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A Culture-Based Study of the Bacterial Communities within the Guts of Nine Longicorn Beetle Species and their Exo-enzyme Producing Properties for Degrading Xylan and Pectin

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In this study, bacterial communities within the guts of several longicorn beetles were investigated by a culture-dependent method. A total of 142 bacterial strains were isolated from nine species of longicorn beetle, including adults and larvae. A comparison of their partial 16S rRNA gene sequences showed that most of the bacteria constituting the gut communities can typically be found in soil, plants and the intestines of animals, and approximately 10% were proposed as unreported. Phylogenetic analysis demonstrated that the bacterial species comprised 7 phyla, and approximately half were *Gammaproteobacteria*. *Actinobacteria* were the second most populous group (19%), followed by *Firmicutes* (13%) and *Alphaproteobacteria* (11%). *Betaproteobacteria*, *Flavobacteria*, and *Acidobacteria* were minor constituents. The taxonomic compositions of the isolates were variable according to the species of longicorn beetle. Particularly, an abundance of *Actinobacteria* existed in *Moechotypa diphysis* and *Mesosa hirsute*, which eat broadleaf trees; however, no *Actinobacteria* were isolated from *Corymbia rubra* and *Monochamus alternatus*, which are needle-leaf eaters. Considerable proportions of xylanase and pectinase producing bacteria in the guts of the longicorn beetles implied that the bacteria may play an important role in the digestion of woody diets. *Actinobacteria* and *Gammaproteobacteria* were the dominant xylanase producers in the guts of the beetles.

Keywords: gut bacteria, longicorn beetle, 16S rRNA gene, culture-based isolation

Insects are the most abundant and diverse species on earth, and survive successfully in various niches. The keys to their successful survival through a long evolutional period include their explosive production of offspring from a single female, and superior adaptation capabilities to diverse environments and food sources. Additionally, the diversity of the insect gut microbial community also plays an important role in host survival. The digestive tracts of insects contain a complex biota, comprising both resident and transient members from bacterial, fungal, protozoan, and archaeal strains. Insects rely on microbes for various physiological functions, including food digestion, nutrition, nitrogen fixation, and pheromone production (Lilburn *et al.*, 2001; Lemke *et al.*, 2003; Dillon and Dillon, 2004). The symbiotic relationships between insects and gut bacteria have been studied extensively in several systems, particularly in termites and aphids, which feed on wood and plant phloem, respectively (Breznak, 1982; Chen and Purcell, 1997; Lilburn *et al.*, 1999; Ohkuma, 2003). With the assistance of molecular biological techniques such as total DNA isolation accompanied by 16S rDNA amplification and computational sequence data analysis, the microbial community structures of several insect gut microbiota have been reported, which were studied restrictively in culturable microorganisms (Amann *et al.*, 1995; Brauman *et al.*, 2001; Friedrich *et al.*, 2001; Egert *et al.*, 2003). Although it's already known that lesser amounts of all microbial species within an environmental sample can rapidly grow into colonies on artificial growth media (Hugenholtz *et al.*, 1998; Randon *et al.*, 1999; Hugenholtz, 2002), we believe that culture-based screening that is focused on the dietary preference of the insect is a valuable tool for finding unique exo-enzyme producing microorganisms. Furthermore, several extracellular hydrolytic enzyme producing bacteria from the digestive tracts of insects, and a spider, were identified and reported (Lee *et al.*, 2004a; Heo *et al.*, 2006; Kwak *et al.*, 2007; Park *et al.*, 2007).

Longicorn beetles are widespread throughout the world. Approximately 20,000 species have been reported, including nearly 300 species in the Korean peninsula (Kim, 2002). Adult longicorn beetles usually emerge from May to August in Korea, and oviposit in the bark of trees. Adults eat the leaves or bark of live trees; larvae usually eat the bark of trees in their early larval stage, and then eat woody parts in their late larval stage while making a tunnel into the tree. As a consequence, they cause damage to the tree and are primarily considered to be pests (Lee, 1987). Although endogenous cellulases have been reported in several insects and termites (Watanabe *et al.*, 1998; Tokuda *et al.*, 1999; Girard and Jouanin, 1999; Lee *et al.*, 2004b), it is believed that the digestion of wood constituents such as cellulose, hemicellulose (xylan), lignin, and pectin is catalyzed by the digestive enzymes produced by symbiotic microorganisms harbored in the guts of insects (Brune and Friedrich, 2000; Suh *et al.*, 2003). In the course of developing useful digestive

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enzymes related to the degradation of cellulose, hemicellulose, lignin, and pectin from insect symbiotic microorganisms, we have isolated many culturable bacterial strains from various insects that feed on wood. Here, we initially report on the gut microbes isolated and identified from nine longicorn beetles, as well as their xylanase and pectinase producing properties, which are required to degrade and adsorb the carbohydrates from the wood.

Materials and Methods

Isolation and cultivation of bacteria

Moechotypa diphysis, *Mesosa hirsute*, *Anoplophora malasiaca*, *Corymbia rubra*, *Prionus insularis*, *Olenecamptus clarus*, and *Massicus radde* adults were collected from several sites in Korea from May to August, 2005, and maintained alive at 4°C until use. *Monochamus alternatus* and *Psacothea hilaris* larvae and adults were provided by Dr. Seol from the Rural Development Administration, Korea. The insects were identified by comparison with the literature, and their guts were isolated by a previously published method (Park *et al.*, 2007). The isolated guts were homogenized in phosphate buffered saline, diluted to 10^{-5}, and plated on both oligotropic media and complete media. The plates were incubated at 25°C for 2 or 4 days; then single colonies were transferred to fresh agar plates and pure colonies were stored at -70°C in 10% glycerol.

PCR amplification of the colonies and sequence analysis

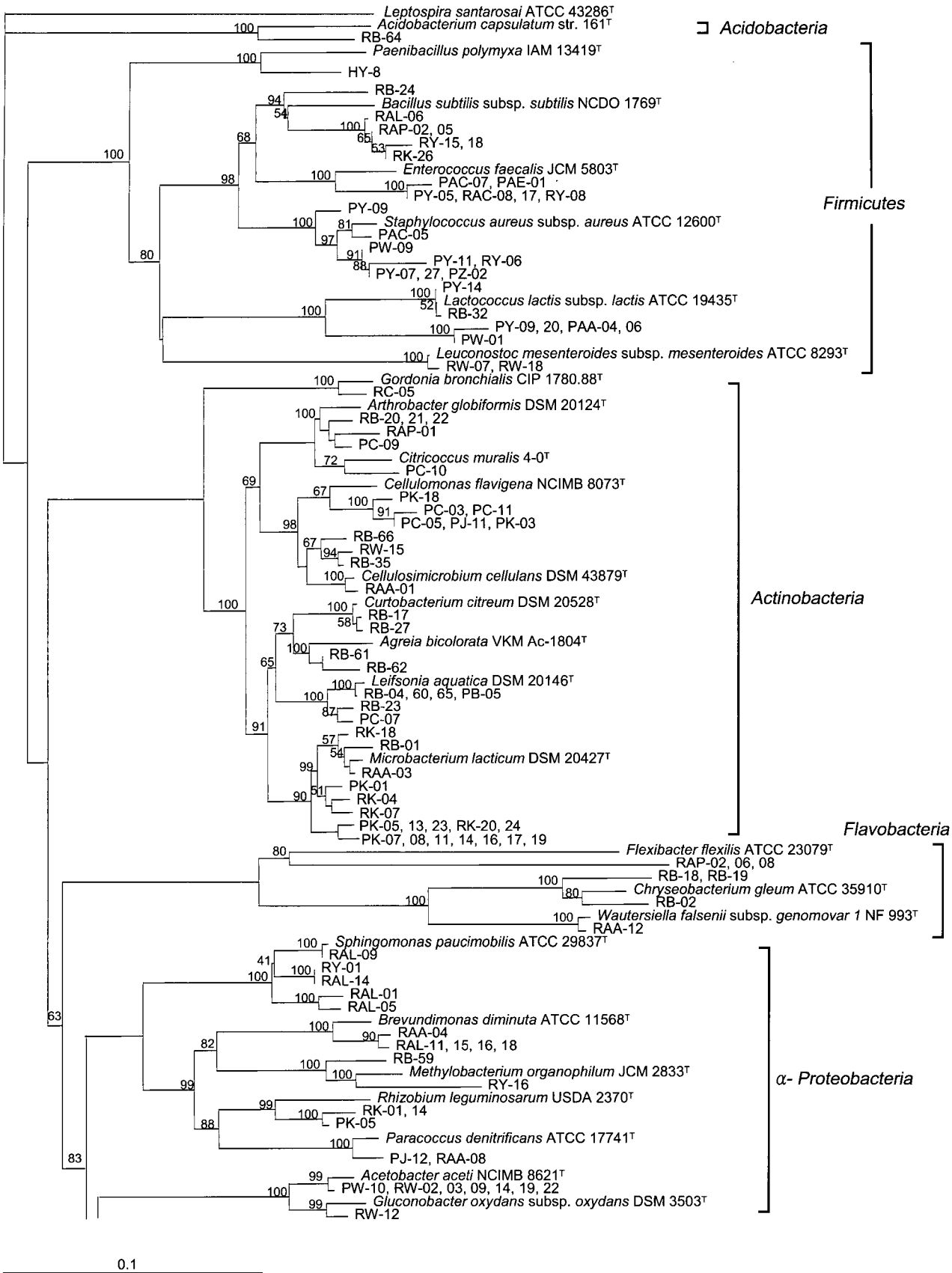
Bacterial 16S rRNA genes were amplified by the colony PCR method. Briefly, a small portion of pure colony was transferred to a PCR premix tube using a toothpick, and PCR was performed using two primers designed to anneal to conserved regions in the bacterial 16S rRNA genes. The forward primer (27F: 5'-agagtttgatcmtggctcag-3') corresponded to positions 8 to 27 of the *Escherichia coli* 16S rRNA gene, and the reverse primer (1492R: 5'-ggttaccttgttacgactt-3') corresponded to the complement of positions 1492 to 1510 (Lane, 1991). Some bacterial strains could not be amplified by the colony PCR method; in that case, genomic DNA was isolated and used as a template for PCR analysis. Amplification was performed under the following conditions: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 2 min; and a final elongation step at 72°C for 7 min. The purified PCR products were sequenced with the 27F primer. The sequences were aligned with those in the GenBank database using a Blast search algorithm (Altschul *et al.*, 1997).

Phylogenetic analysis of the partial rRNA gene sequences

The test sequences were compared to the 16S rRNA gene sequences of the bacterial type strains using the EzTaxon server (Chun *et al.*, 2007). The test sequences were initially aligned with sequences retrieved from the web-site using the CLUSTAL X program (Thompson *et al.*, 1997). Subsequently, alignment was refined manually using the PHYDIT program,

Table 1. Bacterial isolates from the guts of longicorn beetles that showed 16S rRNA sequence homology below 98% as compared to the most related strain

Serial no.	Most related species (accession no.)	Identity (%)	Different/sequenced	Sources
RAP-06	*Flexibacter sancti* ATCC 23092^T (M62795)	85.88	187/1324	*Prionus insularis*
RB-64	*Acidobacterium capsulatum*^{T} (D26171)	92.36	79/1034	*Moechotypa diphysis*
RB-62	*Leifsonia poae* VKM Ac-1401^T (AF116342)	95.98	56/1394	*Moechotypa diphysis*
PJ-11	*Rahnella aquatilis* DSM 4594^T (AJ233426)	96.47	30/851	*Mesosa hirsute*
PK-10	*Stenotrophomonas maltophilia* LMG 958^T (X95923)	96.55	19/550	*Mesosa hirsute*
PC-10	*Citricoccus alkalitolerans* YIM 70010^T (AY376164)	96.88	28/898	*Moechotypa diphysis*
RB-02	*Chryseobacterium taiwanensis* BCRC 17412^T (DQ318789)	96.99	41/1363	*Moechotypa diphysis*
RW-15	*Cellulosimicrobium cellulans* DSM 43879^T (X83809)	97.09	40/1376	*Massicus raddei*
RB-18	*Chryseobacterium taeanense* PHA 3-4 (AY883416)	97.09	40/1376	*Moechotypa diphysis*
PK-06	*Yersinia intermedia* ATCC 29909^T (AF366380)	97.09	22/757	*Mesosa hirsute*
RB-66	*Cellulomonas denverensis* W6929^T (AY501362)	97.13	40/1392	*Moechotypa diphysis*
RB-25	*Pseudomonas agarici* LMG 2112^T (Z76652)	97.33	37/1387	*Moechotypa diphysis*
PAL-07	*Rahnella aquatilis* DSM 4594^T (AJ233426)	97.42	26/1006	*Corymbia rubra*
RAC-13	*Pantoea agglomerans* ATTC27155^T (AB004691)	97.48	24/952	*Monochamus alternatus*
RB-26	*Burkholderia glathei* LMG 14190^T (U96935)	97.69	32/1386	*Moechotypa diphysis*
RC-02	*Erwinia persicina* ATCC 35998^T (U80205)	97.72	22/964	*Moechotypa diphysis*
RK-03	*Serratia proteamaculans* DSM 4543^T (AJ233434)	97.76	23/1025	*Mesosa hirsute*
PW-03	*Rahnella aquatilis* DSM 4594^T (AJ233426)	97.77	19/852	*Massicus raddei*
RB-01	*Microbacterium lacticum* DSM 20427^T (X77441)	97.84	30/1391	*Moechotypa diphysis*
RB-08	*Pectobacterium cypripedii* ATCC 29267^T (U80201)	97.87	30/1409	*Moechotypa diphysis*
RB-35	*Isoptericola variabilis* DSM 10177^T (AJ298873)	97.89	13/617	*Moechotypa diphysis*


Leptospira santarosai ATCC 43286T
Acidobacterium capsulatum str. 161T
RB-64
Acidobacteria
Paenibacillus polymyxa IAM 13419T
HY-8
RB-24
Bacillus subtilis subsp. subtilis NCDO 1769T
RAL-06
RAP-02, 05
RY-15, 18
RK-26
Enterococcus faecalis JCM 5803T
PAC-07, PAE-01
PY-05, RAC-08, 17, RY-08
PY-09
Staphylococcus aureus subsp. aureus ATCC 12600T
PAC-05
PW-09
PY-11, RY-06
PY-07, 27, PZ-02
PY-14
Lactococcus lactis subsp. lactis ATCC 19435T
RB-32
PY-09, 20, PAA-04, 06
PW-01
Leuconostoc mesenteroides subsp. mesenteroides ATCC 8293T
RW-07, RW-18
Firmicutes
Gordonia bronchialis CIP 1780.88T
RC-05
Arthrobacter globiformis DSM 20124T
RB-20, 21, 22
RAP-01
PC-09
Citricoccus muralis 4-0T
PC-10
Cellulomonas flavigena NCIMB 8073T
PK-18
PC-03, PC-11
PC-05, PJ-11, PK-03
RB-66
RW-15
RB-35
Cellulosimicrobium cellulans DSM 43879T
RAA-01
Curtobacterium citreum DSM 20528T
RB-17
RB-27
Agreia bicolorata VKM Ac-1804T
RB-61
RB-62
Leifsonia aquatica DSM 20146T
RB-04, 60, 65, PB-05
RB-23
PC-07
RK-18
RB-01
Microbacterium lacticum DSM 20427T
RAA-03
PK-01
RK-04
RK-07
PK-05, 13, 23, RK-20, 24
PK-07, 08, 11, 14, 16, 17, 19
Actinobacteria
Flexibacter flexilis ATCC 23079T
RAP-02, 06, 08
RB-18, RB-19
Chryseobacterium gleum ATCC 35910T
RB-02
Wautersiella falsenii subsp. genomovar 1 NF 993T
RAA-12
Flavobacteria
Sphingomonas paucimobilis ATCC 29837T
RAL-09
RY-01
RAL-14
RAL-01
RAL-05
Brevundimonas diminuta ATCC 11568T
RAA-04
RAL-11, 15, 16, 18
RB-59
Methylobacterium organophilum JCM 2833T
RY-16
Rhizobium leguminosarum USDA 2370T
RK-01, 14
PK-05
Paracoccus denitrificans ATCC 17741T
PJ-12, RAA-08
Acetobacter aceti NCIMB 8621T
PW-10, RW-02, 03, 09, 14, 19, 22
Gluconobacter oxydans subsp. oxydans DSM 3503T
RW-12
α- Proteobacteria
0.1

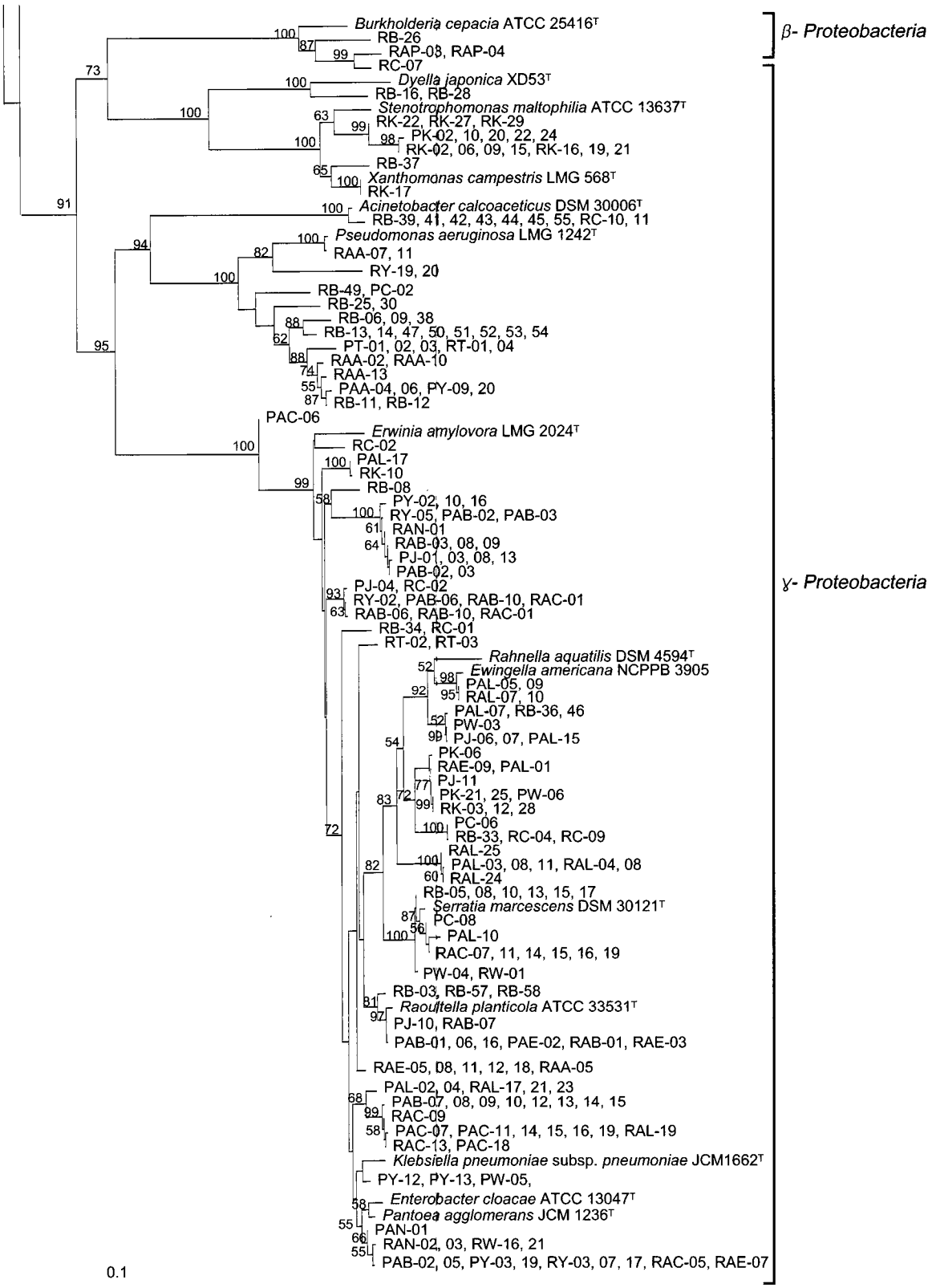


Fig. 1. Phylogenetic association of the 16S rDNA sequences in this study. The phylogenetic tree was built by the neighbor-joining method, using Kimura's two-parameter distance from the partial 16S rDNA sequences. The numbers at the nodes are bootstrap confidence values, and are expressed as percentages of 1,000 bootstrap replications. Bootstrap values higher than 50% are indicated at the main nodes. *Leptospira santarosai* ATCC 43286^T, a member of the *Spirochaetes* group, served as the outgroup.

version 3.0 (Chun, 1995; available at: http://plaza.snu.ac.kr/~jchun/phydit). Ambiguously aligned regions were excluded from the subsequent analysis. A neighbor-joining tree was reconstructed with Kimura's 2-parameter distance model (Kimura, 1980), using the PHYLIP 3.57c package (Felsenstein, 1985). Bootstrap analyses of 1,000 replicates were performed in order to assess the relative stability of the branches.

Enzyme activity test

Xylanase activity was determined with a Congo red stain (Skipper *et al.*, 1985). Cells were grown in M9 minimal media (Difico, USA) containing 0.5% yeast extract and 0.5% birchwood xylan (Sigma, USA). After the colonies were washed with water, the plate was rinsed with Congo red (2 mg/ml) for a few minutes, and then rinsed with 1 M NaCl. The unstained areas were ascribed to indicate the hydrolysis of xylan. Pectinase activity was tested on a R2A agar plate containing 0.3% citric pectin (Avigad and Milner, 1967). After cell growth, 1% *n*-hexadecyltrimethylammonium bromide solution was poured onto the plate, and clear areas around the colonies were monitored. All microbial cells were cultured at 25°C and their enzyme activities were counted as the relative size of the circle indicating activity.

Results

Isolation of bacterial strains

Culturable bacterial strains were isolated from the guts of nine species of longicorn beetles, including adults and larvae, in order to identify and investigate the community structures and to isolate the xylanase and pectinase producing strains. The isolation of pure strains was performed by selecting different colored or shaped colonies upon optical observation of the culture plates in triplicate, and by repeated plating. A total of 342 colonies were isolated, and 142 strains were confirmed as composing the gut bacterial biota after excluding the same clones, as judged by 16S rRNA sequence comparisons. Within these strains, seven isolates were predicted as novel species, demonstrating 16S rRNA gene sequence homology below 97% (RAP-06, RB-64, RB-62, PJ-11, PK-10, PC-10, and RB-02), and approximately 15% of the isolates showed homology below 98% (Table 1).

Population analysis

When the 16S rRNA gene sequences of the isolates were analyzed and their phylogenetic association was built by the neighbor-joining method, 7 distinctive phylogenetic clusters were identified (Fig. 1). Among the isolates, approximately 50% (73 isolates) belonged to *Gammaproteobacteria*. *Actinobacteria* was the second most populous phylum containing 27 isolates, followed by *Firmicutes* (18 isolates) and *Alphaproteobacteria* (15 isolates). *Bataproteobacteria, Flavobacteria,* and *Acidobacteria* were minor constituents (Table 2). The *Gammaproteobacteria* group included *Pseudomonas* sp., *Rahnella* sp., *Serratia* sp., *Pantoea* sp., *Erwinia* sp., and *Enterobacter* sp., which are usually found in soil, plants, and the intestines of animals. The *Actinoabcteria* group was primarily composed of *Cellulomonas* sp. and *Microbacterium* sp.. When the isolates were classified for each species of longicorn beetle to determine the distribution, the number of isolates and phylum compositions were found to vary among the beetles (Table 2). In the cases of *M. diphysis, M. hirsute,* and *P. hilaris*, 41, 24, and 26 bacterial strains were isolated, respectively, and were distributed among most of the bacterial phyla found in this work. There were 16 and 15 bacterial strains isolated from the guts of *M. alternatus* and *C. rubra*, respectively, and no *Actinobacteria* were found in either of the insects.

Exo-enzyme producing properties of the isolates

The digestive activities of the isolates against xylan and pectin were tested on agar plates. The numbers of each enzyme producing strain and the enzyme producing properties of the isolates from the 9 longicorn beetle species are presented in Table 3. The proportion of xylanase producing strains of the isolates, belonging to each insect, varied from 6% to 33%, and reached 22% of the total isolates. A few isolates were identified as pectinase producers at 9% of the total isolates. The taxonomic composition of the xylanase producing strains showed that many of the isolates belonged to *Actionbacteria* (34%), making up 40% of its members; although *Actinobacteria* were only 19% of the total isolates (Table 4). *Gammaproteobacteria* made up 40% of the xylanase producers; however, these producers were only 18% of the total *Gammaproteobacteria* isolates.

Table 2. Distribution of the isolated bacteria belonging to each species of longicorn beetle

Insect sources	Total isolates	*Acido-bacteria*	*Actino-bacteria*	*Firmicutes*	*Flavo-bacteria*	*Alphaproteo-bacteria*	*Betaproteo-bacteria*	*Gammaproteo-bacteria*
Mesosa hirsute	24		7	1		3		13
Corymbia rubra	15			1		5		9
Monochamus alternatus	16			2				14
Psacothea hilaris	26		2	7	1	4		12
Moechotypa diphysis	41	1	16	3	2	1	2	16
Massicus raddei	10		1	3		2		4
Anoplophora malasiaca	2							2
Prionus insularis	5		1	1	1		2	
Olenecamptus clarus	3							3
Total	142	1	27	18	4	15	4	73

Table 3. Distribution of digestive enzyme producing bacteria in the guts of longicorn beetles

Longicorn beetles	Total isolates	Xylanase				Pectinase			
		Isolates	Activity[a]			Isolates	Activity		
			+	++	+++		+	++	+++
Mesosa hirsute	24	3	2	1		1	1		
Corymbia rubra	15	5	3		2	3		1	2
Monochamus alternatus	16	1	1						
Psacothea hilaris	26	4	2	2		1	1		
Moechotypa diphysis	41	10	8	2	1	6	6		
Massicus raddei	10	3	2		1	1	1		
Anoplophora malasiaca	2								
Prionus insularis	4	3	3						
Olenecamptus clarus	3	3	3						
Total	142	32	24	4	4	12	9	1	2

[a]Activities are represented as the size of the hydrolyzed area of substrate: +, 2-4 mm; ++, 4-8 mm; +++, over 8 mm.

Table 4. Distribution of digestive enzyme producing bacteria belonging to each phylum

Longicorn beetles	Total isolates	Xylanase				Pectinase			
		Isolates	Activity[a]			Isolates	Activity		
			+	++	+++		+	++	+++
Acidobacteria	1								
Firmicutes	18	3	2		1				
Actinobacteria	27	11	7	3	1	4	4		
Flavobacteria	4					2	2		
Alphaproteobacteria	15	3	1	1	1				
Betaproteobacteria	4	2	2						
Gammaproteobacteria	73	13	12		1	6	3	1	2
Total	142	32	24	4	4	12	9	1	2

[a]Activities are represented as the size of the hydrolyzed area of substrate: +, 2-4 mm; ++, 4-8 mm; +++, over 8 mm.

Discussion

A total of 342 colonies were isolated from the guts of nine species of longicorn beetle, and 142 strains were confirmed as composing the gut bacterial biota as judged by 16S rRNA gene sequence comparisons and their xylan and pectin degrading properties. Among the isolates, we postulated that more than 10% were unreported bacterial species. Phylogenetic analysis of the isolates revealed that 7 distinctive phylogenetic clusters were identified (Fig. 1), and half of the isolates belonged to *Gammaproteobacteria* and 19% were *Actinobacteria* (Table 2). This culturable gut microbial community we found in longicorn beetles is different from the one found in the mid-guts of gypsy moth larvae, a leaf eater (Broderick *et al.*, 2004), which showed that half of the isolates were low G+C Gram-positive bacteria, although many *Gammaproteobacteria* were also observed. In the case of the saprophagous Coleopteran insect, *Pachnoda ephippiata*, clone library screening established that more than 50% of *Actinobacteria* and *Clostridia* were found in the mid-gut; and in the hind-gut, over 80% of the population was members of *Lactobacillales*, *Clostridiales*, and *Cytophaga-Flavobacterium-Bacteroides* (CFB) (Egert *et al.*, 2003). Our recent culture-independent analysis data also demonstrated that many differences exist in the microbial communities between the longicorn beetle and *P. ephippiata* (unpublished data).

To investigate the differences in the bacterial population based on their diets, we compared the phylum compositions of the insects, which have different diet preferences. In the case of *M. diphysis*, 40 bacterial strains were isolated and were distributed among most of the bacterial phyla found in this work. *Actinobacteria* and *Gammaproteobacteria* composed approximately 80% of the isolates. A similar population ratio was found in *M. hirsute*. Both of these species have a common diet preference that usually involves the ingestion of broadleaf trees such as oak, a queritron, or chestnut trees. On the contrary, no *Actinobacteria* were found in the longicorn beetles that preferred needle-leaf trees, such as *C. rubra* and *M. alternatus*. Nevertheless, additional information is needed to postulate that these bacterial populational differ-

ences originated from the diet preferences of the longicorn beetles.

Although the highest proportion of xylanase producing strains (33%) among the isolates were found in *C. rubra* (Table 3), it was difficult to discriminate a difference among the longicorn beetles related to their diet preference or ecological characteristics because the population density of each strain was not determined. A distinctive distribution of exo-enzyme producing bacteria related to developmental stage (larvae or adult), or the sex of the adult, was also not found (data not shown). Within the bacterial population, the highest proportion for a xylanase producing strain reached 22% of the total population, reflecting their importance in the digestion and adsorption of nutrients from the woody diets of longicorn beetles; this level was below 10% in the case of a tested plant leaf-eater, Lepidoptera (unpublished data). It seems that *Actinobacteria* play important roles in the degradation of hemicellulose because many of the xylanase producing strains belonged to this group, and a relatively high proportion of strong enzyme producing strains were found (Table 4). In termites, it was confirmed that microorganisms producing cellulase, xylanase, or ligninase are involved in the digestion of wood (Cleveland, 1923; Slaytor *et al.*, 1997; Kinya *et al.*, 1998; Zhou *et al.*, 2007).

In this study, we isolated various culturable bacterial strains and tested their xylanase and pectinase producing properties. This is the first report concerning the microbial communities within the guts of longicorn beetles. Finally, gut bacteria, as a source of digestive enzymes, may depend upon the diet preference of the host insect.

Acknowledgements

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