JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2008. Vol. **2**(1), p. 39-47

Preliminary Assessment of Abundantly Growing Bacteria Isolated from *Macrotermes* Gut. Insect order: Isoptera. Species: *Macrotermes convulsionarius* (König)

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(Received: 20 January 2008; accepted: 28 February 2008)

Symbiotic association of microbes and their beneficial roles in termites have been delineated by several studies. Since microbial symbiotic relationship is an essential feature of the termite nutritional ecology and especially important in plant polymer degradation, we have studied the preliminary assessment of the major cultivable bacterial population in *Macrotermes convulsionarius* (könig) gut using traditional and molecular methodologies. Microscopic observation assured that the flagellated bacteria are predominant in the gut. Conventional techniques involving plate count method demonstrated *Bacillus* (45%) *Acinitobacter* (24%), *Salmonella* or *Enterobacter* (15%), and *Enterococcus* (15%). Partial sequence of 16s rRNA gene illustrated that the presence of one or more species within the four groups. A novel species belongs to the family *Enterobacteriaceae* was identified in the gut of termite (access number: AJ620950). Phylogentic tree constructed based on the sequence data further supported the association of these strains to their respective groups. Biochemical studies indicated that the isolates are moderately salt tolerant, possess the enzymes oxidase, catalase and phosphatase and negative to urease and lipase. All the isolates utilize wide range of carbon sources. All culturable isolates in the present study exhibited facultative anaerobic property. These observations suggesting these bacteria are essential to provide anaerobic gradient as well as substrate heterogeneity within the termite gut.

Key words: Cultivable, Bacteria, 16S rRNA, *Macrotermes*.

Termites are one of the most diverse and abundant insects and account for approximately 50% of the total soil macro-fauna in many tropical ecosystems (Wood & Sands, 1978). Termitidae is the largest family, found only in Asia and Africa comprising about 80% of all the termites (Ruelle, 1989). All termites are known to contain diverse bacterial species in their guts (Breznak, 2000; Breznak & Brune, 1994; Brauman *et al.,*1992; Hongoh *et al.,* 2003; Luginbühl *et al.,* 2007). Some of the functions of the gut microbiota are: cellulose digestion, nitrogen fixation, recycling the excretory nitrogen and preventing the entry of foreign bacteria (Ohkuma, 2003; Brune & Friedrich, 2000; Leschine, 1995; Veivers, *et al.,* 1982; Potrikus & Breznak, 1981; Schultz & Breznak, 1978; Potrikus & Breznak, 1977). Importantly, termites are thought to play essential roles in litter fragmentation and recycling of nutrients into soil (Dangerfield *et al.,* 1998). Our understanding of the origin of host-gut microbe interactions in termites is very much in its infancy

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(Bignell, 2000). For example, our understanding of lignin degradation remains incomplete[6] although the mechanisms of cellulose digestion are much clearer. Despite the findings of above important functions in various termites, not much is known about the bacterial diversity of termites from *Macrotermes* of Indian origin. In addition, the gut community varies markedly from species to species (Lilburn *et al.,* 1999). The objective of this preliminary study was to isolate, identify, and characterize the cultivable and predominantly colonizing bacteria from the gut of *Macrotermes convulsionarius* (könig), a dominating termite species from South India. The analysis of 16S rRNA genes, directly amplified from the extracted DNA from termite guts, would give the maximum diversity of the microbial flora. However, the cultivable bacteria would facilitate the understanding of host-gut interaction and also lead to the identification of bacteria of industrial interest. Serial dilution method was adopted to culture the bacteria using the nutrient agar medium and numerically abundant bacteria were characterized using traditional and modern techniques.

MATERIAL AND METHODS

Sampling and enumeration of culturable bacteria

About 10 adult termite workers and few soldiers from three different termite mounds separated by minimum of 200 meters distance were collected from community forest area of CCMB staff quarters, Hyderabad, India. Laboratory rearing of *Macrotermes* without the fungi is a difficult task, (Rouland-Lefevre & Bignell, 2001) thus the isolation of bacteria was performed on the same day. Termites were surface sterilized with 0.1% aqueous mercuric chloride followed by absolute alcohol by soaking them for 10 min in each solution and then dried under sterile condition. Whole gut was isolated from these individuals (triplicates for each colony) using sterilized forceps, homogenized individually under sterile condition in 0.9% NaCl, serially diluted and plated (100 µl) on nutrient agar medium (peptone [0.5% w/v], beef extract [0.3% w/v]), sodium chloride [0.8% w/v]) and incubated at room temperature for 15 days. About 50 mgs of fungal comb material also was homogenized, serially diluted and plated as described above. Common morphotype bacterial colonies between fungal comb and termite gut were carefully excluded, as these colonies would have derived from the ingested fungal comb. Few overlapped bacterial isolates could have been lost in this process but this screening assured that the isolates were typical for the gut of the termite. Bacterial isolates from the triplicates of all the three termite colonies were compared and the common isolates were used for the present study. Based on the colony morphology, 14 different bacteria were collected and clonally purified by repeated streaking on nutrient agar plates. Pure colonies were labeled GK1 to GK14 and subjected to further characterization. Numerical abundance of colonies obtained from the termite gut and its relative proportion to the total colony forming units (CFU) was derived on nutrient agar medium.

Electron microscopy

Isolated intestine gut was flushed twice with 0.5 ml of 100 mM phosphate buffer; pH 7.2, pooled together and centrifuged for 10 minutes at 5,000g at 4°C.The pellet was suspended in 100 µl of phosphate buffer. Images of negatively stained (2% w/v uranyl acetate) bacteria were recorded using JEOL-100CX microscope.

Morphological, biochemical and growth characteristics

Cell morphology was studied using light microscopy. Biochemical characteristics were determined on nutrient agar plates in duplicates according to the methods described by Lanyi (1987); Smibert & Kreig (1994). Tolerance to different salt concentrations was studied by adding different concentrations of NaCl to nutrient agar plates. Growth at different pH was determined using nutrient agar plates buffered with phosphate (pH 5.0 to 8.0) or Tris (pH 9-12). Ability of the bacteria to utilize different carbon compounds was tested by substituting the minimal medium (without citrate) with 0.5% of carbon compound and sensitivity to different antibiotics was performed on nutrient agar medium by disk diffusion method. The sensitivity and resistance of each isolate were determined by the criteria of the National committee for clinical laboratory standards (1997).

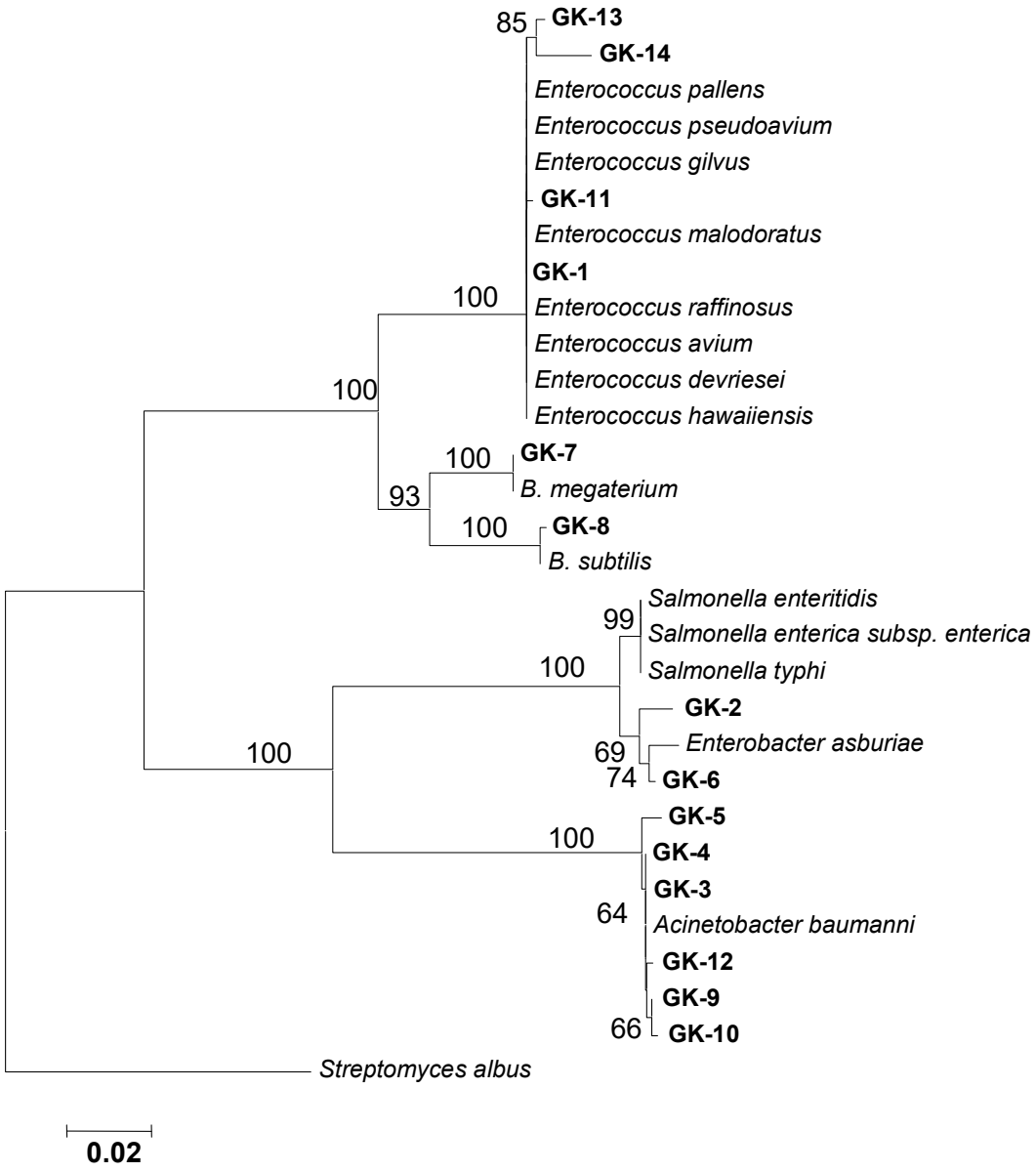

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequence showing the phylogenetic relationship between the bacteria isolated from the gut of *Macrotermes convulsionarius*, a dominant termite of South India, and other closely related microorganisms of the genera *Enterococcus*, *Salmonella, Enterobacter, Acinetobacter* and *Bacillus*. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are given at the nodes.

Besides sequence similarities and phylogenetic analysis, phenotypic characteristics further supported their generic affiliation. One from each group, GK-2, GK-3, GK-7 and GK-14, was characterized in detail with respect to phenotypic properties and the results are shown in table 2. Relative abundance by plate count method was tabulated and presented in table 1.

Table 1. Characteristics of the cultures isolated from gut of *Macrotermes convulsionarius**

Characteristics	GK 2	GK 3	GK 7	GK 14
Size	1 mm	1 mm	1-2 mm	0.2 mm
Shape	Round, Convex, Smooth opaque	Round, Convex, Smooth Opaque	Round, Convex Smooth Opaque	Punctiform Round Shiny Smooth convex
Color	White	White	White	Yellow
Form	Short rods	Cocci	Short rods	Short rods
Arrangement	Single	Single	Single	Single
Motility	Motile	Non-motile	Non-motile	Motile
Salt tolerance (%)				
Tolerance to salt (%)	0 to 12	0 to 6	0 to 10	0 to 8
Optimum temperature	37	37	37	37
Tolerance to pH	5 to 10	5 to 10	5 to 10	5 to 10
Optimum pH for growth	7.5	7.5	7.5	7.5
Biochemical characteristics				
β-galactosidase,Oxidase, Esculine hydrolysis, TSI	+	-	+	+
Catalase, Phosphatase	+/-	+	+	+
Casein hydrolysis,Gelatinase, Arginine dihydrolase, H_2S production Lipase, Urease, Arginine decarboxylase, Methyl red, Indole test, Voges-Proskauer test,Nitrate to nitrite reduction	+ -	- -	- -	- -
Lysine decarboxylase	-	-	-	+
Starch hydrolysis	-	-	+	+
Citrate utilization	+	+	-	-
Utilization of sugars				
Adonitol, Dulcitol, Galactose, Inositol, Inulin, Pyruvate, Raffinose, Rhamnose, Sorbitol, Sucrose, Trehalose, Xylose	+	+	+	+
Arabinose, Fructose, Glucose, Xylitol, Lactose, Maltose, Mannose, Mellibiose, Ribose	+	+	+	-
Citrate, Glycerol	+	+	-	-
Utilization of amino acids				
Alanine, Arginine, Asparticacid, Aspargine, Cystine, Glycine,Glutamic acid, Histidine, Tryptophan	+	-	-	-
Leucine, Serine	-	-	-	-
Lysine, Methionine	+	+	+	+
Phenyl alanine	+	-	-	-
Threonine	+	-	+	-
Tyrosine	+	+	-	-
Sensitivity to antibiotics				
Ampicillin, Bacitracin, Chloramphenicol, Erythromycin, Vancomycin, Fperazone	S	R	S	S
Co-trimoxazole, Kanamycin, Incomycin, Nitrofurantoin, Streptomycin, Tetracycline, Tobramycin, Amikacin, Ofloxacin, Lomefloxacin, Roxithromycin, Ciprofloxacin.	S	S	S	S
Nalidixic acid	S	S	S	R
Pencillin	R	R	S	S

* -, Negative; +, positive; S, sensitive; R, resistant

Table 2. Numerical abundance based on CFU of diluted termite gut homogenate

Bacterial type	Colony count (10^6 CFU gut ^{1})*	n	Fraction of total CFU
Bacillus (GK-7)	6.4 ± 1.2	4	45%
Acinetobacter (GK-3)	4.5 ± 1.7	4	32%
Salmonella (GK-2)	3.2 ± 1.2	4	23%
Enterococcus (GK-14)	2.8 ± 1.9	3	15%

* Average ±SD of n dilution

DISCUSSION

Termites have a major role in controlling carbon and nitrogen fluxes both, in semiarid and humid environments. Termites harbour diverse bacteria in their gut and their possible roles are mentioned above. Investigations of termite gut microbiota by analysis of 16S rRNA gene have been performed in lower and higher termites (Ohkuma & Kudo, 1996; Hongoh *et al.,* 2005; Thongaram *et al.,* 2005). Caste and age dependant microbial association within the colony was shown by terminal restriction fragment length polymorphism (T-RFLP) and clonal analysis of 16S rRNA genes. For instance, the same species of older workers of different colonies comprised almost same kind of bacteria in their gut (Hongoh *et.al.,* 2006). As we have collected adult worker termites from different colonies, it is not surprizing that they share common culturable bacterial population. In the presesnt study, abundant colonies of 14 different mophotypes belonging to four genera were observed and it is not unusual as the recovery of cultivable bacteria was reported to be less (Schultz & Breznak, 1978). Further we have excluded the comon bacteria between fugal comb and termite gut and the resulted four genera gave assurance that the isolates are typical for the gut. Natural variability between individual samples were not observed as we have chosen only the termite gut associated bacteria.

It is evident from table 2 that the majority of isolates were motile and this observation is further supported by electron microscopy (Fig 1). The EM picture showed slightly curved to banana shaped to rod shaped bacteria having multi flagella. From the morphology and the colony numbers it appeared that the species of *Bacillus* and *Acinetobacter* contribute to 45 % and 24%, respectively, where as *Salmonella* or *Enterobacter* and *Enterococcus* hold 15% each (Table1). Also our findings corroborating with earlier report in which it was shown that about 70% of the bacterial isolates belong to *Bacillus* in both wood and soil feeding termites (Bauer *et al.,* 2000; Konig, 2006). We have observed *Proteobacteria* and *Fermicutes* as major groups in the present study. Isolation of bacteria belonging to very few major groups is reported by earlier studies. For instance, Husseneder *et al.,* (2005) have reported the presence of *Fermicutes, Bacteriodetes* and *Proteobacteria*. Similarly, Wenzel *et al.,* (2002) also witnessed the presence of *Proteobacteria, Bacteriodetes, Actinobacteria* and *Fermicutes*. Further, the cultivation independent methods involving 16S rRNA gene cloning also supported the occurrence of above major groups (Shinzato *et al.,* 2007) in higher termites. As 16S rRNA probe has limitations in bacterial identification we have also studied morphological, biochemical, and phylogentic analysis and growth characteristics of the isolates. All non-culturable bacteria which are known to play an important role in the gut of termite is not discussed in this report as the primary concern of this study is on culturable bacteria of termite gut. Ecological implications of these isolates in terms of defense, substrate availability, pH and aerobic gradient are interesting.

At different stages of ligno-cellulose degradation in the termite gut, different substrates might be available to the microbes. When complex carbohydrate polymers are cleaved into simpler compounds, symbiotically associated microbes are expected to utilize the carbon compounds and this

is evidenced from the data that the present bacterial isolates utilized a wide range of carbon compounds, though some variations were observed with respect to amino acid utilization (Table2). Termite gut experiences different concentrations of salts and oxygen and a gradient of pH (Brune, 1998) thus, enable the bacteria to experience such conditions. The bacteria under study can tolerate a wide range of salt concentrations (6 to 10%; Table 2). Termite gut contains an anaerobic gradient system and facilitates the growth of anaerobic or facultative anaerobic bacteria (Bauer *et al.,* 2000; Brune *et al.,* 1995; Brune, 1998). The present bacteria are facultative anaerobes as proved from their growth on arginine dihydrolase medium under both aerobic as well as anaerobic conditions. Role of facultative organisms in the termite gut may be to utilize the oxygen and create anaerobic conditions for the anaerobic microorganisms. Abundance of *bacillus* type in the gut may also involve in the host defense against epithelial colonization of enteric pathogens (Rolfe, 1997). In mammalian intestinal tract, *enterococci* and bacillus are recognized as important surface colonizers involve in defense against pathogens (Tannock, 1997). The termite gut pH is reported to be alkaline; perhaps the highest gut pH in the biological system (Brune & Kuhl, 1996). Therefore, pH is not a limiting factor for the growth of present isolates as they tolerate a wide pH range. Further, the mound soil is known for phosphate eutropic 'hot spot' and is obviously derived from the stored plant matter. The phosphatase from all the isolates (Table 2) and phytase Powar & Jagannatham, 1982) from GK-7 (data not shown) (GK-7 is closely related to *Bacillus subtilis*) could be responsible for phosphate accumulation in the mound soil. Although the industrial enzymes were not presented in the study, extra cellular xylanase from *Bacillus subtilis, Bacillus megaterium* and *Enterobacter* was well established (Roger Bernier *et al.,* 1983; Sindhu et al, 2006; Khandeparkar & Bhosele, 2006). In addition, some of these bacteria from different sources are known to degrade lignin or lignin-related aromatic compounds (Buchan *et al.,* 2006; Hullo *et al.,* 2001). Thus the bacterial isolates, GK-2, GK-3, GK-7 and GK-14 could be significant in degradation of above polymers in the termite gut. All the isolates exhibited diverse morphological, growth and phenotypic characteristics and sensitivity to antibiotics. These parameters could be extrapolated to explain detailed ecological implication of microbial diversity in termite gut but non-culture based molecular techniques are required to substantiate the complete microbial diversity.

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