and 1540. Sequences with high similarity to isolate T202^T were obtained from a BLAST search with GenBank (http://www.ncbi.nlm.nih.gov). The 16S rRNA gene sequence from isolate T202^T was aligned with the multiple alignment program ClustalX (Thompson *et al.*, 1997) against previously determined *Microbacteriaceae* sequences obtained from the public database NCBI (http://www.ncbi.nlm.nih.gov), then edited with GeneDoc (Nicholas *et al.*, 1997). Phylogenetic analyses were performed with the program Mega2 (Mukhopadhyay *et al.*, 2005). To determine consistency between analysis methods, phlyogenetic distance trees were generated using the Kimura two-parameter and Jukes-Cantor models and the neighbor-joining, minimum evolution, and maximum parsimony algorithms (Kimura, 1980; Saitou & Imanishi, 1989).

Results

Enzyme assay

All five MU-conjugated substrates were hydrolyzed by the unfiltered and filtered hindgut enzyme extracts (Figs. 2 and 3). Hydrolysis of MUG was up to 13 times greater than hydrolysis of the other four MU-conjugated sugars by hindgut enzyme extracts. Both

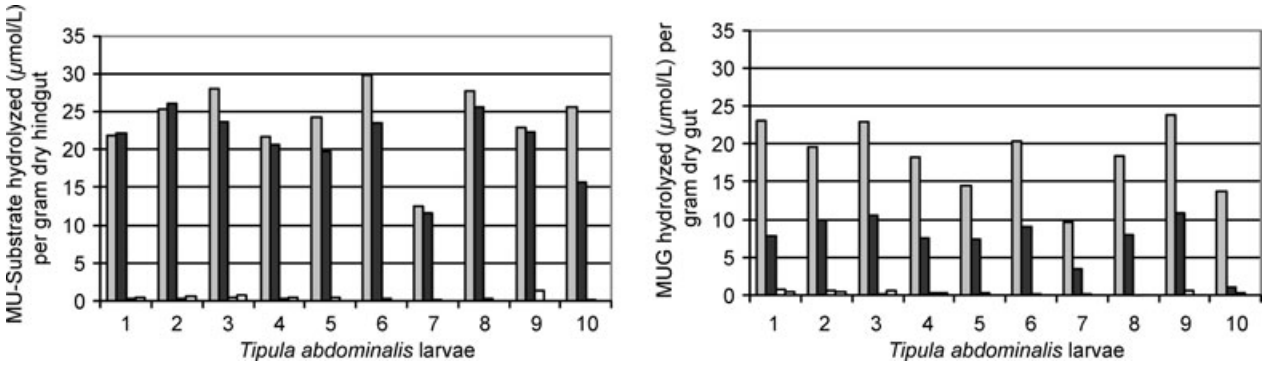

Fig. 2 MUG hydrolyzed (μmol/L/g dry gut weight) by 10 individual *Tipula abdominalis* larvae hindgut enzyme extracts and resuspended guts. Hindgut enzyme extracts and resuspended guts are presented as unfiltered (grey), filtered (black) and boiled (white). Hindgut enzyme extracts from animals 1 and 2, 3–6 and 7–10 were assayed 14, 23 and 28 days after capture, respectively. Resuspended guts from animals 1–3, 4–6 and 7–10 were assayed 14, 23 and 28 days following capture, respectively. All assays were performed between 20 and 30 min.

unfiltered and filtered resuspended hindgut extracts hydrolyzed MUG, MUA and MUX, while only unfiltered resuspended hindgut extracts hydrolyzed MUC significantly more than the negative controls (Figs. 2 and 3). Activity of unfiltered and filtered resuspended hindgut extracts on MUM was similar to negative controls (Fig. 3). All unfiltered and filtered midgut enzyme extracts and resuspended midgut extracts had similar activity to the negative controls (data not shown). No bacterial colonies formed on TSA plates inoculated with filtered or boiled extracts. All enzyme extracts and resuspended gut extracts were assayed the day of dissection since significant enzyme activity was lost when frozen at -20°C overnight and assayed the following day.

Isolation and screening of hindgut microbiota

Of the 198 isolates, 35.3, 39.4, 58.1, 27.3 and 35.9% displayed activity on MUA, MUC, MUG, MUM and MUX, respectively. These numbers are similar to the relative hindgut enzyme extract activity on each of the five MU-conjugated sugar substrates (Figs. 2 and 3). For example, enzyme activity was highest with MUG and lowest with MUM.

Morphological, physiological and chemotaxonomic characterization of isolate T202^T

Isolate T202^T colonies were round, smooth, shiny, convex, opaque, yellow when grown in the light, and white

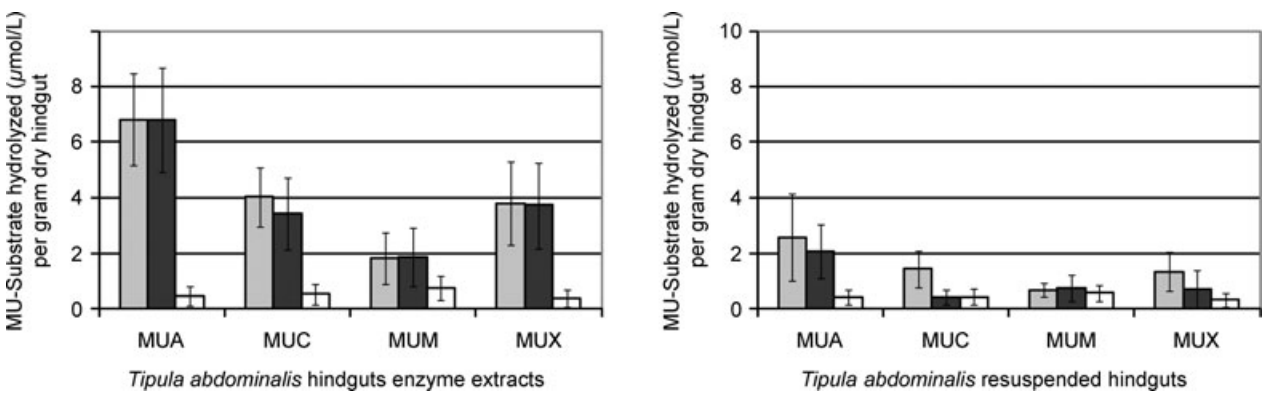

Fig. 3 Average *Tipula abdominalis* hindgut enzyme extract and resuspended hindgut activity (μmol/L/g dry gut weight) on four methylumbelliferyl- (MU)-substrates. Hindgut enzyme extracts and resuspended guts are presented as unfiltered (grey), filtered (black) and boiled (white). Data are for 10 animals and error bars indicate standard deviations. All unfiltered and filtered enzyme extracts and resuspended guts are significantly different ($P \leq 0.001$), determined by Student's *t*-tests, from boiled enzyme extracts except unfiltered resuspended gut activity on methylumbelliferyl-β-D-mannopyranoside (MUM) and filtered resuspended gut activity on 4-methylumbelliferyl-β-D-cellobioside (MUC) and MUM. All assays were performed between 20 and 30 min.

when grown in the dark. Long rods were the predominant morphology in young cultures (width 0.7–1.5 μm, length 3–4.5 μm, 18 h), and short rods predominate older cultures (width 0.4–0.7 μm, length 0.7–1.5 μm, 50 h), yet a marked rod-coccus cycle was not observed. When cells were longer, they often occurred in short chains of 2–4 cells, each at a slight angle to the other at the point of attachment. Gram stain was negative for a young culture (18 h), and positive for an older culture (50 h). Motility was greater in younger cultures than older cultures (18 h and 50 h, respectively). Optimal cell growth was at 21–25°C, pH 7.0, and 0.0–0.5% (w/v) NaCl. Weak growth occurred at 0°C and 37°C, pH 10 and 2.5% (w/v) NaCl. No growth occurred at 42°C, pH 5 and 5% (w/v) NaCl. Cells grew best aerobically, and less growth occurred under microaerobic conditions. Growth was not detected after a 4-week incubation under anaerobic conditions, but growth occurred when transferred to aerobic conditions. Cells did not form endospores. A long-to-short rod cycle, variable Gram stain, lack of sporulation, microaerobic growth, and minimum growth temperatures near 0°C are characteristics found among many members of the *Microbacteriaceae* family (Groth *et al.*, 1996; Kampfer *et al.*, 2000; Mannisto *et al.*, 2000; Sheridan *et al.*, 2003; Zlamala *et al.*, 2002).

Catalase and oxidase tests were positive. Nitrate was reduced to NH_4^+. MR and VP tests were negative, and isolate T202^T did not form H_2S. No hydrolysis of hippurate, gelatin, casein, starch, CMC, xylan or pectin occurred. Isolate T202^T tested positive for the enzyme activities of alkaline phosphatase, β-glucosidase, α-glucosidase, β-glucuronidase, β-galactosidase, and urease. T202^T did not produce α-methyl-D-glucosidase, N-acetyl-glucosaminase, α-galactosidase, leucine arylamidase, citrase or pyrrolidonyl arylamidase. Under aerobic conditions, acid was produced from D-glucose, D-fructose, D-mannose, D-maltose, lactose, D-trehalose, D-mannitol, xylitol, D-melibiose, raffinose, xylose and sucrose. Growth did not occur under anaerobic conditions, therefore acid production from carbohydrates under anaerobic conditions was not detected. Degradation of MUA, MUC, MUG and MUX occurred. MUM was not degraded. Isolate T202^T was resistant to kanamycin (30 μg) and penicillin (10 μg), but not to ampicillin (10 μg), ciprofloxacin (5 μg), erythromycin (15 μg), rifampin (5 μg), polymyxin B (300 μg), streptomycin (10 μg), trimethoprim (5 μg) or vancomycin (30 μg).

The cell wall peptidoglycan was of the type B2γ. The cell wall contained alanine, glycine, D- and L-diaminobutyric acid (DAB), and glutamic acid in the molar ratio 0.6 : 1.0 : 2.5 : 1.0. The predominant sugar in the cell wall was rhamnose, and the minor sugar was mannose. No mycolic acids were present. The most abundant lipids were 12-methyltetradecanoic acid (ai-$C_{15:0}$; 63.5%) and 14-methylhexadecanoic acid (ai-$C_{17:0}$; 21.0%). Other lipids present in significant amounts were 13-methyltetradecanoic acid (i-$C_{15:0}$; 1.35%), hexadecanoic acid ($C_{16:0}$; 5.09%) and 14-methylpentadecanoic acid (i-$C_{16:0}$; 7.03%). Tetradecanoic acid ($C_{14:0}$), 12-methyltridecanoic acid (i-$C_{14:0}$), pentadecanoic acid ($C_{15:0}$), 15-methylhexadecanoic acid (i-$C_{17:0}$), octadecanoic acid ($C_{18:0}$), and cyclohexyl acid w9c-18:1 were each present as $< 1\%$.

Phylogenetic analysis of isolate T202^T

The G + C content of the DNA was 64.9 mol%. Isolate T202^T full 16S rRNA gene sequence (1526 nucleotides) had 96.5% identity to an unidentified actinobacterium (partial sequence) cultured from African millipede fecal pellets. Both an uncultured actinobacterium from an industrial biofilter (partial sequence) (Friedrich *et al.*, 2002) and a *Leifsonia poae* isolate from nematode galls (partial sequence) (Evtushenko *et al.*, 2000) had 96.2% sequence identity to isolate T202^T 16S rDNA.

Results from phylogenetic distance analyses were similar using the Kimura two-parameter or Jukes-Cantor model and the neighbor-joining, minimum evolution, or maximum parsimony algorithm. Phylogenetic trees based on these distance analyses show that isolate T202^T is closely related to the genera *Agreia*, *Frigoribacter*, *Subtercola*, *Rhodoglobus* and *Salinibacterium*, yet forms a distinct lineage among these genera within the *Microbacteriaceae* family (Fig. 4). Isolate T202^T is shown to cluster with the genus *Frigoribacterium*, but the low bootstrap value (Saitou & Imanishi, 1989) indicates that this placement is uncertain. Other characteristics separating isolate T202^T from other genera within this cluster are the cell wall amino and diamino acids, cell wall sugars, fatty acid profile, and menaquinone composition (Table 1). Most notably different are the fatty acids with ai-$C_{15:0}$ composing over 60% of the total fatty acids. The genus *Rhodoglobus* has the closest fatty acid composition with 53.2% ai-$C_{15:0}$ and 22.6% ai-$C_{17:0}$, but this genus has 18.8% i-$C_{16:0}$ compared to 7.0% for isolate T202^T. Also, the genus *Rhodoglobus* has D-ornithine as the diamino acid within the cell wall, whereas isolate T202^T has D- and L-DAB (Table 1).

Discussion

Cellulose and hemicellulose degradation

Cellulase and hemicellulase activity have been successfully demonstrated in hindgut extracts of *T. abdominalis*

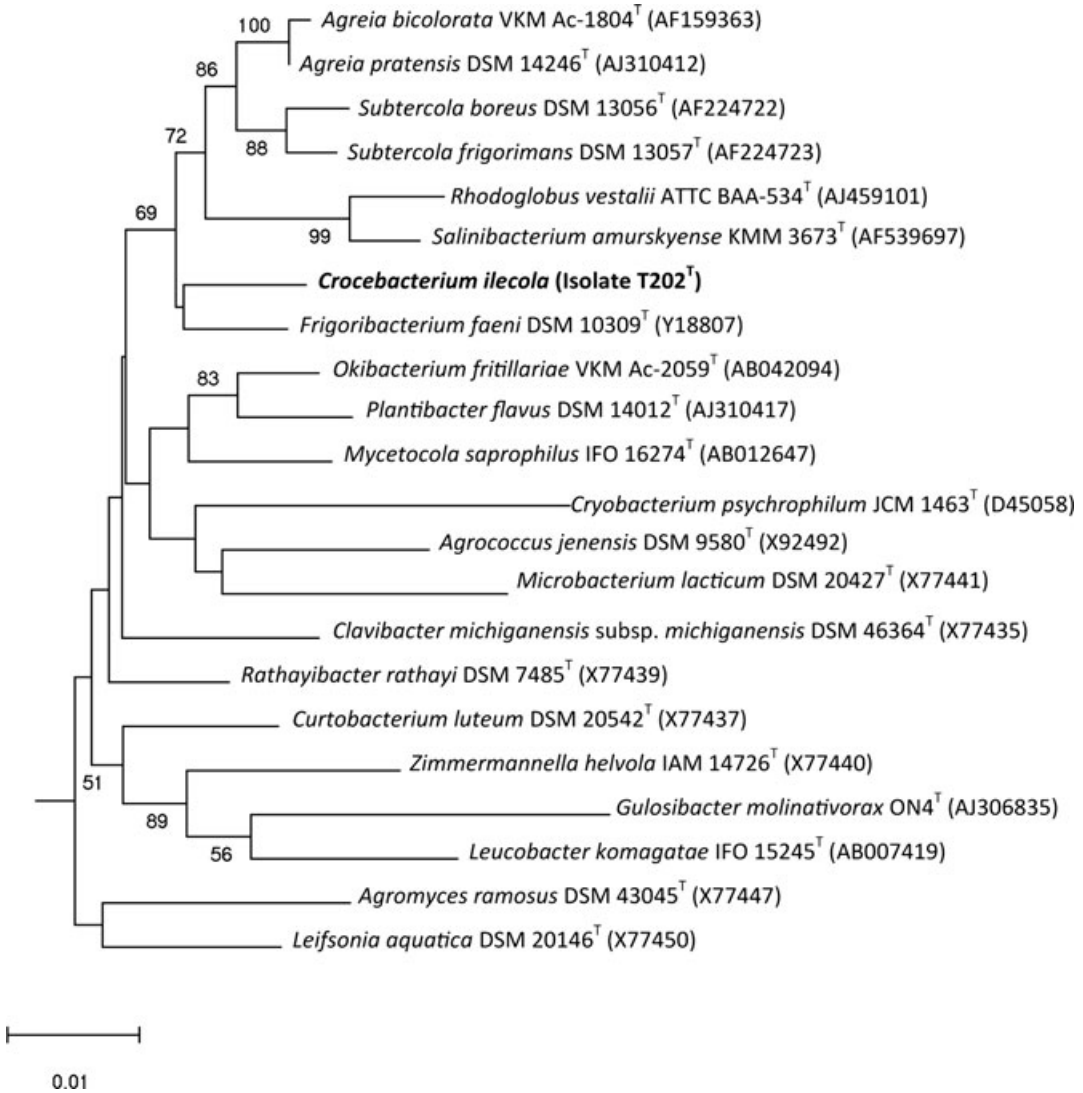

Fig. 4 Phylogenetic tree based on 16S rRNA gene sequences of *Crocebacterium ilecola* gen. nov., sp. nov. and genera of the family *Microbacteriaceae*. Accession numbers for nucleotide sequences are in parentheses. The numbers on the tree indicate bootstrap values, expressed as a percentage of 500 replicates. Only bootstrap values of 50% or greater are shown. Bar indicates 1 nucleotide substitution per 100 nucleotides. *Brevibacter linens* (DSM 20425^T, X77451) served as an out-group sequence to estimate the root position of the tree.

larvae by use of MU-conjugated model substrates. The MU-conjugated sugars model the ends of large polymeric sugars or oligosaccharides. Cleavage of these substrates infers the ability to degrade sugar polymer ends or oligosaccharides produced by the partial degradation of lignocellulosic material. No cellulolytic or hemicellulolytic activity was detected within the midgut, which is not surprising because the pH of the midgut suggests proteolytic, not cellulolytic or hemicellulolytic activity.

Enzyme activity profiles were similar for animals in captivity for 14, 23 and 28 days, therefore demonstrating that captivity between days 14 and 28 had a minimal affect on the detection of hydrolytic enzyme capabilities. Larvae were not assayed on the day of capture; therefore it cannot be determined if a change in hydrolytic enzyme activity occurred between days 0 and 14. It is probable that as food sources change, a shift will occur in the microbial community and/or enzyme production to allow for better degradation of the new food source. In this study, larvae were fed leaves collected from the stream of capture to reduce a potential shift in the microbial community or enzyme production.

Table 1 Distinguishing characteristics of the genera *Agreia*, *Frigoribacterium*, *Rhodoglobus*, *Salinibacterium*, *Subtercola* and isolate T202^T.

Characteristic†	Isolate T202^T	*Agreia*‡	*Frigoribacterium*	*Rhodoglobus*	*Salinibacterium*	*Subtercola*
Peptidoglycan type	B2γ	B	B2β	B2α	B	B2γ
Cell wall diamino acids	D, L-DAB	L-DAB, D-Orn	D-Lys	D-Orn	D-Lys, D-Orn	DAB
Other cell wall amino acids	Ala, Gly, Glu	Ala, Gly, Hyg or Glu	Ala, Gly, Hsr	Ala, Gly, Glu	Ala, Gly, Glu	?Hyg
Cell wall sugars						
major	Rhamnose	Rhamnose	nr	nr	nr	Rhamnose, Xylose
minor	Mannose	Mannose and fucose	nr	nr	nr	Mannose, Glu, Rib
Major fatty acids (%)						
ai-$C_{17:0}$	21.0	14.4–22.1	7.0–14.8	22.6	2.8	3.5–6.8
ai-$C_{15:0}$	63.5	46.7–48.0	37.8–48.3	53.2	40.4	46.1–51.6
i-$C_{16:0}$	7.0	17.2–30.1	10.0–16.7	18.8	34.7	4.2–10.2
i-$C_{15:0}$	1.3	$\leq$ 1.0	1.3–1.8	0.79	6.6	< 1.0–4.3
i-$C_{14:0}$	< 1.0	< 1.0	$\leq$ 1.0	1.1	14.7	< 1.0–6.7
$C_{16:0}$	5.1	2.7–5.2	4.4–26.5	0.55	–	–
Major menaquinones	nr	10	9	11, 12	11	9, 10
G+ C content (mol%)	65	65–67	71.1	62	61	64–68
Temperature optima (°C)	21–25	24–26	4–8	18	25–28	15–17
Temperature range (°C)	1–37	< 37	−2–28	−2–21	4–37	−2–28
Motility	+	+	+	nr	−	−
Colony color	Y, W	Y, O	Y	R	nr	Y

†Peptidoglycan type as designated by Schleifer and Kandler (1972).

‡Data for known genera were collected from Behrendt *et al.* (2002), Evtushenko *et al.* (2001), Kyun Han *et al.* (2003), Mannisto *et al.* (2000), Sheridan *et al.* (2003) and Schumann *et al.* (2003). DAB, diaminobutyric acid; Lys, lysine; Orn, ornithine; Ala, alanine; Gly, glycine; Glu, glutimate; Hsr, homoserine; Hyg, hydroxyglutamate; ai, anteiso-branched fatty acids; i, iso-branched fatty acids; nr, not reported; R, Red; W, white; Y, yellow.

Fermentation of the cellulolytic larval diet is expected within the hindgut since it possesses an enlarged anterior paunch, which is an analogous structure to the termite paunch where fermentation is facilitated by retarding the passage of food through the hindgut (Klug & Kotarski, 1980; Martin, 1987). Also, both the degradation of cellulose to organic acids and the transport of acetate into the hemolymph as a carbon and energy source for *T. abdominalis* have been demonstrated (Lawson *et al.*, 1984; Sinsabaugh *et al.*, 1985). An analysis of enzyme activity from the anterior paunch verses the posterior hindgut is needed to determine where the majority of the cellulose degradation occurs.

Cellulase and hemicellulase activity from hindgut microbiota

Many of the microbial isolates from the larval hindgut demonstrated the ability to degrade cellulosic and/or hemicellulosic material, indicating a possible source of cellulolytic and/or hemicellulolytic enzymes. Isolation of gut-associated microbiota was performed under aerobic conditions on rich media (TSA and LBA). No special efforts were made to enrich for cellulolytic or hemicellulolytic bacteria or to isolate anaerobes because the culturing techniques were originally designed for comparing the quantity of bacteria in the enzyme extracts and resuspended gut extracts to the boiled and filtered controls. It was only after isolation that the magnitude of morphological diversity found by using readily available media, rather than enrichments or specialized media, was realized. Other studies from this research group have cultured bacteria from various areas of the *T. abdominalis* alimentary tract with various types of media under both aerobic and anaerobic conditions (J. Doran-Peterson, unpublished data).

Taxonomic conclusions of isolate T202^T

Isolate T202^T was fully characterized because of its ability to degrade four of the five MU-conjugated substrates tested. Although isolate T202^T has up to 96.2%

sequence identity to other genera within the family *Microbacteriaceae*, this high percent identity is common between members of other genera within this family. For example, the 16S rRNA sequence identities of the genera *Agreia* and *Subtercola* are 96.8%–97.1%, and identities for *Frigoribacterium*, *Rathayibacter*, and *Clavibacter* are 96.1%–97.1% (Evtushenko *et al.*, 2001; Kampfer *et al.*, 2000). These were characterized as separate genera based on polyphasic taxonomic studies rather than 16S rRNA sequence similarity alone. Of the phenotypic properties studied, chemotaxanomic characteristics, such as peptidoglycan composition, and fatty acid profile, were important distinguishing characteristics between these genera (Evtushenko *et al.*, 2002; Tsukamoto *et al.*, 2001). Considering both chemotaxanomic characteristics and 16S rRNA analysis, isolate T202^T clearly forms a separate genus within the family *Microbacteriaceae*. We propose a new genus within the *Microbacteriaceae* family, *Crocebacterium* gen. nov., and the type species, *Crocebacterium ilecola* sp. nov., to accommodate this bacterial isolate.

Ecological implications of isolate T202^T

Many members of the *Microbacteriaceae* family are associated with plants (Behrendt *et al.*, 2002; Dorofeeva *et al.*, 2002, 2003; Evtushenko *et al.*, 2000, 2001, 2002; Sasaki *et al.*, 1998). The source of inoculum for the *T. abdominalis* hindgut is believed to be ingested leaf detritus (Klug & Kotarski, 1980; Lawson *et al.*, 1984); therefore it is not surprising to find members of the *Microbacteriaceae* family within this gut system. Additionally, this bacterium was most likely a gut wall-associated bacterium since it was isolated from the pelleted hindgut (Dillon & Dillon, 2004). Most gut wall-associated bacteria are considered resident rather than transient bacteria because they can resist the flow of material through the gut by attachment or inhabiting small crevices along the gut wall. Because these bacteria are constant inhabitants of the hindgut, they may have a specific role within the microbial community. One possible role is the degradation of cellulosic material in the *T. abdominalis* larval gut (Dillon & Dillon, 2004; Sinsabaugh *et al.*, 1985). Although isolate T202^T was unable to hydrolyze large polymeric carbohydrates such as starch, CMC and xylan, it was able to cleave four of the five MU-conjugated sugars tested. If isolate T202^T is a resident of the hindgut, it may occupy a niche within the microbial community degrading oligosaccharides produced by the partial degradation of lignocellulosic material. To determine whether this bacterial isolate, or any other isolate from the *T. abdominalis* hindgut, is a resident rather than a transient bacterium, studies such as fluorescent *in situ* hybridization may be employed.

Description of Crocebacterium *gen. nov.*

Crocebacterium (Cro.ce.bac.ter.ium. L. adj. *croceus* saffron-colored, golden, yellow; Gr. n. *bakterion* small rod; *Crocebacterium* a small yellow bacterium).

Cells are microaerobic to aerobic, Gram-type positive, high G + C, motile, non-spore forming, irregular rods. Colonies are round, smooth, shiny, convex, opaque and may produce pigments. Growth occurs at mesophilic temperatures and neutral pH. The cell wall peptidoglycan is of the type B2γ, and the diamino acid is DAB (D- and L-forms). No mycolic acids are present. Predominant fatty acids are 12-methyltetradecanoic acid (ai-$C_{15:0}$) and 14-methylhexadecanoic acid (ai-$C_{17:0}$). The type species is *Crocebacterium ilecola*^{T}.

Description of Crocebacterium ilecola *sp. nov.*

Crocebacterium ilecola (i.le.co.la. L. n. *ile* gut; Gr. adj. *–cola* inhabitant; *ilecola* inhabitant of the gut).

In addition to those characteristics described for the genus, *Crocebacterium ilecola* does not have a marked rod–coccus cycle. Cells of younger cultures are motile, long rods, and stain Gram-negative, and cells of older cultures are less motile, shorter rods, and stain Gram-positive. Colonies appear yellow when grown in the light and white when grown in the dark. The DNA G + C content is approximately 65 mol%. Cell wall amino acids are alanine, glycine, D- and L-DAB, and glutamic acid (0.6 : 1.0 : 2.5 : 1.0 molar ratio). Cell wall sugars are rhamnose (major) and mannose. Temperature range for growth is 0–37°C, optimal growth occurs between 21–25°C, and no growth occurs at 42°C. Growth occurs at a pH range of 6–10, optimal growth occurs at pH 7.0 and no growth occurs at pH 5.0. Range of NaCl concentrations for growth is 0–2.5% (w/v), optimal growth occurs between 0.0–0.5% (w/v) NaCl, and growth is inhibited at 5.0% (w/v) NaCl. Catalase and oxidase tests were positive. Nitrate was reduced to NH_4^+. MR and VP tests were negative. Activities were positive for alkaline phosphatase, β-glucosidase, α-glucosidase, β-glucuronidase, β-galactosidase and urease. Under aerobic conditions, acid is formed from D-glucose, D-fructose, D-mannose, D-maltose, lactose, D-trehalose, D-mannitol, xylitol, D-melibiose, raffinose, xylose or sucrose. Hippurate, gelatin, casein, starch, CMC, xylan and pectin are not hydrolyzed. Resistance was observed to kanamycin (30 μg) and penicillin (10 μg), but susceptible to ampicillin (10 μg), ciprofloxacin (5 μg), erythromycin (15 μg), rifampin (5 μg), polymyxin B

(300 μg), streptomycin (10 μg), trimethoprim (5 μg) and vancomycin (30 μg).

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