16S rDNA sequence and phylogenetic position of an uncultivated spirochete from the hindgut of the termite *Mastotermes darwiniensis* Froggatt

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Abstract: We have analyzed the 16S rDNA sequence and the phylogenetic position of an uncultivated spirochete from the hindgut contents of the Australian termite *Mastotermes darwiniensis* Froggatt. The 16S rRNA genes of bacteria from the hindgut contents of *Mastotermes darwiniensis* were amplified by polymerase chain reaction. The amplification products were cloned and sequenced. The sequences were compared to known homologous primary structures. Two of the clones (MDS1 and MDS3) had an insert of 1498 nucleotides showing typical signatures of spirochete 16S rRNA sequences. The sequences of the two clones were most similar to the 16S rRNA sequence of *Spirochaeta stenostrepta* (89.8%) and *Treponema* sp. strain H1 (90.7%). Phylogenetical analysis positioned the hindgut spirochete sequence with that of the free-living anaerobic *Spirochaeta stenostrepta* and *Treponema* sp. strain H1 as its nearest relatives within the cluster of the spirochetes. We conclude that the analyzed SSU rDNA sequences originate from a spirochete related to the genus *Treponema*. It is possibly one of the uncultivated unique spirochetes symbiotic in termite hindguts.

Key words: Termite; Spirochete; 16S rRNA sequence; Phylogeny

Introduction

Termites harbour a specific hindgut flora, which enables them to feed on wood [1,2]. The lower termite *Mastotermes darwiniensis* Froggatt possesses up to six symbiotic flagellates [3–5] and a lot of diverse prokaryotes. The morphologically most remarkable bacteria of the termite hindgut are spirochetes, which range from about 0.2 \( \mu \)m in diameter and 3 \( \mu \)m in length to 1 \( \mu \)m in diameter and 100 \( \mu \)m in length [6,7]. They can be found swimming in the hindgut fluid or being attached to the surface of flagellates such as *Mixotricha paradoxa* [8]. Light and electron microscopical investigations showed that the large spirochetes have complex structural features such as crenulated outer sheaths [7] or microtubule-like structures within the protoplasmic cylinder [9]. According to their morphology and ultrastructure the large termite spirochetes were assigned to the
genera Hollandina, Pillotina, Clevelandina and Diplocalyx [10]. Up to now it is impossible to cultivate these spirochetes outside the hindgut. As a consequence there is no reliable information about their physiological role in the hindgut and their phylogenetic position.

On the basis of small subunit (SSU) rRNA sequences it is possible to reconstruct phylogenetic trees [11] of pro-and eukaryotes. The phylogeny of spirochetes has been examined by Paster et al. [12] and by Rainey et al. [13]. The family Spirochaetaceae comprises the classically defined genera Spirochaeta, Borrelia, Cristispira, Serpulina and Treponema. Based on comparative 16S rRNA sequence analysis they can be divided into at least 4 phylogenetic groups. The first group contains the Serpulina species, the second encloses the borrelias, the third group consists of Spirochaeta species and a further group contains members of the genera Treponema and Spirochaeta. The phylogenetic position of the termite hindgut spirochetes, however, is unknown, because 16S rRNA sequences of these spirochetes are not yet available.

Here we present the 16S rDNA sequence and the phylogenetic position of an uncultivated spirochete from the hindgut contents of the Australian termite Mastotermes darwiniensis showing that the corresponding organism phylogenetically is a member of the Treponema / Spirochaeta group of spirochetes.

Materials and Methods

Preparation of nucleic acids

The termite Mastotermes darwiniensis Froggatt was obtained from the Bundesanstalt für Materialforschung und Materialprüfung (Berlin, FRG) and cultured in glass vessels at 28°C. For the isolation of hindgut contents, the termite was surface sterilized by dipping the decapitated animals in 70% ethanol for about 30 s and rinsing them with sterile water. All further steps were performed sterile. The hindgut was removed from the abdomen and transferred to 100 μl of PBS (130 mM NaCl, 10 mM Na-phosphate, pH 7.2). The paunch was dissected and the suspended contents were centrifuged for 1 min at 1000 rpm to separate large particles. The supernatant was centrifuged for 2 min at 14000 rpm to separate cells from dissolved DNA. The resulting pellet was suspended in 100 μl PBS containing 10 mg ml⁻¹ Poly A to desorb potentially contaminating DNA adsorbed to the surface of the bacteria. The cells were pelleted, washed and finally suspended in PBS. For isolation of DNA, 400 μl of InstaGene DNA purification matrix (Bio-Rad, Hercules, CA) was added and, after incubation at 56°C for 20 min and boiling for 8 min, the suspension was centrifuged and the DNA containing supernatant was used directly for PCR.

Amplification of the 16S rDNA

The bacterial 16S rDNA was amplified in vitro by PCR [14,15] using the primers Eubak3 (ATATGCGCCGAGAAGGAGGTGATCC) and Eubak5 (ATATGCGCCGAGAGTTTGAT(G/T)(A/C)TGCGTCAG), which contained a NotI restriction site to facilitate cloning. The PCR reaction mixture (100 μl) consisted of 20 μl of the DNA preparation, 0.5 μM of each primer, 0.2 mM of each dNTP, 1.5 mM MgCl₂ and 2.5 U of Taq polymerase in 1 x PCR-buffer (50 mM KCl, 10 mM Tris/HC1, pH 8.3). After a denaturation step at 94°C for 5 min, 30 cycles of (a) 1 min at 94°C, (b) 1 min at 52°C, and (c) 3 min at 72°C were performed followed by an extension step at 72°C for 10 min.

Cloning and sequencing of amplification products

The amplification products were purified from nucleotides, primers and Taq-polymerase using Magic PCR Preps (Promega, Madison, WI). The purified products were digested with the restriction enzyme NotI for 4 h at 37°C. The whole restriction mixture was separated on a gel of 1% low melting agarose and the digested PCR products were recovered from melted agarose by Magic PCR Preps. These purified DNA fragments were ligated into pBluescript SK(+) and cloned in Escherichia coli DH5α according to standard methods [16]. For minipreparation of plasmid DNA the alkaline lysis method was used in combination with Qiagen columns (Diagen, Hilden, FRG). The plasmids were digested using
Table 1
Overall 16S rRNA sequence similarities for MDS1 and reference organisms. Mean values are given for the Spirochaeta, Borrelia, Serpulina and Leptospira clusters (S., Spirochaeta, T., Treponema)

<table>
<thead>
<tr>
<th>Organism</th>
<th>16S rRNA similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borrelia</td>
<td>75.7</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>72.6 73.1</td>
</tr>
<tr>
<td>Leptospira</td>
<td>76.6 75.3 75.9</td>
</tr>
<tr>
<td>Spirochaeta</td>
<td>80.8 76.1 74.7 77.2</td>
</tr>
<tr>
<td>MDS1</td>
<td>80.8 77.0 73.7 75.7 81.9</td>
</tr>
<tr>
<td>T. sp. H1</td>
<td>81.2 76.6 73.6 77.0 82.8 90.7</td>
</tr>
<tr>
<td>S. stenostrepta</td>
<td>80.3 76.4 74.6 77.1 82.6 89.9 94.0</td>
</tr>
<tr>
<td>S. zuelzeri</td>
<td>79.8 77.0 74.8 75.7 82.1 88.2 88.3 88.3</td>
</tr>
<tr>
<td>T. bryantii</td>
<td>79.8 76.4 74.1 75.1 80.3 84.8 84.7 84.1 86.1</td>
</tr>
<tr>
<td>T. denticola</td>
<td>78.8 76.2 73.9 75.3 81.0 85.8 86.4 86.0 90.1 85.0</td>
</tr>
<tr>
<td>T. pallidum</td>
<td>79.6 76.0 74.8 76.1 82.4 85.0 86.2 86.4 87.9 83.2 88.3</td>
</tr>
<tr>
<td>T. phagedenis</td>
<td>78.8 76.8 75.7 75.3 81.7 86.3 86.0 87.2 90.7 84.9 92.1 90.0</td>
</tr>
<tr>
<td>T. succinifaciens</td>
<td>74.4 73.0 72.4 73.8 78.4 80.5 81.7 81.8 81.6 83.5 80.0 81.7 81.1</td>
</tr>
</tbody>
</table>

Sau3A and assigned to groups according to the resulting restriction pattern. Of each group at least one plasmid was sequenced using α-35S-dATP and the Sequenase kit (USB, Cleveland, OH).

Phylogenetic analysis

The novel 16S rRNA sequence was added to an alignment of about 1800 homologous primary structures from bacteria [17,18] by using the alignment tool of the ARB program package (Strunk et al., in prep.). Phylogenetic analyses were performed by applying distance matrix (ARB, PHYLIP [19]), maximum parsimony (ARB, PHYLIP) and maximum likelihood methods (fastDNAml [18]) on different data sets. The data sets varied with respect to the reference sequences as well as alignment positions included. Positional variabilities of the individual alignment positions were determined using the respective tool of the ARB package and used as criterion to remove successively highly variable positions.

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper are available at the EMBL nucleotide sequence data base under the accession number X79548.

Results and Discussion

The taxonomy of the spirochetes symbiotic in termite hindguts is based mainly on morphological and ultrastructural features [10], because none of these spirochetes can be cultivated and no SSU rRNA sequences have been published so far. Besides a variety of small spirochetes there are at least 4 different groups of large spirochetes in termites which were assigned to the genera...
Hollandina, Pillotina, Clevelandina and Diplecaryx [10]. From a phylogenetic point of view the question is, whether these complex spirochetes are members of separate phylogenetic groups or possibly just members of the established Spirochaeta/Treponema, Treponema, Borrelia, or Serpulina lineages [12].

In order to answer this question we amplified the 16S rRNA genes of bacteria from hindgut contents of the termite Mastotermes darwiniensis by PCR. The amplification resulted in products of about 1.5 kb in length as determined by agarose gel electrophoresis. After cloning of the amplification products the resulting clones were screened for 1.5 kb inserts by NotI restriction digests. Twenty clones with an appropriate insert were selected for further examination. The plasmids were digested with Sau3A resulting in 7 different restriction pattern groups. The 5' terminal sequence of one group consisting of the two clones MDS1 and MDS3 showed typical signatures of spirochetal 16S rRNA sequences [11] such as a T(U) at position 47 and 50, a A at position 52 and a G at position 53 (positions according to the Escherichia coli sequence [20]). Therefore, the sequences of these two clones were determined completely. The total length of both inserts without the amplification primers was 1498 bp covering the region homologous to bases 28–1528 of the Escherichia coli 16S rRNA sequence. The two sequences were checked for a chimeric origin using the Check chimera program of the Ribosomal Database Project (RDP) [18], which found no chimeric features. As the two sequences were nearly identical (99.8%), for further analysis and for submission to the EMBL data base we used only the sequence of clone MDS1.

The sequence was compared to 16S rRNA sequences available from public data bases [17,18,21] and showed 90.7% and 89.8% similarity to the SSU rDNA sequence of Treponema sp. strain H1 and Spirochaeta stenostrepta as its nearest relatives. After alignment of the MDS1 16S rRNA sequence with about 1800 homologous primary structures, phylogenetic trees were reconstructed applying different treeing methods. Maximum parsimony analyses were performed using the complete sequence data set. Distance matrix trees were reconstructed including all available 16S rRNA sequences from spirochetes as well as representatives of all major bacterial lines of descent as outgroup references. Maximum likelihood analyses were done upon a subset of spirochete sequences including the corresponding sequence from Escherichia coli as an outgroup reference. The tree in Fig. 1 is based on the results of a maximum likelihood analysis. Only alignment positions which share the same character in at least 50% of all available 16S rRNA sequences from the spirochete phylum were included for calculations. The triangles indicate the phylogenetic depths of major clusters of the spirochetes. The tree topology was stable in the majority of the analyses with respect to the relative branching order of the MDS1 sequence and its closest relatives of the treponeme cluster. The monophyletic structure of the spirochete cluster was not supported by part of the distance and parsimony analyses.

As can be seen in Fig. 1 the spirochete, from which our sequence is derived, is located in the treponeme cluster of the spirochetes and has a common ancestor with the free-living anaerobic Spirochaeta stenostrepta. Overall 16S rRNA sequence similarities of MDS1 and the other members of the treponeme cluster are in the range of 80.5% to 94.0% (Table 1). The corresponding values for MDS1 and its closest relatives Spirochaeta stenostrepta and Treponema sp. strain H1 are 89.8% and 90.7%, respectively.

We will develop DNA probes for fluorescence in situ hybridization in order to answer the question of whether the spirochete from which we obtained the SSU rRNA sequence is one of the small spirochetes occurring in the Mastotermes darwiniensis hindgut or whether it is identical to one of the large ones.

A further point of interest is how termite-specific the hindgut spirochetes are. Large spirochete species such as the Pillotina species can be found in Cryptocercus punctulatus as well as in Mastotermes darwiniensis and other termites and thus seem not to be restricted to single insect species. It is remarkable that, recently, the SSU rDNA sequence of a spirochete from the hindgut of Reticulitermes flavipes was analysed (J.A. Brez-
nak, personal communication) and placed nearly at the same position of the spirochetal phylogenetic tree as the spirochetal sequence analyzed in this work. This confirms that closely related spirochetes may occur in different termite species.

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References

5 Yamin, M.A. (1979) Flagellates of the orders Trichomonadida Kirby, Oxymonadida Grassé, and Hypermastigida Grassi & Foà reported from lower termites (Isoptera families Mastotermitidae, Kalotermitidae, Hodotermitidae, Termitopsidae, Rhinotermitidae, and Serritermitidae) and from the wood-feeding roach Cryptocercus (Dictyoptera: Cryptocercidae). Sociobiology 4, 5–119.