An Alkaliphilic and Xylanolytic *Paenibacillus* Species Isolated from the Gut of a Soil-Feeding Termite

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Gut portions of soil-feeding termites belonging to the subfamily Termitinae generally show extensive alkalinity. An alkaliphilic and xylanolytic bacterium, strain SM-XY60, was isolated from the gut of such a soil-feeding termite *Sinocapritermes mushae*. This bacterium was a strictly aerobic endospore-forming rod, capable of growth at pH 6.5 to 10.5, and showing optimal growth at pH 9. The strain grew well on an alkaline medium containing K₂CO₃, but not Na₂CO₃. 16S rDNA analysis and physiological characterization revealed this strain to be a member of the genus *Paenibacillus* but distinct from any known species. The xylanase produced by the alkaline growing cells showed substantial activity and stability at high pH, implying an adaptation of the enzyme to the gut alkaline environment. The isolation of this alkaliphile suggests that the insect gut is of ecological significance for alkaliphiles.

Key words: alkaliphile, *Paenibacillus*, symbiosis, termite, xylanase

Termites (order Isoptera) harbor a diverse variety of microorganisms in their gut. The relationship between termites and the microorganisms in their gut is one of the most fascinating examples of symbiosis, and this relationship contributes to the digestion of ligno-cellulosic matter²⁹. Termites are ordinarily divided into two groups, so-called lower and higher termites. Lower termites harbor symbiotic cellulolytic protists in the gut while higher termites (family Termitidae) do not. Lower termites usually feed on wood, but higher termites show considerable variation in their feeding behavior. Soil-feeding termites feed exclusively on soil, presumably deriving nutrition from the humic compounds therein^{2,3,28}. However, analysis of gut contents

shows that soil-feeding termites feed on heterogeneous matter containing plant tissues and microbial cells besides a large proportion of minerals and humus particles³⁷. Although soil-feeding termites play an ecologically important role due to their great abundance especially in tropical areas, our knowledge about the specific nutritive components utilized and their metabolism within the gut is limited^{2,7}. Nevertheless, it is considered that the gut microflora are responsible for the degradation of soil organic matter.

Interestingly, the hindgut portions of soil-feeding members of the subfamily Termitinae show extreme alkalinity (up to >pH 12), representing one of the most extreme alkaline environments ever found within biological systems^{4,5,9}. It is reported that gut alkalinity is effective in the solubilization of humic materials in the ingested soil²², but aromatic components of humic materials are not substantially

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utilized²¹). Rather, polysaccharides and bacterial components are sufficiently transformed and mineralized^{20,21}).

A great number of alkaliphilic strains from various environments and their alkaline enzymes have been shown to be useful for a wide variety of industrial processes¹⁹). Due to the nature of soil-feeding termites described above, their gut is expected to be an attractive source of alkaliphilic microorganisms capable of degrading ligno-cellulosic substances. Despite the isolation of a large number of bacteria including ligno-cellulose degraders from the gut of various termite species²⁶), there has been no report, to our knowledge, of the isolation and description of alkaliphilic bacteria from the gut of termites. Here, we report the physiological characterization of an alkaliphilic and xylanolytic *Paenibacillus* strain isolated from the gut of a soil-feeding termite, and also describe some properties of the xylanolytic enzyme produced by this strain. Along with an accompanying report³⁹), the isolation of alkaliphiles suggests that the insect gut is of significance to the ecology of alkaliphiles.

Materials and Methods

Isolation and cultivation

A soil-feeding termite *Sinocapritermes mushae* (Termitidae, Termitinae) was collected in Okinawa prefecture, Japan. After the exterior surfaces of the termite had been extensively washed with distilled water, the guts were withdrawn using forceps. The guts were homogenized in distilled water and spread onto xylan-containing alkaline solid medium (polypeptone [Difco] 10 g, yeast extract [Difco] 5 g, NaH₂PO₄ 1 g, MgSO₄·7H₂O 0.2 g, K₂CO₃ 10 g, oat spelts xylan [Sigma] 5 g, and agar 15 g dissolved in 1 L of distilled water). After incubation for two days at 30°C, a xylan-degrading bacterium (designated as strain SM-XY60) was isolated and characterized further. The isolate was cultured at neutral pH with medium containing KCl instead of K₂CO₃. The strain has been deposited into the Japan Collection of Microorganisms as JCM10914.

Physiological and chemotaxonomic characteristics

The characterization of phenotypic features was carried out as described previously^{15,33}). Tests examining the utilization of various substrates as the sole carbon source were performed using GP Biolog microplates according to the manufacturer's instructions. The diamino acid in the peptidoglycan and menaquinones were analyzed as described previously²⁵). The G+C content of the DNA was determined by a previously reported method³⁸).

16S rDNA analysis

The 16S rRNA gene was amplified using as primers 5'-ATTGGATCCGTTTGAGCMTGGCTCAG-3' [27F] and 5'-TGAGTCGACACGGGCGGTGTGTRC-3' [1392R]. Amplified 16S rDNA purified with a Wizard PCR prep DNA purification kit (Promega) was used as a sequencing template. In addition to the sequencing primers previously described^{31,32}), the following primers were used: 5'-ACTCCTACGGGAGGCAGC-3' (350F), 5'-TGCTGCCTCCCGTAGGAG-3' (350R), and 5'-TTACCGCRGCTGCTGGCA-3' (520R). DNA sequencing was performed with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) and with an automatic sequence analyzer (ABI model 377). The nucleotide sequence data determined in this study will appear in the EMBL, GenBank, and DDBJ databases under accession number AB046581. The sequence determined in this study was compared with 16S rDNA sequences in the databases. Sequences were aligned using the CLUSTAL W package⁴⁰), then checked manually. Construction of a neighbor-joining tree³⁴) and bootstrap analysis for evaluation of the tree's topology¹³) were carried with the PHYLIP package version 3.5c¹²).

Enzyme assay

The culture supernatant was concentrated with Centricon-10 (Amicon) before use in the enzyme assay. Enzyme activity was assayed in 10 mM phosphate buffer (pH 7.0) at 50°C using 0.5% oat spelts xylan (Sigma) as the substrate. It was suspended homogeneously by ultrasonic treatment prior to its addition to the reaction mixture. The amount of reducing sugar liberated was determined using 3,5-dinitrosalicylic acid. One unit of enzyme activity was defined as the amount of enzyme that released 1 μ mol of reducing sugar as the xylose equivalent. The protein concentration was determined using a Bio-Rad DC Protein Assay kit with bovine serum albumin as the standard.

Results

An alkaliphilic and xylanolytic strain SM-XY60 was isolated from the gut of the soil-feeding termite *Sinocapritermes mushae*. The bacterium was found to be a strictly aerobic and slightly curved rod forming an ellipsoidal spore in swollen sporangia. The characteristics of strain SM-XY60 are summarized in Table 1. The pH range for growth was 6.5 to 10.5 and optimum growth occurred at pH 9, indicating that the strain was alkaliphilic. The temperature range for growth was 15°C to 40°C. Growth was inhibited in the

Table 1. Characteristics of strain SM-XY60 in comparison to allied *Paenibacillus* species.

Characteristics	SM-XY60	<i>P. lautus</i> ^a	<i>P. glucanolyticus</i> ^b	<i>P. campinasensis</i> ^c
Anaerobic growth	–	+	+	+
Growth at 5% NaCl	–	+	+	+
Growth at pH 5.7	–	+	+	–
Optimum growth:				
pH	9	7	7	10
Temperature (°C)	37	28–30	30	40
Catalase	+	+	+	+
Oxidase	+	–	+	–
Nitrate reduction	–	+	+	ND
Hydrolysis of:				
Casein	+	–	+	+
Starch	+	+	+	+
Gelatin	+	+	+	+
Urea	–	+	–	–
Degradation of:				
CMC ^d	+	+	+	ND
Xylan	+	ND	–	ND
Acid production from:				
D-Glucose	+	+	+	+
D-Xylose	+	+	+	+
Raffinose	+	–	+	–
Sorbitol	–	+	+	+
Cell wall peptidoglycan ^e	meso-DAP	meso-DAP	meso-DAP	meso-DAP
Menaquinone	MK-7	MK-7	MK-7	MK-7
G+C content	49.2	50–52	48	50.9
16S rDNA sequence identity to SM-XY60		96.0–96.6%	95.7–96.1%	94.8%

–, negative reaction; +, positive reaction; ND, not determined.

^a Data from Heyndrickx *et al.*¹⁶⁾ and Nakamura²⁷⁾.

^b Data from Alexander and Priest¹⁾ and Shida³⁶⁾.

^c Data from Yoon *et al.*⁴¹⁾ Acid production from four substrates is incorporated from the data using a Biolog microplate.

^d CMC, carboxy methyl cellulose.

^e meso-DAP, meso-diaminopimelic acid.

presence of 5% NaCl. Strain SM-XY60 grew well in the medium containing 1% K₂CO₃, but, when K₂CO₃ was replaced with 1% Na₂CO₃, growth of the strain did not occur.

Phylogenetic analysis of the partial nucleotide sequence of the 16S rDNA (1375 bp) revealed that strain SM-XY60 belonged to the genus *Paenibacillus* (Fig. 1). However, it was found to represent a lineage distinct from previously known *Paenibacillus* species in the phylogenetic tree. Strain SM-XY60 showed close phylogenetic relationships with *P. lautus*, *P. glucanolyticus*, and *P. campinasensis*, and groupings with the former two species were supported by a high bootstrap value, 92.8%. The strain demonstrated 96.6–96.0% nucleotide sequence identity with *P. lautus*,

96.1–95.7% with *P. glucanolyticus*, and 94.8% with *P. campinasensis*.

The G+C content of the DNA of strain SM-XY60 was 49.2±0.5%, which fell within the range for *Paenibacillus* species (45 to 54%). Also, menaquinone 7 as the principal quinone and meso-diaminopimelic acid in the cell-wall peptidoglycan were characteristic of the genus *Paenibacillus*^{16,36)}.

Table 1 shows comparisons of some representative characters of strain SM-XY60 and the three related *Paenibacillus* species. The absence of anaerobic growth and the growth inhibition in the presence of 5% NaCl were distinct features of strain SM-XY60. The alkaliphilic nature of

Fig. 1. Phylogenetic position of strain SM-XY60 in relation to *Paenibacillus* species based on the 16S rRNA gene sequence. *Alicyclobacillus acidocaldarius* was used as an outgroup for the phylogenetic analysis. Bootstrap values above 500 from 1,000 resamplings are shown for each node. The scale bar represents 0.02 substitutions per nucleotide position.

the growth was absent in the case of *P. lautus*²⁷⁾ and *P. glucanolyticus*¹⁾. *P. campinasensis* was isolated as an alkaliphile⁴¹⁾, but strain SM-XY60 and *P. campinasensis* differed in several physiological features. In the test examining the utilization of various substrates oxidized as sole carbon sources on Biolog microplates, utilization of 45 out of 95 substrates differed between them (data not shown). The results indicated that strain SM-XY60 probably represented a novel species of *Paenibacillus*, though the specific epithet for this was not given in this study.

Xylanase activity in the culture media was detected at levels as high as 0.54 U/mg protein when the cells were grown at pH 10 (Fig. 2). The effect of pH on the activity and stability of the enzyme is shown in Fig. 3. The xylanase was

stable over a broad range of pH and showed substantial activity, more than 80% of the level observed under optimum conditions, between pH 5 and pH 9.5. Xylanase activity was hardly detected (less than 0.07 U/mg protein) when the cells were grown at neutral pH although the growth rate of the cells did not differ significantly from that at alkaline pH. The enzyme was stable at 40°C for two hours and the optimal temperature for activity was 55°C.

Discussion

A large number of lignocellulose decomposers that contained novel species have been isolated from the gut of termites including soil-feeders^{26,35,42)}. However, to our

Fig. 2. Cell growth and xylanase production of strain SM-XY60. The cells were grown at pH 10.0 (solid circles). Xylanase activity in the culture medium was measured (solid squares).

Fig. 3. Effect of pH on the xylanase activity. The enzyme activity was measured under various pH conditions at 50°C. The optimal activity (2.8 U/ml) was obtained at pH 6.0 and was taken as 100%. The pH of the reaction mixtures was adjusted with acetate buffer (pH 4.0–6.0), phosphate buffer (pH 6.0–8.0), Tris-HCl buffer (pH 8.0–9.0) or glycine-NaOH buffer (pH 8.5–11.0), and relative activity is shown (open circles). The pH stability of the enzyme was investigated by incubating the enzyme under various pH conditions at 30°C for two hours. The residual activity relative to that observed at pH 6.0 (2.3 U/ml), taken as 100%, is shown (open squares).

knowledge, this is the first report of the isolation and characterization of an alkaliphile from the termite gut. Although Brune⁷⁾ mentioned the presence of a number of alkaliphiles in extremely alkaline gut regions, no taxonomic or physiological description of them was given. In the accompanying paper³⁹⁾, the presence of *Paenibacillus* species related closely to strain SM-XY60 was revealed in the gut of some Termitinae termites in Thailand by culture-based and culture-independent analyses. The species represented by

strain SM-XY60 is probably shared by some members of the subfamily Termitinae whose guts have extreme alkaline regions. On the other hand, in an extensive survey of the 16S rDNA clones of the gut microbial community of a lower termite *Reticulitermes speratus*^{17,18,32)}, we failed to detect a 16S rDNA sequence affiliated to *Paenibacillus*. There are two reports about the isolation of *Paenibacillus* from lower termites^{35,42)}. One report is on a xylanolytic *Paenibacillus macerans* and the other, a cellulolytic *Paenibacillus* sp. but the two species are distantly related to strain SM-XY60. Strain SM-XY60 was distinct from any previously known *Paenibacillus* species with respect to both 16S rDNA sequence similarity and several physiological features. Thus, this strain should represent a novel species of *Paenibacillus*. The gut community of termites consists of a large number of yet-unknown microorganisms^{29,30)}. Considering the immense diversity of termites particularly soil-feeders, their guts are likely to be valuable sources of lignocellulose-decomposing alkaliphiles which, along with their enzymes, may be useful in applied science.

Although the extent to which gut symbionts contribute to digestion remains unknown, the gut alkalinity may affect their physiological and biochemical properties. The xylanase produced when strain SM-XY60 was grown at alkaline pH showed not only stability over a broad range of pH but also significant activity at high pH (approximately 80% of the optimal level at pH 9.5). The enzyme was produced in substantial amounts only under alkaline conditions. These enzymatic characters are beneficial for adaptation to alkaline environments in the termite gut. This strain also showed cellulolytic activity, though it was weak, under both alkaline and neutral conditions. Probably, the strain plays a role in the digestion of hemicellulose and cellulose in the ingested soil.

The ion content in the gut may also affect the physiology of the isolate. In some soil-feeding termites, portions of the gut corresponding to regions of high pH are known to contain large amounts of K^+ ⁶⁾. In caterpillars, whose gut is also known to be an alkaline environment, elevation of the gut pH is evidently linked to potassium transport and the net accumulation of K_2CO_3 ¹¹⁾. The finding that strain SM-XY60 grew well in the alkaline medium containing K_2CO_3 but not Na_2CO_3 may be a reflection of the chemical conditions in the gut. Generally, alkaliphiles have been isolated in alkaline media containing Na_2CO_3 ¹⁹⁾, although some are known to grow in alkaline media containing K_2CO_3 ^{23,24)}. The dependence of growth on K_2CO_3 instead of Na_2CO_3 may require special attention in studies involving the isolation of alkaliphiles from the gut of certain arthropods. In the

accompanying paper³⁹), some of the alkaliphiles isolated from several termite species indeed preferred K⁺ to Na⁺ for their growth.

One may consider that the strict aerobic phenotype of strain SM-XY60 is a contradiction to its inhabitance within the gut. However, a study using a microelectrode has revealed that the gut environment is micro-oxic and that oxygen penetrates the gut through the gut periphery⁸). Such oxygen is consumed through the autoxidation of humic substances in the case of soil-feeding termites²²) and probably through the rapid and continuous activities of gut symbionts in most termites⁸). In fact, a large number of strict aerobic bacteria have been isolated from termite guts²⁶).

Isolation of the alkaliphile suggests that the gut of certain termites is a niche for alkaliphiles. In addition to soil-feeding termites of the subfamily Termitinae, extensive alkalinity of the gut is a common feature of the termites of this subfamily, regardless of their feeding behavior⁵). An elevation of gut pH to some extent is also evident in other subfamilies of higher termites except fungus-growing termites (subfamily Macrotermitinae). Moreover, high pH values within the gut have been reported for certain lepidopteran, coleopteran and dipteran larvae^{10,11,14}). Considering the abundance and diversity of arthropods as well as termites, the arthropod guts may be a rich reservoir of alkaliphiles.

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