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Cellulolytic ability of *Bacillus amyloliquefaciens* in the gut of *Reticulitermes speratus kyushuenesis* Morimoto[1)]

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Reticulitermes speratus kyushuenesis Morimoto의 장내세균 *Bacillus amyloliquefaciens*에 의한 Cellulose 분해 능력

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ABSTRACT

A new rod-shaped endospore-forming bacterium isolated from the hindgut flora of the termite, *Reticulitermes speratus kyushuenesis* Morimoto is described. The isolate stained Gram positive, but the KOH test and the test for L-alanine aminopeptidase were negative. The length of a single cell varies from 2.5-9.0㎛, and the cell is about 0.5-0.7㎛ thick. The isolate had a high cellulolytic ability and was identified as *Bacillus amyloliquefaciens*.

Key words : *Bacillus*, hindgut flora, termites, *Reticulitermes speratus kyushuenesis*

INTRODUCTION

Termites are generally distributed world wide and since they mineralize a broad range of organic compounds, especially heavy degradable polymers such as wood, they are ecologically important insects (Johnson & Whitford 1975; Abe 1982; Park 1993). Termites are classed as either lower termites or higher termites, and the latter include about 75% of the known species (Kuhnigk *et al.* 1995). They harbour in an enlarged part of the hindgut (paunch) symbiotic microorganisms, which consist in the case of the lower termites of bacteria and flagellates; the higher termites lack the flagellates (Breznak 1984). Yeasts and fungi have also been found. Although termites consume about 3-7 x 10^{15}g of lignocellulose/year and therefore play an important role in recycling cellulolytic material, the

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mechanisms of lignin breakdown are still unknown (Breznak & Brune 1994; Varma et al. 1994).

The species of the symbiotic flora and their role in the degradation of organic matter have only been partly elucidated. During the course of studies on the degradation of lignin monomers and other aromatic compounds, we found in the termite gut an endospore-forming bacterium, which showed some phenotypic resemblance to the group of *Bacillus* species, and it was tentatively identified as *B. amyloliquefaciens*. This paper describes the conditions in the termite gut, an essential prerequisite for an understanding of the metabolic processes occurring in the hind gut, and the characteristics of the microorganism.

MATERIALS AND METHODS

Organisms

Reticulitermes speratus kyushuenesis Morimoto was collected at the southern part of Korea, Kyungnam. The animals were kept in a container at 28℃, and fed on filter paper or news paper. The termites were acclimated to laboratory condition for at least 1 month prior to experiments. Termites had reached at least the third instar and weighed 10-15mg.

Condition in the gut

Termite guts were exposed by tearing the abdomen along the midline with fine forceps (Heath 1927). The paunch was ruptured onto a microscope slide. The fluid contents were immediately diluted with solution similar to hindgut fluids as described by Yamin (1981), ommiting nutrient components and bubbling with nitrogen gas prior to use.

Culture conditions and media

Hindgut microorganisms were cultured in medium I (Yamin 1981: modified), containing 10.8 mM K_2HPO_4, 6.9 mM KH_2PO_4, 21.5 mM KCl, 24.1 mM NaCl, 5.3 $MgSO_4$, 0.53 mM $CaCl_2$, a vitamin and trace element solution (10ml/L; Balch *et al.* 1979), and yeast extract (1g/L), pH 7.2. The following aromatic compounds were added after sterilization (1 mM each): benzoic acid, 4-hydroxybenzoic acid, vanillic acid, coumaric acid, and ferulic acid. Stock cultures of the isolates were usually maintained on tryptic soy agar (Difco) and transferred monthly.

Characterization and identification of the isolate

The colonial morphology was studied on tryptic soy agar and on nutrient agar (Difco). The cell and spore morphologies were examined after growth on nutrient agar supplemented with $MnSO_4$ (50mg/L), $CaCl_2 \cdot 2H_2O$ (100mg/L), and $MgSO_4 \cdot 7H_2O$ (500mg/L) by phase contrast microscope (Olympus, Japan). Motility was determined microscopically. Spores were stained by the method of Schiffer-Fulton (Gerhardt *et al.* 1981). After Gram staining (modified Hucker method, Gerhardt *et al.* 1981), the isolate was characterized by several biochemical tests such as the KOH test (Suβmuth *et al.* 1987), tests for oxidase and L-alanine aminopeptidase activity (Merck, Bactident 13000, Darmstadt, Germany), and growth on different selective media. The isolate was further characterized with rapid tube test for cellulose production (Smith 1977), which is the modified Pettersson medium without carbon source, using agar at a concentration of 0.75%, and dispensed into screw-capped tubes (16 x 75mm, 2ml per tube).

The isolate showing high cellulase activity was then identified using Biolog System (Microstation System™, 1993).

RESULTS AND DISCUSSION

Condition in the gut

The digestive system consisted of the foregut (consisting of the crop and the gizzard), the midgut and the hindgut. The hindgut divided into five successive segments, the first proctodeal segment, the second segment called the enteric valve, which controls the entrance into the third segment known as the paunch and in which the symbiotic microorganisms are abundant. The last two segments are the colon and the rectum (Fig. 1). An important function of the enteric valve is known to prevent the return of the paunch contents to the midgut or foregut (Noirot and Noirot-Timothée 1969). The Malphigian tubules entered the gut at the junction of the midgut and the first proctodeal segment, just in front of the proctodeal segment.

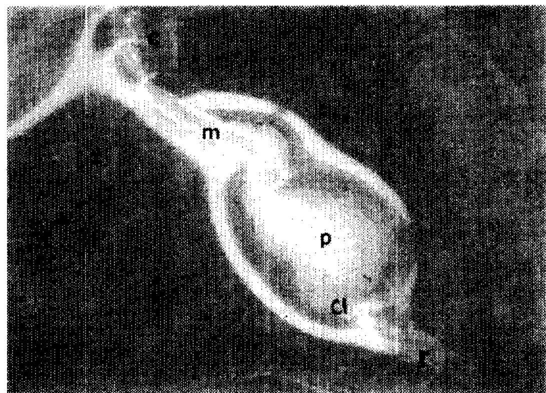

Fig. 1. Morphology of gut of *Reticulitermes speratus kyushuenesis*. c: Crop, m: Midgut, p: Paunch, cl: Colon, r: Rectum.

The termite hindgut has generally been assumed to be anaerobic. Several pieces of evidence have led to this concept: for example the sensitivity of termite protozoa to oxygen (Cleveland 1925; Trager 1934; Hungate 1939; Mauldin *et al.* 1972), studies on the metabolism of cellulose by termites in which acetate and hydrogen were reported as end products, and *in situ* formation of methane by hindgut bacteria (Hungate 1939, 1943; Cook 1943; Breznak 1975).

Table 1 shows the pH of the different sections of the gut of *R. speratus kyushuenesis* in this study, and other termites in other studies. pH values of the hindgut and the paunch were 6.8 and 7.2, respectively. In other studies, the hindgut including the paunch and the colon of most species had a pH in the range of 6.0-7.5, except for the soil-feeding species where conditions were more alkaline. Thus the conditions in the hindgut are favourable for the growth of many microorganisms which are usually tolerant of pH values ranging from 6 to 9. The midgut, in which enzyme secretion occurs, also had a pH around neutrality (O'Brien & Slaytor 1982).

Table 1. pH of the guts of *R. speratus kyushuenesis* and other known termites.

Species	Foregut	Midgut	Hindgut	Paunch
Reticulitermes speratus kyushuenesis	5.6	6.8-7.2	6.8	7.2
Reticulitermes lucifugus[1]	5.2	6.5-7.0	-	7.0-7.5
Kalotermes flavicollis[2]	5.2-5.4	6.8-7.5	5.0-7.5	-
Zootermopsis angusticollis[3]	5.2-6.8	5.2	3.0-6.8	-
Zootermopsis nevadensis[1]	-	7.0-7.5	-	7.0-7.5
Microtermes edentatus[4]	8.8-9.6	8.8-9.6	6.0-9.6	7.2-7.6
Microtermes arboreus[1]	-	7.0-8.0	-	6.4-7.0
Cubitermes severus[1]	-	6.5-7.5	-	9.0-10.0
Nasutitermes costalis[1]	-	5.5-6.0	-	9.0-9.5
Nasutitermes exitiosus[5]	2.0-2.8	6.8-7.5	-	5.5-6.5

1. Bignell & Anderson (1980), 2. Noirot & Noirot-Thimothée (1969), 3. Randall & Doody (1934, cited by Noirot & Noirot-Thimothée 1969), 4. Kovoor (1967), 5. McEwen et al. (1980).

Isolation of pure culture

The termite workers were surface sterilized by dipping the decapitated animals in 70% ethanol for about 30s and rinsing them with sterile water. The gut was removed with sterile forceps. The hindgut (paunch) contents were injected via a

syringe into liquid medium I (5*ml*) with or without yeast extract (1g/L) supplemented with a mixture of different aromatic compounds (Kuhnigk *et al.* 1995). The incubation of the inoculated medium and different serial dilutions thereof was performed under aerobic conditions at 28℃. Pure cultures were obtained by serial dilutions and by several subsequent platings onto solid medium I. On tryptic soy agar, raised opaque colonies with erose to lobate margines were formed. The diameter of the colonies was ca. 3mm after 2 days.

Characterization and identification of the isolate

Isolate is a rod-shaped motile bacterium occurring singly, in pairs, or in chains (Fig. 2). It stained Gram positive, but the KOH test and the test for L-alanine aminopeptidase were negative. The length of a single cell varies from 2.5-9.0㎛, and the cell is about 0.5-0.7㎛ thick. The cell wall is double layered, and the inner layer should represent the murein sacculus and the outer layer is most probably an S-layer (Kuhnigk *et al.* 1994). The cells produce subterminal oval endospores with swollen sporangia. Parasporal crystals are not found. According to the several biochemical tests, the isolate could be assigned to the Genus Bacillus, and distinguishing features of the isolate are shown in Table 2.

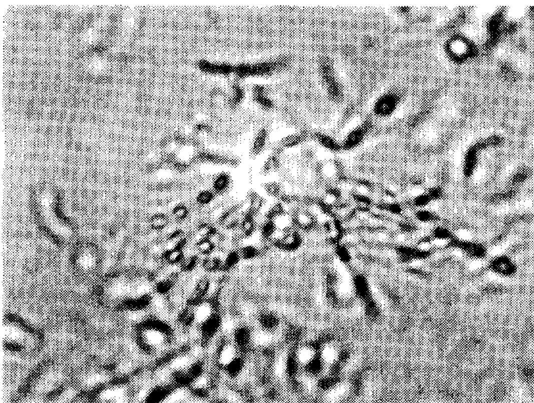

Fig. 2. Isolates of the symbiotic bacteria in the hindgut of *R. Speratus kyushuenesis*.

Table 2. Differential characters of the isolate and other similar endospore-forming bacterial.

Characteristic	Geneus				
	Isolate	*Bacillus*	*Sporolactobacillus*	*Clostridium*	*Sporosarcina*
Rod-shaped	+	+	+	+	−
Diameter over 2.5㎛	−	−	−	−	−
Filaments	−	−	−	D	−
Rods or filaments curved	−	−	−	D	NA
Cocci in tetrads or packets	−	−	−	−	+
Endospores produced	+	+	+	+	+
Motile	+	+	+	+	+
Stain Gram positive at least in young cultures	+	+	+	+[2]	+
Strict aerobes	D	D	−	−	+
Facultative anaerobes or microaerophilis	D	D	+	−[3]	−
Strict anaerobes	−	−	−	+	−
Homolactic fermentation	D	D	+	−	−
Sulfate actively reduced to sulfide	−	−	−	−	−
Catalase	+	+	−	−	+
Oxidase	D	D	ND	−	+
Marked acidity from glucose	+	+	+	D	−
Nitrate reduced to nitrite	D	D	−	D	D

1. Symbols : +, 90% or more of strains positive; −, 10% or less of strains positive; D, substantial proportion of speciesdiffer; NA, not applicable; ND, not determined.
2. Rarely Gram-negative
3. Rarely aerotolerant

For the cellulolytic ability of the isolate, the medium was allowed to solidify with the tubes in a vertical position after sterilization at 121℃ for 15min. A second batch of modified Petterson medium (A) was prepared by using two-thirds the specified volume of water. The remaining one-third volume of water (B) was mixed with enough cellulose-azure (Calbiochem, San Diego, California) to provide a 2% suspension when A and B were combined. A and B were sterilized separately, and then mixed while still hot, and the sterile mixture was pipetted onto solidified basal medium at a rate of 0.5*ml* per tube. The caps were tightened, and the tubes of medium

were stored at 4℃ until needed. The medium was surface inoculated with isolates, and tubes were inspected periodically for evidence of dye release by examining basal layers for blue coloration. The amount of dye released, and the speed of release, appeared to be related to the degree of cellulolytic ability of individual cultures. Among the tested isolates, the highly active species produced the first detectable effect within 2 days, and color density of the basal layer increased rapidly during subsequent incubation. None of the tubes inoculated with noncellulolytic species showed any evidence of dye release, and appeared similar to the uninoculated control. Figure 3 demonstrates the appearance of selected cultures after incubation of the two isolates (Negative and Positive) and control for 10 days. Although this method was designed as a qualitative test for fungi which are known to be highly active in the degradation of cellulose, it can be used during screening procedures to estimate relative cellulolytic ability by symbiotic bacteria.

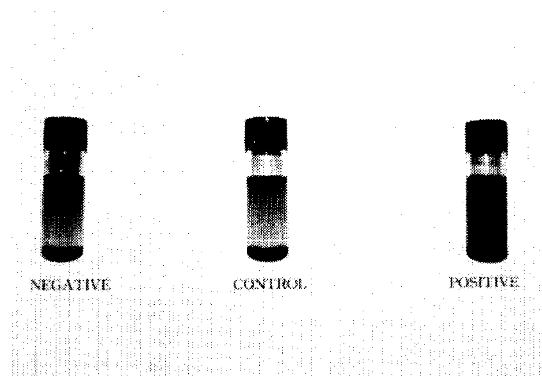

Fig. 3. Cultures of isolates on modified Petterson medium containing cellulose-azurs in the upper layer. Negative: noncellulolytic species, Control: not inoculated, Positive: highly active species.

The isolate which had high cellulolytic ability was then identified using a Biolog System. To identify the isolate, it was incubated on Biolog Universal Growth Medium (BUGM) added with glucose (1%) for 24 hrs. The isolate was identified as *Bacillus amyloliquefaciens*.

적 요

흰개미 장에서 분리한 간상의 내생포자를 생성하는 장내세균을 시험한 결과, 그람 양성균이었으나, KOH 시험이나 L-alanine aminopeptidase 시험에서는 모두 음성으로 나타났다. 공시균의 크기는 대략 2.5-9.0㎛ 였으며, 두께는 대략 0.5-0.7㎛ 였다. Cellulose 분해능력이 뛰어난 공시균을 Biolog System을 이용하여 동정한 결과 유사계수 0.755로서 *Bacillus amyloliquefaciens*.로 동정하였다.

검색어: Bacillus, 장내세균, 흰개미, *Reticulitermes speratus kyushuenesis*

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