

THE FORMOSAN SUBTERRANEAN TERMITE AS A MODEL FOR AN EXOTIC ECOSYSTEM

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ABSTRACT

We have selected the Formosan subterranean termite as an astrobiological model, since it is a readily accessible source of exotic and novel microorganisms. Using both classical and molecular microbiological techniques we sought to identify bacteria with roles in the nitrogen and sulfur cycles within the termite's internal microbial ecosystem. Our investigation into the gut bacteria of this insect has yielded five species of bacteria capable of growth with very low levels of usable nitrogen. In addition we have determined the presence of sulfate-reducing bacteria in the hindgut of this insect. These microorganisms can aid astrobiologists in several key areas, including the growth of microorganisms under nutrient poor conditions, the ability of microorganisms to use anaerobic metabolic pathways, and the development of techniques to identify novel organisms.

INTRODUCTION

There has been growing interest in the nascent field of astrobiology, a discipline that includes the study of the possibility of life outside of Earth. A leading candidate for investigation is the Jovian satellite Europa. The principle reason for the attention that this moon has received is a growing body of evidence that liquid water may be present. Currently, there have been several terrestrial ecosystems that have been proposed as analogs to Europa, these include undersea rift vents and Lake Vostok in the Antarctic. However these ecosystems are expensive and difficult to access.

We used of the Formosan Subterranean termite (*Coptotermes formosanus* (Shiraki)) as an accessible model for an exotic exobiological ecosystem, with a focus on the nitrogen and sulfur cycles. *C. formosanus* is limited to a monotonous cellulose diet that is a very poor source of nutrients. Therefore survival of these insects is incumbent on the presence of symbiotic protists and bacteria that enable the termites to digest their food and provide nutrients. Researchers have discovered the presence of several types of bacteria that inhabit the gut of termites that are capable of fixing nitrogen (Ohkuma 1996). In addition, sulfate-reducing bacteria have also been isolated from several species of termites (Kuhnigk 1996). Because termites are a vast reservoir of novel microorganisms, the use of *C. formosanus* as a research animal could yield previously unknown microbes with roles in the nitrogen and sulfur cycles. Elucidation of novel communities of microbes in the sulfur and nitrogen cycles will allow researchers to expand their understanding of these critical environmental processes, In turn this will expand the range of diverse organisms available to astrobiologists who wish to model putative ecosystems outside of Earth.

Another space-based application of microbiology concerns the future colonization of the solar system. Long term human presence in space requires the creation of self contained and self

sustaining habitable structures. Ideally, such a habitat would be capable of recycling of nitrogen, carbon, sulfur, and oxygen. In terrestrial ecosystems, these cycles are largely mediated by microorganisms. A greater understanding of microbes responsible for these cycles would provide a "tool kit" for the future long-term habitation of space. It should be noted that the primary reason for the failure of the Biosphere project was due to a misunderstanding of microbial ecology.

Our investigation into the presence of nitrogen fixing bacteria was done using both classical and molecular techniques. Polymerase chain reaction (PCR) was used in an effort to detect the *nifH* gene, which is responsible for the fixation of nitrogen. Specialized media was used for the purposes of cultivating suspected nitrogen fixing and sulfate reducing bacteria. Characterization of isolated microbes was performed using both classical and molecular techniques. Molecular identification of isolates employed PCR to amplify 16SrDNA. Results from the PCR were compared to a computerized on-line database (i.e. BLAST) to establish the relationship of our isolates to known microorganisms. Classical tests used in characterization included gram staining, fermentation of twelve carbohydrate sources, assay for motility, the methyl red test, and nitrate reduction test.

MATERIALS AND METHODS

Termites:

All termites used in this study were Formosan subterranean termites (*C. formosanus*) collected from traps located on the UH-Manoa campus.

Microbiological media:

Nitrogen fixing media (NF) is a media prepared without an added nitrogen source, it was derived from Cote and Gherna (Cote and Gherna 1994) with the following modifications per liter of media: 1mL heme solution, 40 μ L vitamin K solution, with glucose, cellobiose, and acetate as carbon sources. Sulfate reducing media (SR) was derived from Cote and Gherna (Cote and Gherna 1994) with the following modifications per liter of media: 75 μ L RPMI 10X vitamin solution and titanium citrate at 1-2mM as an additional reducing agent.

Isolation of bacteria:

Termites were surfaced sterilized by application of 80% ethanol. Hindguts were removed with sterile forceps and homogenized in 1mL of sterile distilled H₂O. All termites were dissected within eight hours of collection. Suspected nitrogen-fixing bacteria were cultivated using serial dilutions onto NF solid media and incubating the plates at 30°C for 7 days in an anaerobe jar using CO₂/H₂ gas generator packets. Sulfate-reducing bacteria were cultivated by enrichment in SR media, using 100 μ L of inoculum from a termite hindgut homogenized in 1mL of sterile distilled H₂O. Bacteria grown in liquid SR media were incubated at 30°C for three to five days in an anaerobe jar using CO₂/H₂ gas generator. Bacteria grown in semi-solid media (i.e. 0.1% agar) were incubated at 30°C under atmospheric conditions, since the agar retarded the penetration of O₂ into the media.

Assay for the production of acetic acid:

formosanus using culture independent techniques (Shinzato 2001). The closest characterized relation is a 91% match to bacteria of the genus *Streptococcus*.

Isolate 2. Gram variable cocci, with lanceolate to coccobacillary cells typically found in pairs or alone. Non-motile. Anaerobe. Ferments a wide range of carbohydrates. Methyl red positive. Does not reduce nitrates. 16SrDNA identification indicates 99% relation to sequence from obtained from *C. formosanus* using culture independent techniques (Shinzato 2001). The closest characterized relation is a 92% match to bacteria of the genus *Enterococcus*.

Oval. Gram positive, cocci to short rod shaped bacteria. Cells typically occur in clusters but chains can be observed. Non-motile. Aerotolerant anaerobe. Catalase negative. Ferments a wide range of carbohydrates. Methyl red positive. Does not reduce nitrates. 16SrDNA identification indicates a 96% relation to bacteria of the genus *Lactococcus*.

Poly. Gram-positive regular rods, pleimorphic in older cultures. Non-motile. Facultative anaerobe. Ferments a wide range of carbohydrates. Catalase positive and oxidase positive. Methyl red negative. Reduces nitrates. 16SrDNA identification indicates a 99% relation to the bacteria *Cellulomonas hominis*.

Cocoid. Gram variable cocci, typically occur alone or in pairs. Non-motile. Anaerobe. Ferments a limited range of carbohydrates at a slow rate (i.e. seven days). Catalase negative. Methyl red negative. Affinity to other organisms is not known but cocoid is suspected to be a lactic acid bacteria (i.e. order Lactobacillales).

It should be noted that a less than 99% match in the 16SrDNA sequence could serve as cutoff point for the identity of an organism at the species level, while a match of less than 97% can serve as a cutoff at the genus level (Drancourt 2000). For instance, Isolate 1 with a 91% 16SrDNA sequence match with various *Streptococcus* species is in all likelihood a novel genus of bacteria.

Results of the PCR of the *nifH* gene:

In spite of thirty attempts, we were unable to successfully amplify the *nifH* gene. Amplified products from the positive control never attained the sizes reported in the literature. Several changes were made to the initial PCR conditions including increasing/decreasing $MgCl_2$ concentration, primer concentration, and changing the cycling temperatures.

Characterization of sulfate-reducing bacteria enriched from termite guts:

Bacteria were recovered that morphologically resembled bacteria of the genus *Desulfovibrio*. These bacteria were highly motile vibrios. When these bacteria formed chains, their "corkscrew" structures were suggestive of spirochetes. These organisms stained gram negative. Reduction of sulfate was detected through the use of the addition of an iron salt such as $FeCl$ and observing a black precipitate or through the use of an acidic cupric chloride solution. The characteristic "rotten egg" smell of hydrogen sulfide was also helpful in detecting the presence of sulfate-reducing bacteria. Sulfate-reducers were only found in media which was anoxic or contained anoxic regions. Isolated colonies could not be recovered from organisms present in the liquid and semi-solid media, because we were unable to create a solid media that would remain completely anoxic. Enzymatic assays revealed that lactic acid was oxidized to acetic acid. Other bacterial isolates also occurred along with sulfate-reducers in the SR media, these include gram-positive cocci and gram-negative rods.

Acetic acid production was assayed using enzymatic test kits (r-Biopharm). A 100 μ L sample was removed from SR media in which sulfate-reducing bacteria were detected and transferred into a liquid SR media. The SR media was incubated under anaerobic conditions for five days.

DNA extraction:

DNA from gram-negative bacteria was typically extracted by boiling a bacterial sample in sterile distilled H₂O for 15 minutes. In some cases (e.g. positive controls) a more pure DNA sample was desired, so Promega DNA extraction kits were used. In the case of gram-positive bacteria, the thick cell wall of these organisms often called for a more rigorous extraction technique to obtain a high yield of DNA (Ausebel 1994).

PCR methods:

Amplification of the *nifH* gene was attempted using the primers IGK, KAD, YAA, and GEM (Ohkuma 1996). Three PCR conditions used were as follows: #1) 30 cycles at 94°C for 30 sec, 48°C for 30sec, and 72°C for 2min (Ohkuma 1996). #2) 30 cycles at 94°C for 30 sec, 52°C for 30sec, and 72°C for 2min. #3) 30 cycles at 94°C for 30 sec, 58°C for 30sec, and 72°C for 2min. PCR condition #1 was obtained from the literature, while PCR condition #2 and #3 were experimental conditions derived from computer programs. Reaction mixtures were as follows. Milli-Q H₂O 27.2 μ L, 10X buffer 5 μ L, 25mM MgCl₂ 3 μ L, 2mM dNTPs 5 μ L, 10 μ M forward primers (IGK or YAA) 2.4 μ L, 10 μ M reverse primers (GEM or KAD) 2.4 μ L. Template DNA 5 μ L. *Taq* polymerase 0.2 μ L. Primer concentration was 10 μ M. *Klebsiella pneumonia* served as a positive control, while *Enterococcus faecalis* served as a negative control. Amplification of 16SrDNA gene was done using the primers 8f and 926r (Lane 1991). PCR conditions were as follows: initial denaturing at 94°C for 3 min followed by 30 cycles at 94°C for 30 sec, 55°C for 30sec, and 72°C for 2min. PCR cycling conditions were obtained from the literature (Hugenholtz 1998). Reaction mixture was as follows: Milli-Q H₂O 28.2 μ L, 10X buffer 5 μ L, 25mM MgCl₂ 3.2 μ L, 2mM dNTPs 5 μ L, 10mM forward primer 8f 1.5 μ L, 10mM reverse primers 926r 1.5 μ L. Template DNA 5 μ L.

PCR product sequencing:

Sequencing of PCR products was done by the BIOCORE facility at UH-Manoa using an Applied Biosystems sequencer.

Identification of bacteria using 16srDNA sequences:

Taxonomic identification of bacteria from 16srDNA sequences was obtained by using the online resource BLAST (Altschul 1997), maintained by the National Center for Biotechnology Information.

RESULTS

Characterization of bacterial isolates recovered from NF media:

Five bacterial types were isolated from NF media; we called these bacteria "isolate 1," "isolate 2," "oval," "poly," and "coccoid." Some important characteristics of these isolates are as follows:

Isolate 1. Gram-positive irregular rod, with cells that can be curved or tapered. Non-motile. Anaerobe. Catalase negative. Ferments a limit range of carbohydrates. Methyl red negative. Does not reduce nitrates. 16SrDNA identification indicates 99% relation to sequence obtained from *C.*

DISCUSSION

We were unable to demonstrate the isolation of nitrogen fixing bacteria from the gut of *formosanus* because of the inability to amplify the *nifH* gene. While five species of bacteria were recovered on NF media it must be emphasized that these organisms are not necessarily capable of nitrogen fixation. For trace amounts of usable nitrogen may be present in the agar and the milli-Q H₂O used to prepare the media. In spite of the difficulties encountered in the PCR, it can certainly be established that a number of bacteria found in the hindgut of *C. formosanus* are capable of survival at very low levels of usable nitrogen. Given the hostile environment associated with current candidates for astrobiological exploration (e.g. Mars) the ability to grow in nutrient poor environments will certainly be necessary for biological life should it be discovered elsewhere in the solar system.

An important role that sulfur-reducing bacteria can play in the internal ecology of *C. formosanus* is the cycling of carbon. We observed that in SR media inoculated with sulfate-reducing bacteria, lactic acid was being oxidized into acetic acid. Since *C. formosanus* is host to high levels of lactic acid bacteria (i.e. 10⁵ CFUs), sulfate-reducing bacteria play a useful role in converting one organism's "waste" into a carbon source that it can be assimilated by the termite and its internal symbionts. The utility of these intriguing microbes as astrobiological models lies in their sulfate-reducing metabolism. Since much of the oxygen present on Earth is derived from photosynthetic organisms, it appears doubtful that candidate astrobiological systems would have oxygen present at the levels necessary to support aerobic respiration. However, recent evidence from the Galileo space probe has demonstrated that sulfur compounds are present on Europa, raising the possibility of sulfur and sulfate driven ecosystems. It should be noted that sulfur and carbon cycles are critical to the health of terrestrial ecosystems, and a greater understanding of the microorganisms involved in these cycles would aid in the development of long term human habitation of space.

We have found that *C. formosanus* plays host to a diverse group of novel bacteria species and genera. The microbes we have worked with span a wide array of different metabolisms and phylogenies. Indeed, *C. formosanus* and other termites serve as a fertile "testing ground" for the implementation of molecular techniques to identify unknown microorganisms, including those that cannot be cultivated outside their host. The utility of molecular techniques to astrobiologists is that any microorganisms discovered outside of Earth will certainly be novel and probably will be difficult, if not impossible, to cultivate in the lab.

CONCLUSION

The Formosan subterranean termite harbors a diverse internal community of microorganisms, many of which are species and genera new to science. Included in this population are bacteria that are able to reduce sulfate, oxidize lactic acid to acetic acid, or grow on media with very low levels of usable nitrogen.

Further research goals could include the assay for nitrogen fixation by means other than PCR, such as using gas chromatography. Another area of interests for future research would include investigating the possibility of sulfur oxidizing bacteria in the gut of *C. formosanus*. Finally, a greater investment in equipment for isolating strict anaerobes, such as a nitrogen gas tank, would be useful for the isolation of the sulfate-reducing bacteria in pure culture.

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