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Isolation and Characterization of Six Microorganisms from the Digestive Tract of the Cricket *Gryllus bimaculatus*

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We describe the isolation and characterization of six different intestinal microorganisms from the digestive tract of the cricket *Gryllus bimaculatus*. Based on 16S rRNA gene sequences, we obtained six isolates belonging to four different genera: Staphylococcus, Bacillus, Citrobacter, and Proteus. All the isolates were resistant to ampicillin. Ampicillin is an irreversible inhibitor of the enzymeetranspeptidase, which is needed to make bacterial cell walls. None of the isolates were resistant to kanamycin, which binds to the 30S subunit of the bacterial ribosome and then inhibits total protein synthesis. Gram staining was conducted, in addition to morphological classification under a microscope. Four gram-positive isolates and two gram-negative isolates were detected. The gram-positive isolates were GL1 (round shaped, 2 am in diameter), GL2 (rod shaped, 2.5 μm in length), GL3 (rod shaped, 2 μm in length), and GL4 (round shaped, 1.5 μm in diameter). The gram-negative isolates were GL5 (rod shaped, 2 μm in length) and GL6 (rod-shaped, 2.5 μm in length). Notably, two of the isolates, GL2 and GL4, secreted specific extracellular proteins. These were determined by MALDI-TOF-MS spectral analysis to be a 87 kDa collagenase, 56 kDa hypothetical protein, and 200 kDa hypothetical protein. The six isolates in this study could be used for various biotechnological applications and pest management, both in the field and in greenhouse systems. In addition, it would be interesting to determine the relationship between these isolates and their host.

Key words : Cricket *Gryllus bimaculatus,* intestinal microorganisms, MALDI-TOF-MS, 16S rRNA gene

Introduction

The mammalian gastrointestinal tract is inhabited by a variety of microbiota with benefits for a range of physiological functions, such as obstructing pathogen invasion and inducing maturation of the immune system [25]. Additionally, the microbiota plays an important role in human metabolism by supplying limited nutrients, such as vitamins and small fatty acids [8]. It is suggested that the evolution of the association between insects and bacteria began over 250 million years ago, resulting in the establishment of the current mutually beneficial interactions between them [17]. The earliest report of an association between the insect gut and bacteria was based on observations of the olive fly, *Bactrocera oleae*, in 1909. Insects have ten times more intestinal bacteria of various types in their digestive tract than the total number of cells in their bodies [18]. Recently, most studies in this field have focused on the molecular systematics of intestinal bacteria, which is closely related to the growth and development of the insect [6]. Many entomologists are interested in the industrial use of biologically active substances produced from insect intestinal microbiota because these are recognized as new genetic resources for the field of biotechnology [13]. Representative examples of these reported to date include both arazyme and metalloprotease produced by *Serratia proteamaculans* in *Nephila clavata* [14], ligninase produced by *Sympetrum depressiusculum* [11], and xylanase produced by *Paenibacillus sp.* HY-8 in *Moechotypa diphysis* [10]. Although there are already studies on gut microbiota in some crickets, this is the first report of gut microbiota in the two-spotted cricket *Gryllus bimaculatus* used in this experiment [9]. A detailed review of the gut microbiota of insects published by Engel & Moran contributed greatly to

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the development of this field [20, 23].

A hemimetabolous insect species, the two-spotted cricket *Gryllus bimaculatus* (Orthoptera: Gryllidae), is one of the most economic importance cricket species in the world. It is easily bred outdoors, not requiring special conditions, and has recently been used as a model animal for tissue regeneration, neurophysiology, and behavioral research [16]. Crickets are rich in proteins and various lipids and have recently been used as animal feed for chicken and fish [1]. Therefore, there is a high demand for these insects, necessitating the mass rearing of healthy crickets. To achieve this, maintenance of healthy intestinal microbiota in the digestive tract is particularly important. Here, we identified microorganisms in the digestive tract of the cricket because such microorganisms are reported to have essential functions in the growth and survival of the host insects.

Materials and Methods

The fifth-instar larvae of the two-spotted cricket *G. bimaculatus* used in this study were gifts from the Rural Development Administration (Jeonju, South Korea). *G. bimaculatus* adults were reared at 28-30°C with humidity of 70% under a 10:14 hr light:dark photoperiod in plastic containers (55×39×31 cm). The crickets were fed a commercial feed for rats and rabbit (1:6) with an unlimited water supply. The artificial diet (67% cellulose powder, 3% casein and 30% dextrin) was fed to induce synchronous growth for 2 days before isolating intestinal microorganisms [22].

In a sterilized chamber, a sample of *G. bimaculatus* was anesthetized with CO_2 and then surface-sterilized by washing for 5 min in 0.5% sodium hypochlorite solution and rinsing with sterile water, providing complete disinfection of the cricket. An incision was then made in the abdomen, after which the digestive tract was immediately taken out using sterile tweezers. Intestinal microbiota in cold phosphate-buffered saline (PBS) was serially diluted in Luria-Bertani (LB) medium and cultured at 37°C for 24 hr. Isolated colonies were subcultured 3-5 times on LB medium until single colonies were identified. LB medium containing antibiotics was used to screen anti-antibiotic susceptibility. To yield a DNA template, a single colony was suspended in 200 µl of LB medium and cultured at 37°C for 24 hr. Each final PCR (100 µl) contained 5 U of Expand DNA polymerase (SolGent, Seoul, Korea), 2 µl of DNA template, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM (each) dATP, dCTP, dGTP and dTTP together with forward primer 518F 5′-CCA GCA GCC GCG GTA ATA C-3′ and reverse primer 805R 5′-GAC TAC CAG GGT ATC TAA TC-3′ (SolGent, Seoul, Korea) for the16S rDNA amplification. Reaction mixtures were first incubated for 5 min at 95°C. Then 35 cycles were performed as follows: 15 s at 94°C, 15 s at 50°C, and 1 min 30 s at 72°C. Reaction mixtures were then incubated at 72°C for an additional 5 min. PCR products were sequenced using primers of 518F and 805R (described above). The BLAST program was used for sequence homology searching against sequences of bacteria available in the databank (http://www.ncbi.nlm.nih.gov/); subsequently, a phylogenetic tree was constructed using the neighbor joining method [19]. To differentiate between the two categories of bacteria, Gram staining was performed. Each single colony of bacteria was smeared on a sterile glass slide containing a drop of sterile distilled water. The smear was covered with crystal violet, which served as the primary stain for 1 min, followed by the addition of Gram's iodine. This was then decolorized using 95% ethanol and counterstained with safranin for about 2 min. After a brief wash with distilled water, the sample was blotted dry for examination. Gram-stained isolates were viewed under a microscope (CX31; Olympus, Tokyo, Japan) at 1,000× magnification and photographed using a digital camera (Samsung, Seoul, South Korea).

Each secreted protein in the LB was collected by acetone precipitation, followed by SDS-PAGE. The resulting bands (secreted proteins) were cut out from the Coomassie Brilliant Blue (CBB)-stained gels and digested with trypsin (Promega, Madison, WI, USA), mixed with cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid, and subjected to MALDI-TOF analysis (Microflex LRF 20; Bruker Daltonics, Bremen, Germany). Spectra were collected from 300 shots per spectrum over the m/z range 600-3,000 and calibrated using a two-point internal calibration using trypsin auto-digestion peaks (m/z 842.5099, 2211.1046). A peak list was generated using Flex Analysis 3.0. The threshold used for peak-picking was as follows: 500 for a minimum resolution of monoisotopic mass and 5 for S/N. The search program MASCOT, developed by Matrix Science (http://www.matrixscience.com/), was used to identify proteins by peptide mass fingerprinting. The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of one missed cleavage, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, monoisotopic masses, and mass tolerance of ±0.1

Da. All chemicals used in this study were of analytical grade; 4-sulfophenyl isothiocyanate, a-cyano-4-hydroxycinnamic acid, sodium bicarbonate, and ammonium bicarbonate were purchased from Sigma (St. Louis, MO, USA).

Results and Discussion

New microorganisms are classified by several criteria, such as morphology, Gram staining, the component ratio of biomolecules, and behavior. However, 16S rRNA gene sequence analysis is now considered one of the best methods to determine bacterial systematics, since the gene is common among all microorganisms, shares the same biological functions, is highly conserved within a species and among species of the same genus, and rarely undergoes horizontal transfer [26]. Here, we isolated six members of the intestinal microbiota from the two-spotted cricket *G. bimaculatus* on LB medium, which is most commonly used for stable culture of intestinal microorganisms such as *E. coli*. These six isolates were named GL1–6 (from G. *bimaculatus* and LB medium used for the isolation). To clarify the evolutionary positions of these six isolates, we performed 16S rRNA gene sequencing, the results of which are shown in Fig. 1. The GL1–6 isolates most closely matched with *S. sciuri* strain DSM 20345 (99% DNA identity) [21], *B. cereus* ATCC 14579 (99% DNA identity) [4], *B. amyloliquefaciens* subsp. plantarum strain FZB42 (99% DNA identity) [5], *S. gallinarum* strain VIII1 (99% DNA identity) [3], *C. youngae* strain GTC 1314 (98% DNA identity) [12], and *P. mirabilis* strain ATCC 29906 (99% DNA identity) [2], respectively. Molecular phylogenetic trees of these isolates were constructed by the neighbor-joining method.

The morphological features of the six isolates were characterized by Gram staining. The results are shown in Fig. 2: four isolates, GL1–4, were Gram-positive, while GL5 and GL6 were Gram-negative. These results are consistent with similar strains classified as 16 rDNA. The isolates typically demonstrated the following morphological features: GL1 was round and approximately 2 μm in diameter; GL2 was rod-shaped and approximately 2.5 μm in length; GL3 was rod-shaped and 2 μm in length; GL4 was round and 1.5 μm in diameter; GL5 was rod-shaped and 2 μm in length; and GL6 was rod-shaped and 2.5 μm in length. The present findings were consistent with the results of other studies that isolated and identified the following microbiota from various organisms by Gram staining: a *S. sciuri* strain isolated from *Xylosandrus germanus* (Blandford), a *B. cereus strain* isolated from silkworm *Bombyx mori*, an *S. gallinarum* strain isolated from the sweet potato whitefly *Bemisia tabaci*, and a *P. mirabilis* strain isolated from the normal microbiota of the human gastrointestinal tract.

We tested antibiotic resistance in the six isolates using two different antibiotics: ampicillin, which irreversibly inhibits the enzyme transpeptidase, essential for bacterial cell wall construction, and kanamycin, which binds to the 30S subunit of prokaryotic ribosomes and causes both mistranslation and inhibition of translocation during protein synthesis [15, 21]. For this experiment, an LB plate covered with one of the six isolates was incubated at 37°C for 24 hr with a disc containing antibiotics placed on the LB surface. As shown in Fig. 3, on an LB agar plate, clear zones around a disc containing kanamycin meant that the isolate was completely prevented from growing in the presence of this antibiotic. However, no clear zones were observed for any of the isolates for LB plates containing either control or ampicillin disks. In summary, all six isolates tested were resistant to ampicillin but not kanamycin. Furthermore, when crickets were injected with kanamycin, we can observe some died soon afterwards, in contrast to untreated crickets (data not shown). These observations suggest that the intestinal microbiota is important for ensuring the survival and growth of the host cricket.

To characterize the proteins secreted from the isolates identified in this study, each LB culture was subjected to SDS-PAGE after concentration by acetone precipitation. Two notable secretory proteins (estimated molecular masses of 87 and 56 kDa) were detected from GL2 and one protein (200 kDa) was detected from GL4. Upon CBB staining, interesting bands were cut out from the gel and identified by MALDI-TOF. We found that GL2 secretes both an 87-kDa collagenase and a 56-kDa hypothetical protein, while GL4 secretes a 200-kDa hypothetical protein. Collagen, a major component of the extracellular matrix, is broken down by collagenase into collagen peptides. In this respect, it is interesting that the intestinal microorganisms of insects produce and secrete collagenase, suggesting that directly secreted collagenase helps to prevent the pathogenesis of bacteria in the intestine or more sensitive to bacteria membrane than intestinal epithelium [7].

In summary, we isolated and characterized six types of ampicillin-resistant microorganisms from the intestine of the hemimetabolous insect *G. bimaculatus*. These microorgan

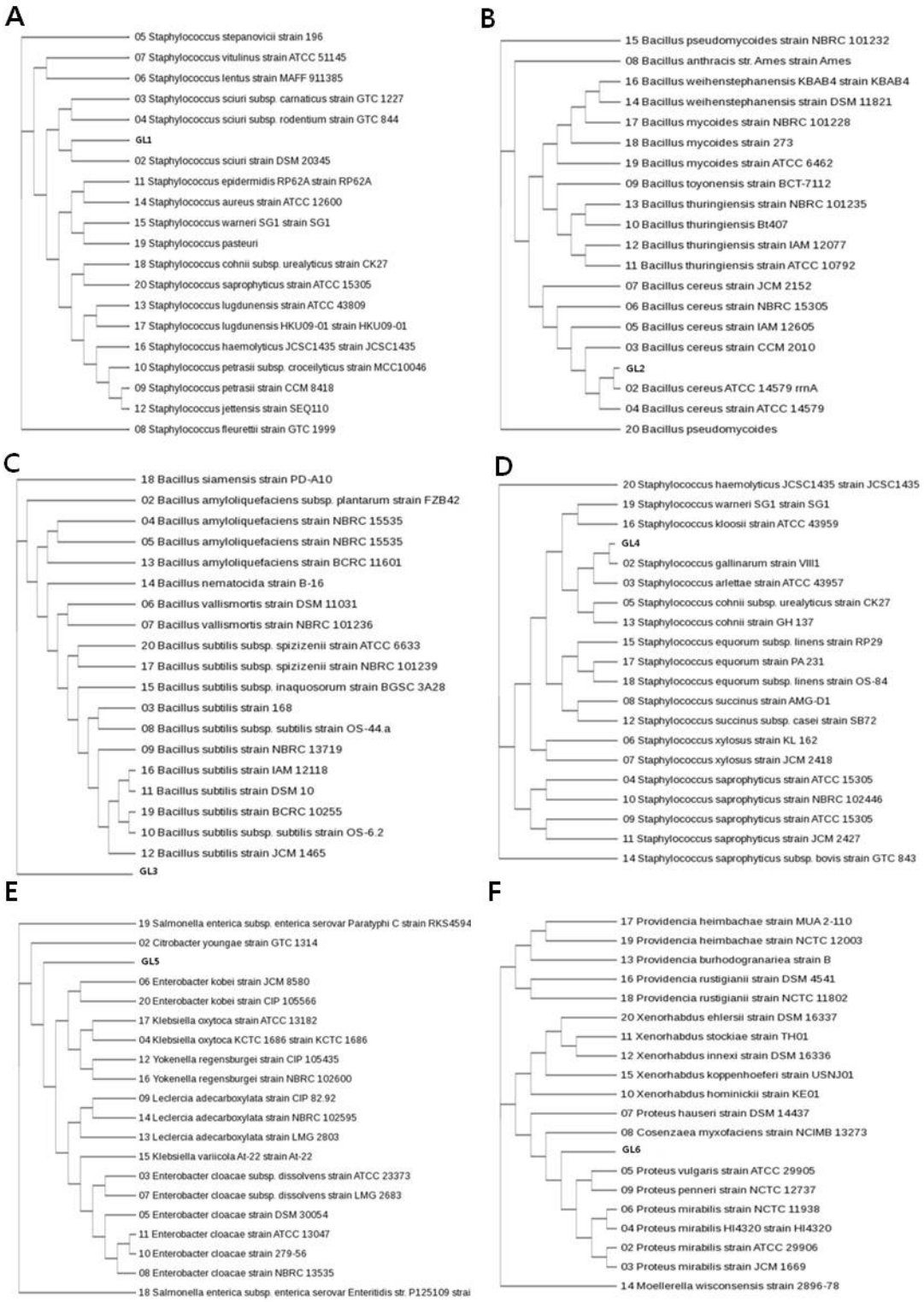

Fig 1. Phylogenetic tree of isolates. The phylogenetic tree was constructed using the neighbor-joining algorithm of 16S rRNA gene sequences. (A) Isolate GL1 is most closely related to *S. sciuri* strain DSM 20345, (B) isolate GL2 GL1 is most closely related to *B. cereus* ATCC 14579, (C) isolate GL3 is most closely related to *B. amyloliquefaciens* subsp. plantarum strain FZB42, (D) isolate GL4 is most closely related to *S. gallinarum* strain VIII1, (E) isolate GL5 is most closely related to *C. youngae* strain GTC 1314, and (F) isolate GL6 is most closely related to *P. mirabilis* strain ATCC 29906.

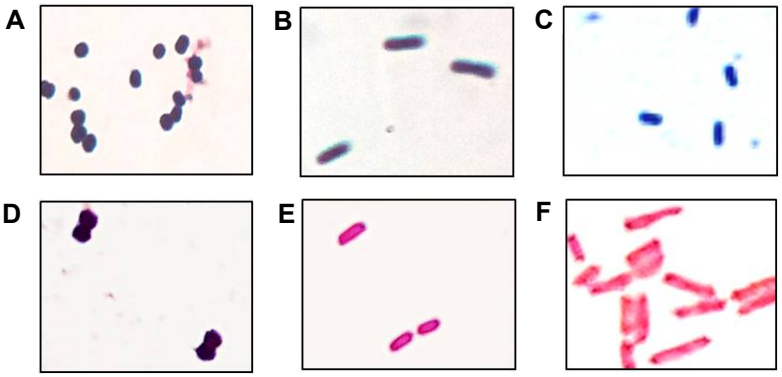

Fig 2. Microscopic observation of isolates. Four Gram-positive isolates: round GL1, 2 μm in diameter (A); rod-shaped GL2, 2.5 μm in length (B); rod-shaped GL3, 2 μm in length (C); and round GL4, 1.5 μm in diameter (D). Two Gram-negative isolates: rod-shaped GL5, 2 μm in length (E); and rod-shaped GL6, 2.5 μm in length (F).

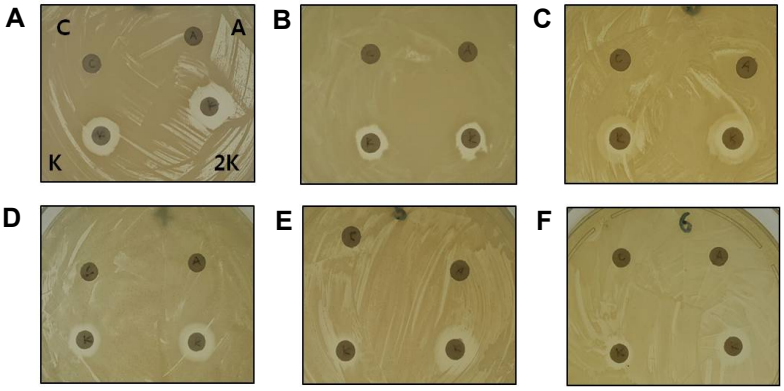

Fig 3. Antibiotic susceptibility. Isolates were streaked on LB plates with antibiotic-impregnated disks. Clear rings around the discs show that isolates did not grow, reflecting antibiotic susceptibility. In the panel, C, control (PBS); A, 100 μg/ml ampicillin; K, 50 μg/ml kanamycin; 2× K, 100 μg/ml kanamycin.

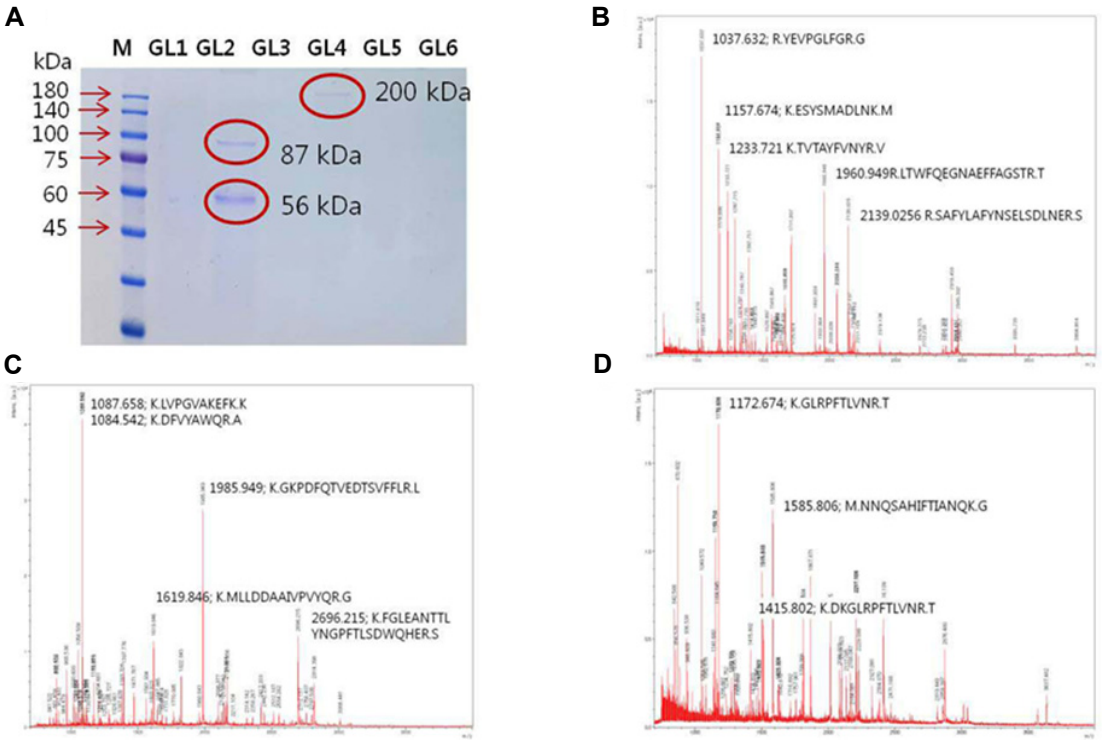

Fig. 4. SDS-PAGE of secreted proteins. SDS-PAGE (A) and MALDI-TOF-MS spectral analysis of proteins secreted from the isolates (B – D). The resulting extract was purified on reverse-phase cartridges. The ion peaks possibly corresponding to the secreted proteins are labeled at their peaks. (B) 87-kDa collagenase, (C) 56-kDa hypothetical protein, and (D) 200-kDa hypothetical protein.

isms include GL1, which is most closely related to *S. sciuri* strain DSM 20345 (Gram-positive); GL2, which is most closely related to *B. cereus* ATCC 14579 (Gram-positive); GL3, which is most closely related to *B. amyloliquefaciens* subsp. plantarum strain FZB42 (Gram-positive); GL4, which is most closely related to *S. gallinarum* strain VIII1 (Gram-positive); GL5, which is most closely related to *C. youngae* strain GTC 1314 (Gram-negative); and GL6, which is most closely related to *P. mirabilis* strain ATCC 29906 (Gram-negative). We classified these bacteria according to the analysis of their 16S rRNA genes.

In the near future, we will test the association of intestinal bacterial biota with the health of the host, including determining the relative abundance of each bacterial strain, which is a critical factor for host health. These studies will hopefully reveal the roles played by each of these microorganisms in the digestive tract in the survival, growth, and protection against pathogen infection of the host cricket. The information on the microorganisms reported here may also inform useful genetic resources in biotechnology.

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초록 : 쌍별귀뚜라미(*Gryllus bimaculatus*) 소화기관에서 분리한 6종류의 특성규명

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우리는 처음으로 쌍별귀뚜라미 소화기관에서 6종류의 미생물을 분리하고 특성을 규명하였다. 이들은 16S rDNA을 기준으로 분류한 결과 4종류(*Staphylococcus*, *Bacillus*, *Citrobacter*, *Proteus*)에 속하였다. 분리된 6종류의 미생물은 공통적으로 ampicillin에 저항성을 보이지만 kanamycin 저항성은 보이지 않았다. 이들을 Gram염색하여 미생물의 형태적 특징을 확인하였다. Gram-positive한 rod-shaped GL2와 round-shaped GL4는 다른 분리 균 보다 많은 양의 세포외분비물을 만들었다, 이들을 MALDI-TOF-MS spectral analysis결과 87-kDa collagenase, 56-kDa & 200-kDa hypothetical protein이였다. 새롭게 분리된 6종류의 미생물은 귀뚜라미의 생리에 미치는 영향과 이들의 생물공학적 혹은 해충 방제에 이용될 수 있는 연구가 기대된다.