16S rRNA TYPING OF CELLULOLYTIC BACTERIA FROM THE TERMITE *ODONTOTERMES FORMOSANUS*

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ABSTRACT

Odontotermes formosanus termites are recognized as one of the major ecosystem engineers in tropical soils. This termite depends on intestinal microorganisms for cellulose digestion. Nine bacterial isolates from termite gut were identified using sugar fermentation and biochemical tests and confirmed by 16S rRNA gene sequencing. The aerobes isolated from the termite gut were Bacillus sp., Citrobacter freundii and Pseudomonas aeruginosa. The facultative anaerobes isolated were Salmonella entrica, Enterococcus casseliflavus, Staphylococcus gallinarum and Serratia marcescens. DNA from these bacterial cultures was extracted for molecular identification by 16S rRNA gene amplification. The cellulolytic activities of these bacteria were assessed by congo red assay. This study revealed the presence of cellulolytic aerobic and facultative anaerobic bacteria in the gut of termite Odontotermes formosanus which could be manipulated for their cellulose digestion in rumen

Key words: *Odontotermes formosanus,* 16S rRNA gene, Polymerase chain reaction, Cellulolytic bacteria

INTRODUCTION

Odontotermes formosanus, a higher termite, comes under the order *Blattaria* and subfamily *Macrotermitinae* (or Blattodea; cockroaches). The termite gut microbe ecosystem is one of the most fascinating examples of symbiosis between an animal and microbe, and among a diversity of microbes (Kane 1997, Radek 1999) such as bacteria, protist, fungi, archae and flagellates (Breznak and Brune, 1994, Ohtoko *et al,* 2008). Significant cellulolytic activities have been reported in the symbiotic fauna of higher termites (Tokuda *et al.* 2007) and these gut microbes possess wood decomposition enzymes that can be used in wood digestion to turn agricultural plant wastes to useful products (Sanderson, 1996; Bignell *et al.* 1997). Since termites are reported to have strong cellulolytic bacterial flora, these bacteria could be manipulated for improving the digestion of

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fibrous animal feed stuffs. Keeping this in mind, in this study, nine species of cellulolytic bacteria from the gut of *Odontotermes formosanus* were isolated by culturing in a medium containing carboxymethyl-cellulose and cellobiose and characterized for their possible manipulation.

MATERIALS AND METHODS

Isolation of microbes from termite gut : Soil termites were collected and identified as *Odontotermes formosanus* based on their shape and parts. The termites were washed with sterile distilled water, dried on a filter paper and externally sterilized with 70% ethanol. The entire gut was removed with sterile forceps and crushed. The guts were homogenized in 1 ml of sterile distilled water, centrifuged at 12,000 rpm for 5 min to remove large gut debris. The supernatant was serially diluted and aliquots of 0.3 ml were then plated on Nutrient agar, Streptomyces agar, Clostridial agar and Bacteroides agar. Colonies were picked up from the agar plates after incubating at 37°C overnight and inoculated into respective broths.

Identification and Characterization of bacteria isolated from termite gut: Gram's staining was done for morphological identification of bacteria isolated from termite gut. Termite gut bacteria were characterized by sugar fermentation and biochemical tests. Biochemical tests were done by biochemical test kit (Hi media, India) for characterization of both aerobic and anaerobic bacteria. Anaerobic isolates were characterized using Anaero Test kit (Neugen, India) as per the manufacturer's instructions. Carbohydrate utilization of aerobic termite gut bacteria was identified using Hi- Carbo test kit (Hi media, India).

Assesment of cellulolytic activity of the isolates: The cellulolytic activity of the isolated bacteria was tested by the Congo red assay described by Ramin *et al* (2008).

16S rRNA gene amplification and sequencing: Genomic DNA was isolated from all the nine bacterial isolates by CTAB method (Ramin *et al.,* 2009) and checked in 1% agarose gels and stored at -20°C. 16S rRNA gene was amplified by polymerase chain reaction using B8F [ATGGGCGCCTTCTACAGTCC] and B1500R [GGTCCTCACACGGTGCTGCA] primers and the genomic DNA isolated from the bacterial isolates as described earlier (Ramin *et al* 2009) using Red dye master mix 2X [Amplicon]. Purified PCR products were sequenced using Big-Dye terminator v 3.1 kit (Applied biosystems, USA) with both the forward and reverse primers. The gene sequences obtained were compared with other sequences available in the NCBI GenBank database using BLAST sequence- homology search to find matches with other known bacteria.

RESULTS AND DISCUSSION

Isolation and identification of termite gut microbes: Improving the utilization of low quality fiber is always a concern in ruminant nutrition and there has been a constant lookout for improving the digestion of cellulose and hemicelluloses. Termites are terrestrial eusocial insects which harbor a variety of microbes in their gut and provide carbon, nitrogen and energy source to the host. Hydrolysis of cellulose and hemicellulose is one of the important metabolic activity attributed to the gut microbes of termites.

Many bacterial genera have been identified in the termite, *O. formosanus* using classical microbiology and 16S rRNA sequencing (Leadbetter and Breznek 1996; Schafer *et al.* 1996; Ohkuma and Kudo 1996). In this study, termites collected from soil were identified as *Odontotermes formosanus* based on morphological characters. Nine bacterial species were isolated from the gut of *Odontotermes formosanus*. Out of the nine isolates, five were aerobic and four were facultative anaerobic bacteria. Identification of the isolated bacteria was done by morphology, Gram's staining and motility tests and the results are presented in Table 1 and 2. Three Gram positive rods isolated from the termite gut belonged to the genus *Bacillus* and identified as *B. cereus, B. thuringiensis, and B. pumilus.* All these three were aerobic and produced white colonies. Their biochemical characters were identical and utilized citrate, urease, and ornithine and reduced nitrate.

The Gram negative, facultative anaerobic rod isolated from the termite gut was identified as *Serratia marcescens*, which belonged to the family *Enterobacteracea.* Adams and Boobathy (2005) have also reported the presence of *Serratia marcescens* in termite gut.

Another Gram negative rod obtained in this study was *Pseudomonas aeruginosa.* Two more Gram positive isolates from termite were identified as *Citrobacter freundii [GenBank accession number: JN866823]* and *Salmonella enterica. Pseudomonas aeruginosa* and *Citrobacter freundii* are nitrogen fixing bacteria in the gut of termites (Brauman *et al.* 1992) and as in our study, nitrogen fixing bacteria have been isolated and identified as *Pseudomonas aeruginosa* and *Citrobacter freundii* from different types of termites (Potrikus *et al.* 1977). Among the two Gram positive cocci, the one found positive for MR test, citrate utilization, H_2S production and nitrate reduction was identified as *Enterococcus casseliflavus* and the other which utilized ornithine and positive for urease and VP test as *Staphylococcus gallinarum [GenBank accession number: JQ279067].*

Assessments of cellulolytic activity of the termite gut bacteria: All the bacterial isolates were subjected to congo red assay for checking their cellulose degrading ability. The results indicated that irrespective of the species, all the nine isolates recovered from the gut of the termite were found to have cellulose degrading ability with zone of clearance in the congo red assay (Plate 1). Carboxymethyl cellulase activity has been reported in *Bacillus* sp (Varma *et al.* 1994) and the three *Bacillus sp* isolated in this study were found to have cellulolytic activity as assessed by congo red assay. Similarly, termite gut bacteria belonging to the genus *Enterobacter* have also been reported to act on four different compounds namely, cellulose, hemicelluloses, aromatic compounds and lignin (Borji *et al.* 2003). Two of our isolates, *Serratia marcescens* and *Citrobacter freundii* belonging to the genus *Enterobacter* also had cellulolytic activity. Like *Salmonella typhimurium,* which possesses cellulase synthase genes (Yoo et al., 2004), the *Salmonella entrica* isolate obtained in this study was also found to have cellulolytic activity. Though there has been no report on the cellulolytic activity of the other isolates namely, *Pseudomonas aeruginosa, Staphylococccus gallinarum* and *Enterococcus casseliflavus*, these isolates too had cellulase activity which probably could be attributed to the symbiotic relationship existing between the

microbial population and the termite leading to the cellulolytic activity.

16S rRNA gene sequencing : The 16S rRNA gene was amplified using B8F and B1500R primers using the genomic DNA isolated from *Bacillus cereus, Bacillus thuringiensis, Bacillus pumilus, Serratia marcescens, Pseudomonas aeruginosa, Citrobacter freundii, Enterococcus casseliflavus, Staphylococccus gallinarum* and *Salmonella entrica* by PCR which resulted in the amplification of an expected amplicon of 1500 bp (Plate 2). Homology analysis of the sequences showed 90-100% homology with the available 16S rRNA sequences in the GenBank (Table 3).

Among the nine isolates, the 16S rRNA gene of *Bacillus thuringiensis* had 100% homology with an isolate reported from USA. *Bacillus cereus* and *Pseudomonas aeruginosa* had 98% homology with a Pakistan and Chinese isolates respectively, available in GenBank. The homology of *Citrobacter freundii* with a Chinese isolate was found to be 97%.

Studying the microbial diversity of termite gut by culture dependent and culture independent methods is essential to assess the variations existing in different ecological and geographical areas (Husseneder *et al*. 2003). This would not only give an insight about the native population but also those introduced from other ecological zones. In this context, the present study has led to the isolation and identification of nine different bacterial isolates with cellulolytic activity from the gut of *Odontotermes formosanus* under tropical Indian conditions. It is also evident from the study that many of the isolates recovered from the termite were found to be closely related to similar isolates from USA, China and Pakistan.

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Table 1: Characteristic features of aerobic bacteria isolated from termite gut

Isolates / Characteristic features	**1**	**2**	**3**	**4**	**5**
Gram's staining	+	+	+	-	-
Morphology	Bacilli	Bacilli	Bacilli	Rods	Rods
Motility	+	+	+	+	+
Pigment production	No	No	No	Blue-green	No
Indole	-	-	-	+	+
Methyl red	-	-	-	-	+
VP	-	-	-	+	-
Citrate	+	+	+	-	+
H_2S	-	-	-	-	-
Urease	+	+	+	+	-
ONPG	-	-	-	+	+
Nitrate	+	+	+	+	+
Ornithine	+	+	+	+	+
Sugar fermentation					
a).Lactose	-	-	+	-	+
b).Xylose	-	-	+	-	-
c).Raffinose	-	-	+	-	-
d).Cellobiose	-	+	+	+	+
e).Esculin	+	+	+	+	-
f).Fructose	+	+	+	+	+
Identified as	*Bacillus cereus*	*Bacillus thuringiensis*	*Bacillus pumilus*	*Pseudomonas aeruginosa*	*Citrobacter freundii*

Note: + = Positive, - = negative, VP= Voges Proskauer, ONPG= O-nitrophenyl-â-D-galacto pyranoside

Table 2: Colony characters and staining characters of facultative anaerobic bacteria isolated from termite gut.

Isolates / Characteristic features	**6**	**7**	**8**	**9**
Gram's staining	-	-	+	+
Morphology	Rod	Rod	Rod	Rod
Motility	+	+	+	-
Pigment production	Pink Colour	No	No	No
Indole	-	-	-	-
Methyl red	-	-	+	+
VP	-	-	-	+
Citrate	+	-	+	-
H_2S	-	+	+	-
Urease	-	-	-	+
ONPG	+	-	-	-
Nitrate	-	-	-	-
Ornithine	+	+	+	+
Sugar fermentation				
a).Lactose	-	-	+	-
b).Xylose	-	-	+	-
c).Raffinose	-	-	+	-
d).Cellobiose	-	+	+	+
e).Esculin	+	+	+	+
f).Fructose	+	+	+	+
Identified as	*Serratia Marcescens*	*Salmonella Entrica*	*Enterococcus casseliflavus*	*Staphylococcus gallinarum*

Note: + = Positive, - = negative, VP= Voges Proskauer, ONPG= O-nitrophenyl-β -D-galacto pyranoside

Table 3: GenBank Database homology of the bacterial isolates from *O. formosanus*

Isolate	Closest match in GenBank	Closest match in Gen Bank with accession number	Homology (%)	E-value
Bacillus cereus	*Bacillus cereus* isolate BRL02-43 16S rRNA Partial sequence (Pakistan)	DQ339674.1	98	0.0
Bacillus thuringiensis	*Bacillus thuringiensis* 16S rRNA Partial sequence (USA)	NZ-ACMY01000010.1	100	0.0
Bacillus pumilus	*Bacillus pumilus* 16S rRNA Partial sequence (China)	JF3431931.1	93	0.0
Pseudomonas aeruginosa	*Pseudomonas aeruginosa* LESB58 complete genome (China)	FM209186.1	98	0.0
Citrobacter freundii	*Citrobacter freundii* 4-7-47 CFAA Cont 1.6, Whole genome (China)	ADLG01000006.1	97	0.0
Serratia marcescens	*Serratia marcescens* N28b waa gene cluster (China)	U52844.3	90	0.0
Enterococcus casseliflavus	*Enterococcus casseliflavus* 16S rRNA Partial sequence (UK)	JF803544.1	94	0.0
Salmonella enterica	*Salmonella enteric* 16S rRNA Partial sequence (UK)	CP002614.1	90	0.0
Staphylococcus gallinarum	*Staphylococcus gallinarum* 16S rRNA Partial sequence (Japan)	JF803547.1	96	0.0

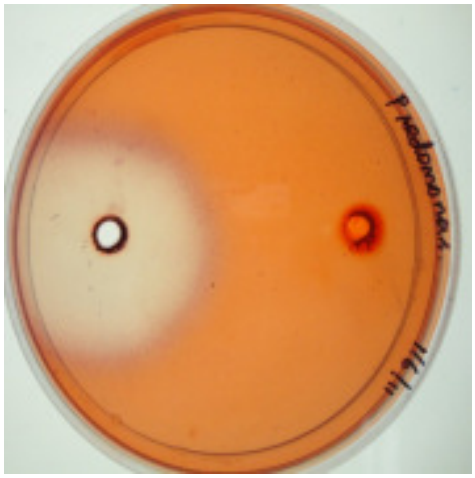

Fig 1. Congo red assay with the bacteria isolated from termite gut showing a zone of clearance indicating cellulolytic activity. T: Test sample, C: Control.

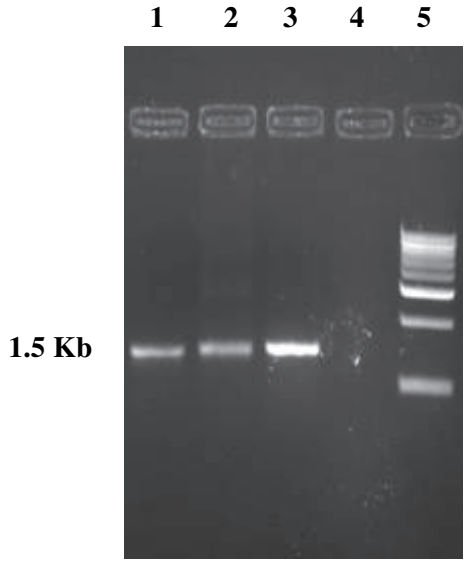

Plate 2. Agarose gel (0.8%) electrophoresis showing 1.5 Kb PCR products amplified using B8F and B1500R primers and genomic DNA isolated from termite gut microbes (Lanes 1-3) and 1kb ladder (Lane 5)

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