An Attempt at Isolating and Characterizing Segmented Filamentous Bacteria from the Termite Gut

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Abstract

An attempt was made to identify and isolate segmented spore forming bacteria (SFB) from the gut of termites found locally in Woods Hole, MA. Ten individual termites were selected at random and their guts removed and homogenized in BSS buffer. Genomic DNA was the isolated with the MBIO DNA extraction kit. Several PCR (16S rDNA gene) reactions were then carried out which included a SFB specific reverse primer, general bacterial primers, primers for both DGGE (GC clamp) and t-RFLP (Fluorescent marker). Attempts were also made to culture spore forming bacteria by pasteurizing 10 termite guts (homogenized) to kill all non-spore forming bacteria and inoculating a sterile media, which was diluted to extinction. The SFB specific primer yielded no PCR product. All other PCR reactions appeared to produce suitable product for further analysis. Two DGGE experiments were carried out; both of which produced inconclusive results. The t-RFLP sent back from the lab was incomplete and thus no data was presentable. The PCR using general primers yielded enough product to clone using the TOPO cloning kit. Each clone represent four individual types of bacteria, three of which belonged to the ε proteo bacteria

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Segmented filamentous bacteria (SFB) are a gram positive endospore-forming, nonpathogenic species of bacteria commonly found attached to the intestinal walls of many animals (including humans) and insects via a holdfast. These bacteria have been isolated, but not cultivated from many animals including mice, rats, pigs and chickens. Attempts have already been made to characterize these bacteria from different sources on the basis of their 16S rDNA genes (Snel, J. 1995). Although there were differences between the SFBs, they were all phylogenetically closely related to the *Clostridium* genus. Snel and colleagues have since proposed a generic name for these bacteria, which is *Candidatus* Arthromitus.

These bacteria have not, as yet, been isolated from the gut of termites. The aim of this project was to use specific primers (Snel *et al*, 1995) to try and amplify 16S rDNA from the termite gut for subsequent genetic and molecular analysis. It was hoped that any PCR product could have cloned and phylogenetic analysis carried out in order to determine the relationship between SFBs from the termite gut and those previously isolated from other animals.

The microbial diversity of the termite gut is well known and a part of this project was to demonstrate this by using two relatively new molecular techniques denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (t-RFLP). These methods are principally a form of community fingerprinting and can provide valuable information on bacterial communities. Recently, t-RFLP has been

shown to be an automated and sensitive approach to the characterization of microbial communities by using capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection (Markus, M. *et al* 1999). This method uses the PCR reaction with one or two fluorescently labeled primers. The PCR product is then digested with one or two restriction enzymes such as Rsa1. Digesting with these enzymes generates a number of fragments, which vary in length depending on the DNA sequence of the bacteria being investigated and the restriction enzyme being used. The fluorescent end labeled fragments may now be viewed via capillary electrophoresis and laser-induced fluorescence. For mixed microbial communities, this has the advantage that each end-labeled fragment will be specific for each microbial species.

In contrast, whilst denaturing gradient gel electrophoresis (DGGE) is still dependent on the PCR reaction, it does not use labeled primers, but instead uses a primer with a GC rich clamp. PCR product is loaded into a vertical polyacrimide gel and run through an increasing gradient of urea and formamide (i.e. 30% - 70%) at 60°C for approximately 5 hours. As the double stranded DNA moves through the gel it is gradually denatured whilst being held together at one end via the strongly bonded GC clamp, preventing total denaturation. DNA that has the same or similar base composition will move through the gel at the same speed giving rise to the bands that are seen when the gel is dyed with a DNA stain (under UV lighting).

Both of the above methods, whilst being very informative about the microbial community being studied, are governed by the PCR reaction which is well known to introduce bias into the reaction. Reaction bias may be introduced due to the fact that certain microbes will be over shadowed by those bacteria present in higher numbers and as a result may never be seen in subsequent analysis. Certain bacterial species have high 16s rDNA gene copy numbers, such as the spore forming *Clostridia,* and as a result will be amplified in

preference to those bacteria will fewer copy numbers. This bias may be reduced if species specific primers are used, essentially narrowing down the amount of DNA amplification in the reaction. Providing this is taken into account, the above techniques are a useful tool in studying differences in bacterial communities.

Methods & Materials

DNA extraction from termite gut

Ten individual termites were selected at random for DNA extraction. These were then placed on ice, in order to anesthetize them, prior to extracting individual stomachs. With the aid of a dissecting microscope individual termites were placed on para-film and held with fine tipped forceps by their head. It was then possible to remove the stomach by gripping the tip of the hindgut and gently pulling away from the head. The gut tissue was then pulled out onto the parafilm where the paunch was isolated. The paunch was then cut open with a sterile razor blade and washed with BSS buffer consisting 1.8mMk2HPO4, 6.9mMKH2PO4, 21.5mMKCL, 24.5mMNaCl (autoclaved) whilst cooling 1.5gms/500ml of methyl cellulose plus DTT was added and kept under Nitrogen flux to maintain anoxic conditions.

Genomic DNA isolation & PCR amplification

DNA was extracted from ten termite guts by using the UltraClean Soil DNA Kit, Mo Bio Laboratories, Inc., P.O. Box 606 Solana Beach CA., U.S.A. The genomic DNA extracted was run on a 1% agarose gel to determine whether the extraction had indeed worked.

Four sets of PCR reactions were run using different primers specific for the 16s RNA subunit. For the first reaction a spore forming filamentous (SFB) reverse primer SFB1008 5'-GCGAGCTTCCCTCATTACAAGG- 3' (Snel, J. *et al* 1995) and general forward primer 8F were used. For the second reaction general bacterial primers 8F and 1391R were used. The third set of primers consisted of a fluorescently labeled forward primer and the general bacterial primer1391R for subsequent use in t-RFLP analysis. The final PCR reaction consisted of the DGGE primers with a GC clamp attachment. All of the PCR reactions with the exception of the DGGE PCR were carried out with a thermal program,

which comprised 30 cycles with a initial melting temperature of 94°C for 5 minutes, 94°C for 30sec, 58°C for 30sec 72°C for 1 min and a final extension temp at 72°C for 7 min. The DGGE PCR consisted of an initial melting temperature of 94°C for 5 min and ten cycles of 95°C for 1 min, 66°C for 1 min, 72°C for 3 min and a final 15 cycles at 95°C for 1 min, 56°C for 1 min, 72°C for 3 min with a final extension temperature of 72°C for 5 minutes.

Cloning

Cloning was carried out on the PCR product produced with the general bacterial primers using the TOPO cloning kit. The PCR product was first ligated by adding 2ul of fresh PCR product, 2 ul of sterile water and 1ul PCR2.1-TOPO vector which was then incubated at room temperature for 5 minutes. A negative control was carried by adding 4ul of sterile water to 1ul of PCR2.1-TOPO vector. After the incubation period it was necessary to transform the vector into E *coli* cells supplied with the kit. This was carried out by adding 2ul of the TOPO cloning reaction into a vial of one slot competent cells and mixed. This was incubated on ice for approximately thirty minutes. The cells were then heat shocked in a water bath heated to 42°C for 30 seconds. This was then transferred to ice once more and 250ul of SOC solution was added. The tubes were then placed into 250ml Erlenmyer flask and horizontally shook for 30 minutes at 37°C. The reaction product was then plated out on LB media which had been overlaid with 20ul of X-Gal and incubated at 37°C over night.

Denaturing Gradient Gel Electrophoresis (DGGE)

A gradient ranging from 30% to 70% denaturant was made up to a final volume of 30ml (one gel).

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Table 1 Ingredients for the 30% to 70% denaturing gradient gel for DGGE

UREA	2.14g (30%)	5g (70%)
FORMAMIDE	2.04ml	4.76ml
TAE (X50)	0.34ml	0.34ml
ACRYLAMIDE	2.77ml	2.77ml
ddH$_2$O	10.15	5.17

Ten milliliters of the two solutions were then added to their respective wells with the addition of 100ml of 10% bromophenol blue to the 70% denaturant solution. This was to provide a visual indictor to the degree of gradient reached in the gel. Prior to pouring the gel 100ul of 1% APS and 4ul of TEMED was added to provide effective polymerization. The gel was allowed to polymerize for approximately 2 hours prior to loading. The DGGE water bath contained 7 litters of dH$_2$O and 140ml of TAE buffer, which was heated to 60°C prior to loading of the gel. 50ul of PCR product and 5ul of loading buffer was injected into each well, which had all been previously flushed with buffer from the water bath to remove much of the urea which would have leaked into the wells. The DGGE was the run at 200 volts for approximately 5 hours before being removed for staining and subsequent viewing under UV light.

t-RFLP

The PCR product from this reaction was then digested using the enzyme RsaI prior to being sent for analysis. 5ul of PCR product, 1ul RsaI enzyme, 1ul Buffer, 1ul BSA (1ug/ul) and 2.5ul of sterile dH$_2$O. This was placed into a water bath @ 37°C for 3 hours before being sent for analysis.

Culture Media

The media used to try and isolate the SFBs was a broad-spectrum media designed to grow any spore forming bacteria that survived pasteurization. The ingredients were 0.1g/l cellulose, 50mM (final) Sodium Acetate, 1 liter H_2O, 10ml FW (100X), 10ml NH_4CL (100X), 1ml Sulfate (1M), 1gm Yeast cells, 10ml rumen fluid, 1ml EDTA trace elements, 10ml MOPS (1M) pH 7.2, 0.1gm Chitin. This was then autoclaved prior to adding 0.5ml Vitamins and 2ml bicarb. The media was aseptically aliquotted in 9ml volumes to ten capped test tubes. 1ml of 10 homogenized termite guts were then diluted to extinction into the test tubes and left to grow aerobically at room temperature.

Epifluorescent microscopy

This particular microscope was supplied by the Ziess Gruppe, Unternehmensbereich mikroskopie D-07740 Jena (model Axioplan 2). This method of microscopy was used in order to provide information on the different bacterial species with respect their ability to auto-fluoresce (F420 for methanogens) as well as using specific fluorescent dyes such as Acridine orange to give total cell counts, due to its ability to stain DNA.
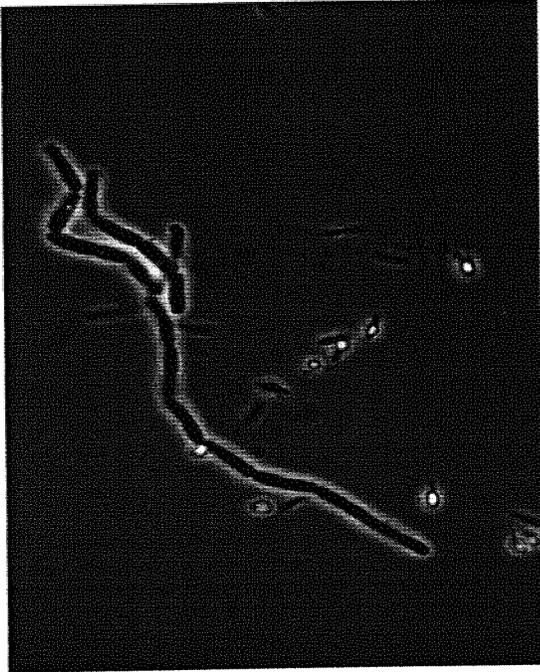
Results

Microscopy

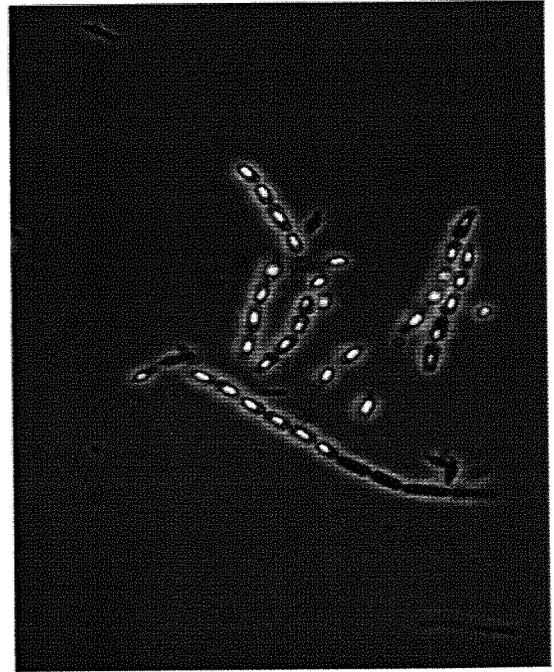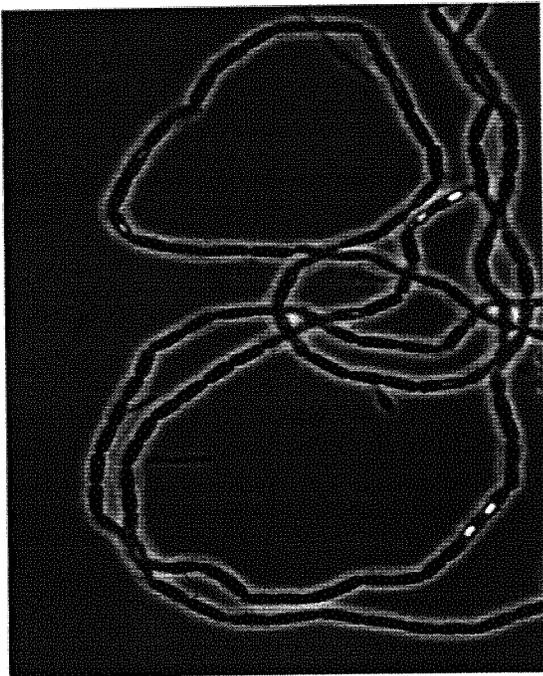Figure 1 Isolated bacteria from culture media

Figure 2 Isolated bacteria from culture media

Figure 3 Isolated bacteria from culture media
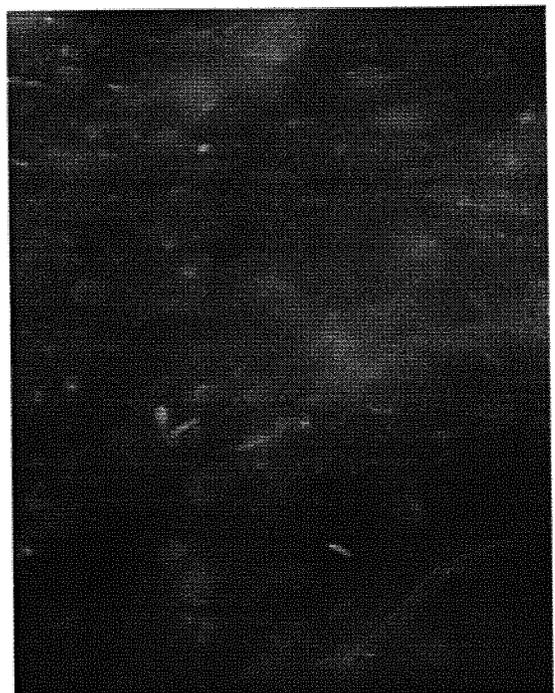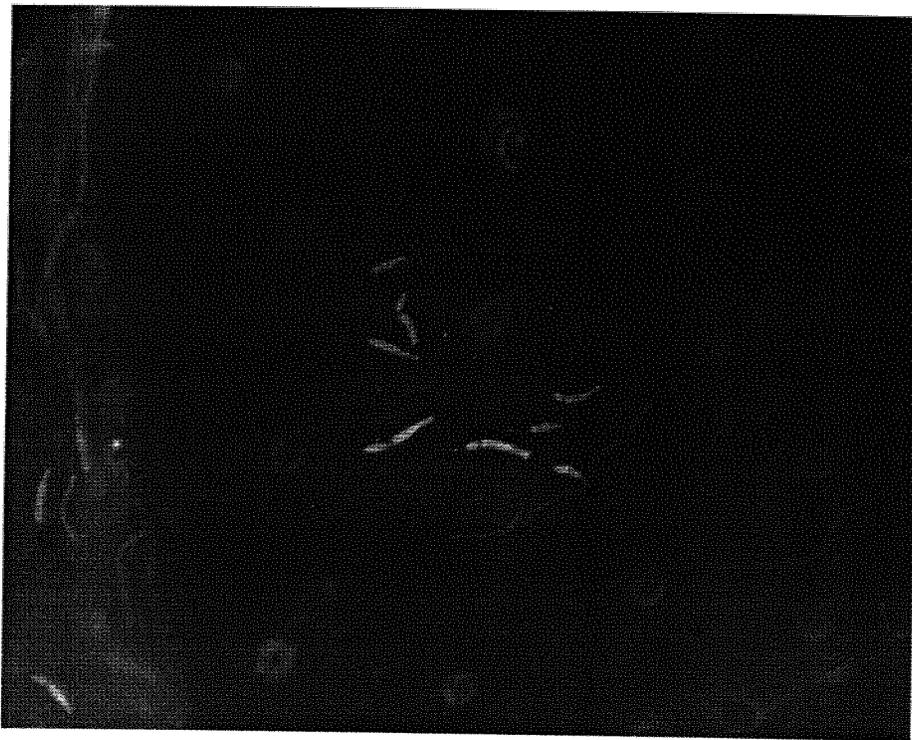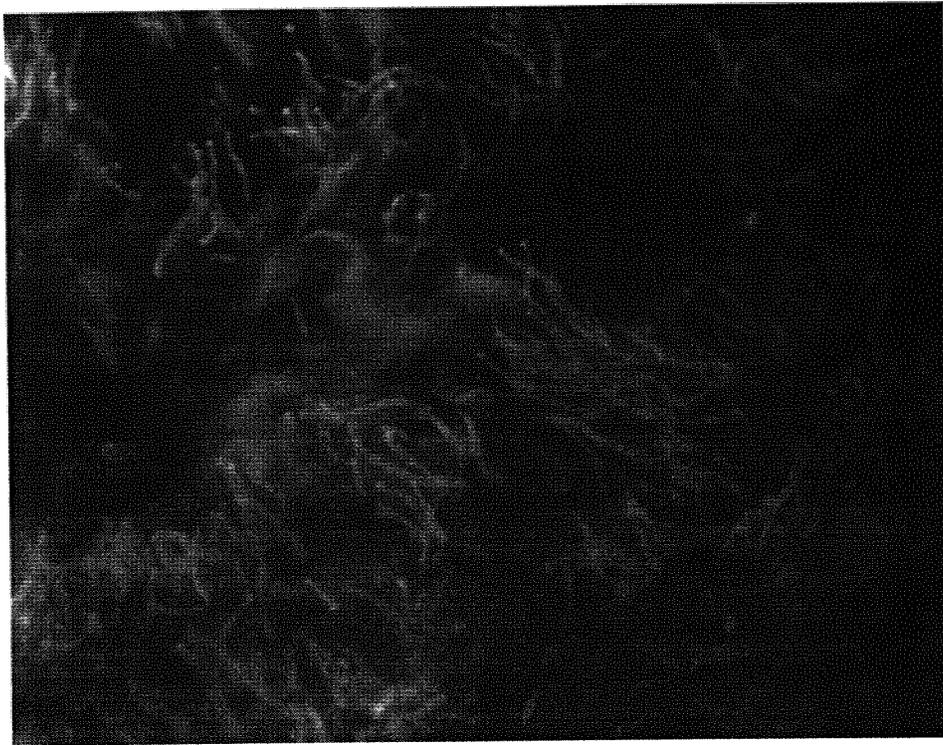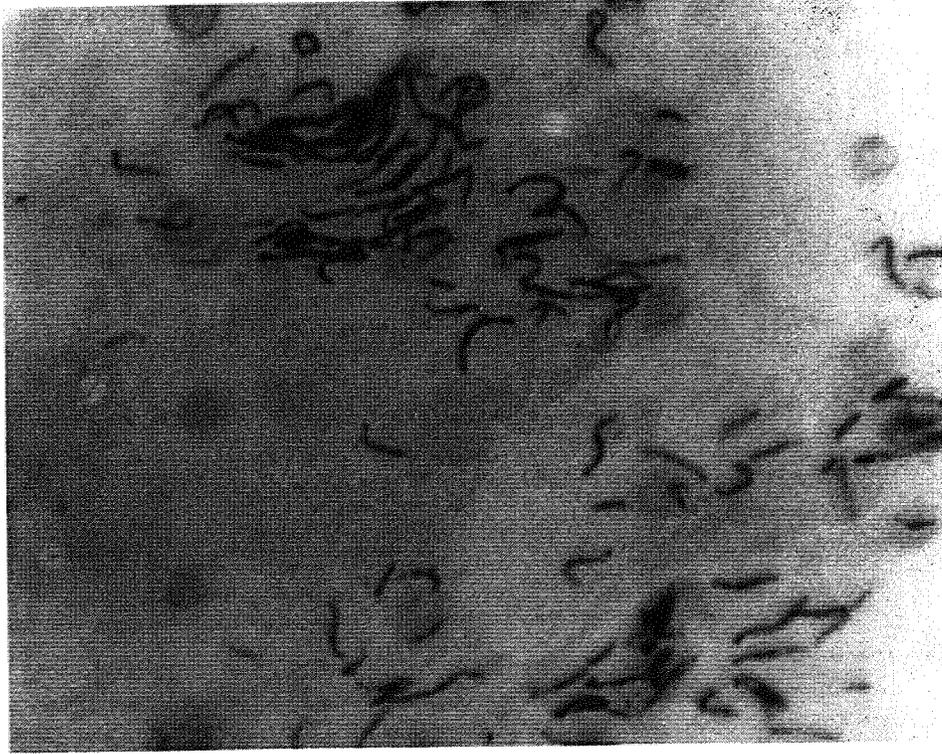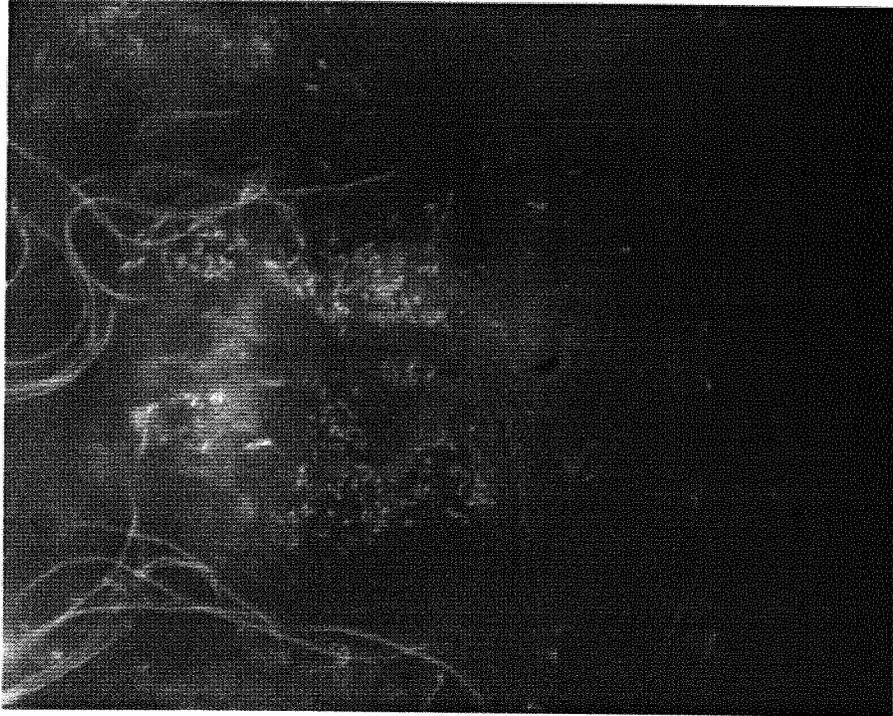

Figure 4 Auto fluorescence of unknown
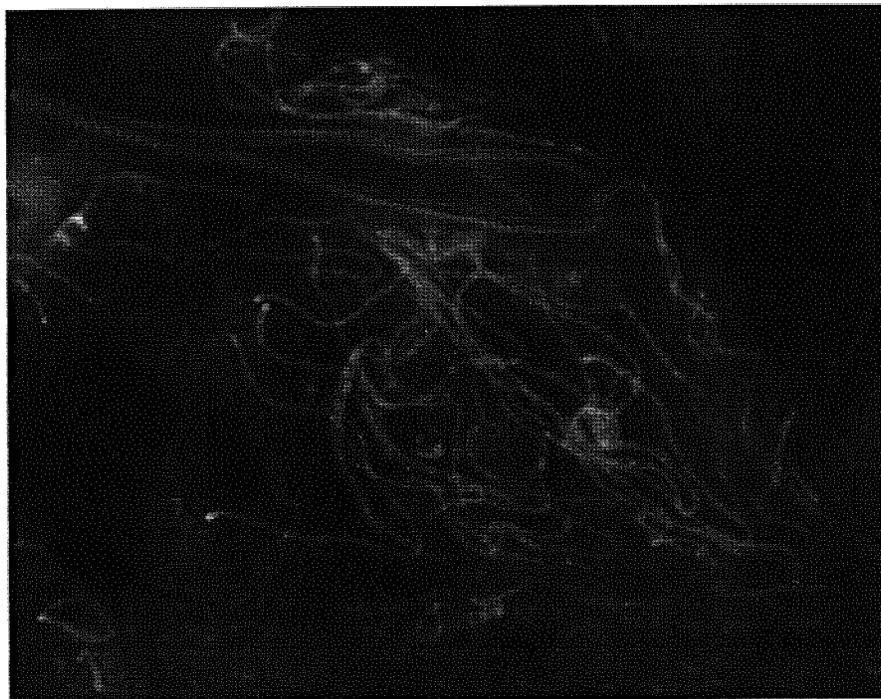bacteria viewed with the DAPI filter at x100

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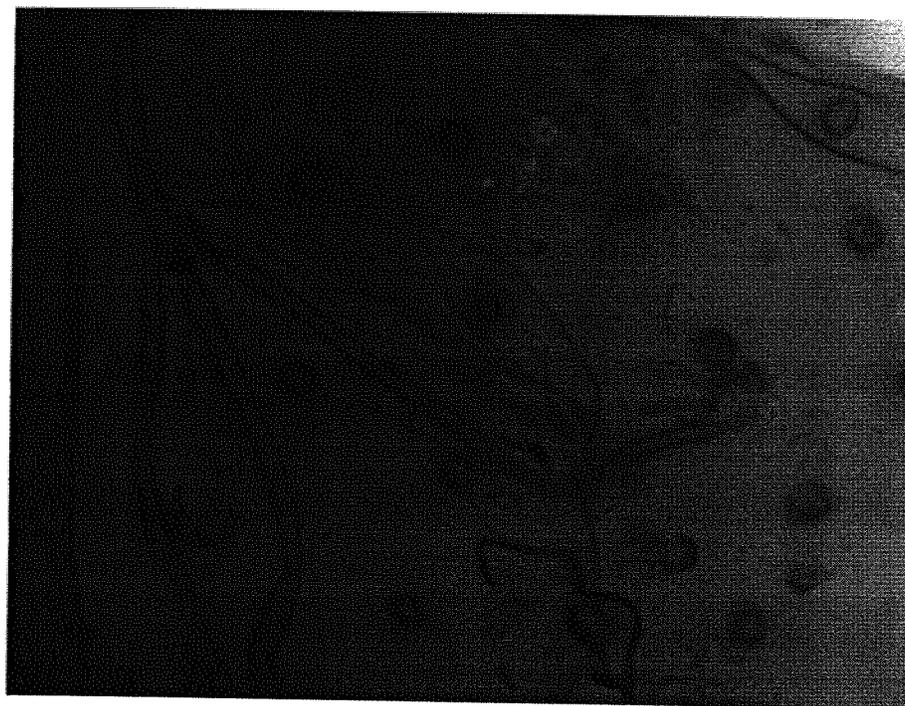
Figure 5 Digital image of auto fluorescing bacteria using DAPI filter

Figure 6 Digital images of unknown bacteria, which appear to be attached to the gut wall via a holdfasrt

Figure 7 Auto-fluorescence of methanogens using Lucifer yellow filter

Figure 1 Auto-fluorescence of methanogens using Lucifer yellow filter

Isolation of SFBs

Spore forming bacteria were isolated from the gut of the termite (figure 1-3, page 4), although they do not appear to be the segmented filamentous type. It is not apparent at this stage if there was more than one type of spore forming bacteria present or whether the different morphologies seen were different stages in the life cycle of one idependent species. There was insufficient time to extract DNA from these isolates for phylogenetic or ARDRA analysis.

PCR

There was no observed PCR product for the SFB specific primer set in any of the reactions carried out. However, there was product produced with general bacterial primers and also those for t-RFLP and DGGE.

DGGE

The two DGGE gels that were run (30% to 70% denaturant) produced very dubious results. Although several bands could be viewed there was a great deal of smearing in each of the lanes. The three dilutions of DNA sample used showed very different results indicating the possible bias produced during the PCR amplification reaction. Therefore niether of the two DGGE gels have been used to show bacterial population variability.

Phylogenetic classification using 16S rRNA gene

Digests of the four clones selected were sent for sequencing to Michigan State university, Ohio, USA. The returned sequences were assessed with the software programme 'ARB' (technical University, munich, Germany). The phylogenetic analysis of this gene suggests that three of the bacteria (MD11, MD13 & MD14) are ε proteobacteria, and are closely related to similar organisms found as symbionts in tubeworms. The fourth bacteria

(MD12) is believed to be a member of the Cytophagales species, and is closely related to bacteria isolated from antartic ice cores.

t-RFLP

The digested PCR product was sent to XXXXXX for analysis. The following results were sent and are presented below.

Disscussion

The purpose of flushing the termite gut with BSS solution was to remove any non-adhering bacteria, which would therefore make the SFBs and other bacteria attached to the gut wall the more dominant species. This was necessary to help reduce the amount of bias that may be encountered with PCR reactions. Bacteria that represent a large percentage of the total population or those with high 16S rDNA gene copy numbers will be amplified to a greater extent during PCR reactions in preference to those present in lower numbers or with low 16S gene copy numbers. Unfortunately, this did not help in the amplification of SFB DNA. Whether there were any of this species present at all or the fact that they were present but were not compatible with the primers used (Snel J., *et al* 1995).

Instead of continuing with the SFB specific primers, general bacterial primers were used so that enough suitable PCR product could be used for cloning purposes. Of the three dilutions of DNA used (10^{-1}, 10^{-2} & 10^{-3}) all produced a PCR product viewed on a 1% agarose gel stained with ethidium bromide. It was only possible to isolate four clones for phylogenetic analysis due to time constraints, although it would have been possible to have used a lot more. It was for the same reasons that ARDRA was not used on the colonies selected prior to sequencing.

It is hard to speculate on why the DGGE did not work too well or whether it was the PCR that was the problem. It would usually take many trials of both the PCR and DGGE before optimization would have been achieved. Therefore, it is fair to assume that with time scale of this project such optimization could never have been achieved. However, the main purpose with using DGGE was to gain working knowledge of the system, which was achieved. It would have been interesting to have compared the DGGE results with those achieved with t-RFLP and to see the differences in sensitivity between

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the two. It has been mentioned in previous papers that t-RFLP is more sensitive than DGGE. It is thought that DGGE will only produce bands for those bacterial populations representing 10% and more of the total bacterial population, probably due to the biases observed during amplification. Muyzer and colleagues showed that t-RFLP had a slightly higher resolution that DGGE and was therefore useful as a rapid and sensitive analytical tool.

The Epi-fluorescent microscope is a very useful tool for viewing bacteria. Without any staining methanogens were easily viewed with the Lucifer yellow filter proving that this is a very useful and rapid detection instrument. By using dyes such as DAPI and Acridine orange total cell counts can be made of samples where cultivation based quantitative measurements are not practical.

Future work may consist of using anaerobic media or O_2 gradient tubes, as it is hypothesized that these bacteria may be micro-aerophiles and may exist in very low O_2 conditions in the termite gut. Total DNA sequencing of the clones is hoped to be carried out by Jarred Leadbetter, Caltech, USA.

Acknowledgements

I would like thank all the staff here at the microbial diversity for a truly wonderful time. I feel honored to have been included as a student on this course in view of the experience and knowledge of the staff and of my piers. Everyone here made this experience what it was....... fantastic! I hope we will all meet again in the not too distant future, THANKYOU ALL SO MUCH.