Abstract:

This project sought to investigate the spatial organization of a granule forming microbial community in the fermented milk product, Kefir. A 16s gene library was constructed from whole-community DNA extractions, and indicated that a Lactobacillus species previously identified as a member of the Kefir community was likely a major player in the community structure. Kefir grains were thin-sectioned by a cryosectioning method in to allow direct microscopic examination of the wall structure. Fluorescent In Situ Hybridization with a general bacterial probe was utilized with epi-fluorescent and confocal microscopy to demonstrate that the kefir grains are hollow, with a wall composed primarily of bacterial cells, with yeast embedded in the wall surrounding an interior lumen.

Introduction:

Kefir is a food product produced by the fermentation of milk by both lactic acid bacteria and yeast (4). While the nutritional benefits of kefir are perhaps most responsible for its prominence as a probiotic, the fact that it develops granules over the course of the fermentation raises considerable interest from a microbiological standpoint. Current literature suggests that the granules are composed of an exopolysaccharide, Kefiran, produced by a Lactobacillus species (9). The production of this polysaccharide creates the potential for spatial organization within the kefir grain, and it can be hypothesized that the various organisms responsible for the production of the final kefir product exist in specialized niches within this matrix. While various studies have examined the gross community structure of kefir, the spatial organization of this community has not, to the best of my knowledge, been examined.

The overall goal of this study was to examine the physical structure of the kefir grain, and determine both the identity and the spatial organization of the microbes which composse the grain. As gross morphological differences can be observed between fresh and aged grains, new and old grains were examined for both phylogenetic and structural differences which might suggest the presence of a successional community within the kefir grains.
Materials and Methods:

Kefir Cultures:

Kefir cultures were graciously maintained and provided by Kritee, who also provided significant guidance in the determination and selection of aged and fresh kefir granules for investigation.

DNA Extraction:

As the kefir grains are composed of a dense mixture of EPS and living cells, nucleic acid extraction was expected to be challenging. An initial DNA extraction was performed in duplicate on approximately 500mg of aged kefir grains utilizing the Mo-Bio Ultraclean Soil DNA extraction kit according to the manufacturers directions, with several deviations. The bead-beating step of the kit was extended to three minutes in an effort to completely macerate the EPS matrix, and maximize the cell lysis. Following lysis, the sample was spun for an additional minute, for a total of two minutes, to maximize the removal of EPS and protein from the supernatant. The DNA produced by this method was utilized to construct the initial clonal library of the aged kefir grains.

Considering the reported diversity of the kefir grains and the high amount of initial biomass extracted, the total DNA yield of this extraction protocol seemed poor (See Results). As an attempt to mitigate this, a modified phenol-chloroform extraction protocol was performed (ref cold spring harbor). The solutions used are detailed in Appendix 2. In this protocol, duplicate 500mg samples were added to a Mo-Bio dry bead-beating tube, with 250uL Minimal Salt Solution and 500uL Lysis Buffer. This solution was then briefly vortexed, and bead beat for two minutes. Following bead beating, the solids were pelleted from solution by centrifugation for five minutes at maximum speed. The lysate was then transferred to 750uL of Phenol:Chloroform:Isoamyl Alcohol and mixed by inverting several times. The aqueous and organic phases were separated by centrifugation for three minutes at maximum speed, at which point the aqueous phase was transferred to a fresh 750uL of Phenol:Chloroform:Isoamyl Alcohol. The mixing and separation steps were repeated. Following centrifugation, the aqueous phase was transferred to a fresh microcentrifuge tube, and the DNA was precipitated by the addition of 150uL of sodium acetate and 1.5mL of absolute ethanol, followed by centrifugation for 10 minutes at max speed. The supernatant was decanted, and the remaining pellet resuspended in 100uL Tris buffer at pH 8.0.

Despite the apparent high yield of DNA from the phenol-chloroform extraction protocol, concerns about phenol contamination precluded its further usage.

Due to continued concern about DNA yield from the extraction protocols, a balance was struck using the lysis buffer from the phenol-chloroform protocol with the Mo-Bio extraction kit according to the manufacturers directions. This protocol was utilized to extract a second aged kefir community, as well as a fresh kefir community. In this case, approximately 150mg of kefir material was extracted for both the fresh and aged communities.

Small Subunit RNA Gene Amplification:

The Polymerase Chain Reaction (PCR) was utilized to amplify the small subunit ribosomal RNA gene from the community DNA extractions. The Promega Master Mix Kit was utilized according to the manufacturers directions for all reactions. For the amplification of the DNA extracted from the initial aged kefir grains, reactions were set up using 2uL of undiluted template DNA, as well as 2uL of template DNA which had been
diluted 1:10 in nuclease free water. For the amplification of DNA from the second second set of aged grains, as well as the initial set of new grains, 2uL of undiluted template DNA was used, as well as 2uL of template DNA diluted 1:100 in nuclease free water. In addition, a 'poison control' reaction was set up, in which 1uL of undiluted template DNA was mixed with *E. coli* genomic DNA in order to ensure that PCR inhibitors were not present.

**Construction of Clonal Libraries:**

A clonal library was constructed of the initial aged kefir grains was constructed utilizing the Invitrogen TOPO TA kit and pCR-4-TOPO vector according to the manufacturers instructions. 2uL of the PCR product generated by the 1:10 template dilution was utilized. 40uL and 80uL of transformed cells were spread-plated on LB-Kanamycin plates as suggested by the manufacturer. 48 clones were selected, and sequenced by the Bay Paul Center.

A further two libraries were constructed from the PCR product generated from the undiluted template from the fresh and aged kefir grains. The libraries were prepared as previously described, however, the pCR-2.1-TOPO vector was utilized instead of the pCR-4-TOPO vector. Successfully transformed cells were identified by white colonies on LB-Kanamycin plates spread with IPTG and X-gal. A total of 29 colonies from the aged kefir were picked for sequencing, and 31 colonies from the fresh kefir were picked for sequencing.

**Thin Sectioning of Kefir Grains:**

Clusters of kefir grains (see fig 1) were selected from the culture and rinsed repeatedly in PBS pH 8.0 until the majority of loose material was removed. Following rinsing, the grains were fixed for four hours in 4% formaldehyde buffered in PBS as suggested by Amann et al. (1). Following fixation, the grains were rinsed three times in PBS pH 8.0 to remove residual formaldehyde, then transferred to OCT media in preparation for cryosectioning as suggested by (11). Complete infiltration of the grain by the OCT media was determined by the sinking of the grain in the media, and generally took 4-6 hours. Following infiltration, the sample was mounted on a base of OCT medium at -25°C in the cryostat at the MBL Central Microscopy Facility. Complete freezing of the samples took 4-6 hours, after which sections could be cut. Sections of 5uM and 20uM thickness were cut on the CMF cryostat, and mounted on gelatin coated slides. Sections were dehydrated in a 5-100% ethanol series as suggested by (11).

**Fluorescent In Situ Hybridization:**

FISH was carried out directly as suggested in the MBL Microbial Diversity Laboratory Manual. Formamide concentrations used were in accordance with suggestions in their original publications. The probes EUB338 (1), GAM42a(10), and EBAC1790 (2) were used. The use of LGC0355 (6) was planned, but did not arrive in time to be utilized. All probes were 5' labeled with Cy3. All sections were counterstained with DAPI at 10ug/uL.

**Imaging:**

Samples were imaged by epi-fluorescence on a Zeiss Axio-Imager Z1 upright microscope equipped with appropriate filter cubes and a Zeiss Axiocam mRm camera. Epi-fluorescence images were acquired and processed with Zeiss Axiovision software. Confocal microscopy was performed with an Olympus FluoView 300 laser scanning microscope system.
equipped with Argon Ion and Helium-Neon lasers. Confocal images were processed with Olympus FluoView software and DAIME (3).

Results and Discussion:

DNA Extraction and Clone Libraries:

The initial DNA extraction from the aged kefir grains gave results that can at best be considered questionable. While microscopy indicated that the cell density of the kefir grains was extremely high, the extraction yielded a rather meager 21.9ng/uL for one sample, and 9.1ng/uL for the other. While this DNA was successfully amplified and cloned, it is not certain if the extracted DNA is representative of the total community DNA present. The phenol:chloroform extraction appeared to give a much higher total DNA yield, with the fresh grains yielding 118.7ng/uL, and the aged grains giving 154.4ng/L. However, the potential for phenol contamination of the extracted DNA resulted in this product not being used. The usage of an additional lysis buffer resulted a somewhat better yield than was initially achieved, with 42.4ng/L obtained from the fresh grains, and 32.5ng/uL obtained from the aged grains. This DNA was successfully amplified (data not shown) and cloned.

The sequences returned from the clone library did not indicate the diversity suggested by the available literature. The ARB software package was used to align the 16s sequences against the SILVA 16s database. The aligned sequences identified by addition to the SILVA guide tree by the ARB maximum parsimony tool. Nearest neighbors were selected, and a tree was generated by the FastDNA ML maximum likelihood method contained in the ARB software. The tree can be found in Appendix 1. Analysis of the libraries demonstrated a surprising lack of diversity, with all of the clones from both the first and second libraries generated from the aged kefir grains grouping extremely close to each other, and with very high identity to *Lactobacillus kefiranofaciens*. While the presence of this organism is not in and of itself a surprise, the complete lack of diversity is troubling, and suggests that the DNA extraction may not have been representative of the community. Alternately, the *L. kefiranofaciens* species may have been in the vast majority, and too few clones were screened to detect any other species.

Perhaps more troubling, however, was the fact that all of the clones generated from the fresh kefir grains showed high identity with *E. coli*. Unfortunately, there was not sufficient time to properly explore this, or to pick a further set of colonies. As such, the observed results can best be explained as cross-contamination of the PCR reaction with the *E. coli* genomic DNA used in the poison control.
Thin Sectioning and Fluorescent *In Situ* Hybridization

Prior to the usage of FISH on the kefir thin sections, the sectioning process was optimized for epifluorescent microscopy by the use of DAPI as a general stain. This allowed for a number of revelations about the structure of the kefir grains, most notably that the grains were found to be hollow, with the perimeter of the grain being a solid mass of bacterial cells. Interestingly, it appeared that the interior wall of the grain was embedded with yeast cells, as shown in figure 2. This work also provided an opportunity to optimize the thickness of the sections. Due to the shallow depth of field inherent to an upright microscope at higher magnifications, it was found that sections thicker than 5 microns could not be reliably imaged, as only a limited portion of any section would be in focus at any given time, as demonstrated by figure 3. However, achieving 5 micron thick sections with the cryosectioning procedure chosen for this project was unreliable at best, with very few cut sections actually being suitable for analysis. However, FISH utilizing a Cy3 labeled EUB338 probe together with DAPI as a general was quite successful, and clearly discriminated between the yeast and bacterial cells (figure 4).

While the combination of 5uM sections and conventional epifluorescent microscopy gave considerable enlightenment as to the gross structure of the kefir grain, little was indicated about the overall three-dimensional structure. While FISH with the available probes could not be compared to a general DAPI stain due to the lack

![Figure 2: DAPI Stained Kefir Grain, with bacterial and yeast cells. 5uM Section, 400x magnification.](image)

![Figure 3: 20uM Kefir grain section demonstrating limited depth of field at 400x](image)

![Figure 4: 5uM Kefir grain section labeled with EUB338-Cy3 and DAPI Stained. False-Colored 400x](image)
of appropriate optical filters on the confocal microscope available, this limitation was balanced by the ability to visualize thicker sections, as well as the ability to optically section these thicker sections at a far finer level than could be achieved by the cryostat. Use of the confocal system allowed for the determination that the wall of the kefir grain appears to be a nearly solid mass of bacterial cells, organized in a linear fashion with defined edges. It was hoped that the identity of these wall cells could be determined via FISH, however, a *Lactobacillus* probe ordered for this purpose did not arrive in time to be run.

Confocal microscopy also revealed one of the potential artifacts of either the cryosectioning or FISH process, this being the loss of material from the surface of the section. It was noted during the optical sectioning and analysis of the confocal images that the top surface of the sections was quite irregular. As the sections are through the grains, not of the surfaces, it was expected that the surfaces be fairly uniform. A number of potential explanations for this phenomenon can be proposed, with most logical explanation being that some amount of loosely attached material is eroded away from the surface over the course of the washing and incubation steps inherent to FISH. This effect can be seen in figure 6, which was digitally sectioned to create figure 5.

Based on the finding of a nearly monophyletic group of *E. coli* in the clone library generated from the fresh kefir grains, the gamma proteobacteria probe GAM42a and the enterobacteria probe EBAC1790 were used in an attempt to localize the cells responsible for the clones. However, no signals were detected in hybridizations using either probe.
Conclusions:

Based on the combination of the clone library from the aged kefir grains with epi-fluorescent and confocal microscopy, a number of tentative conclusions can be arrived at. First, it can be determined that the kefir grains have an organized structure at the microbial level, and are not simply an amorphous mass of cells. More specifically, the grains appear to be ellipsoid 'shells' composed of bacteria, with yeast lining the interior lumen space. While far less definitive, it appears that this outer shell is composed primarily of *Lactobacillus* species. This statement, however, requires far more support when taken in light of the high microbial diversity in kefir reported by other investigators. While far too few sections were observed to make a definitive statement, it was noted that yeast appeared to be far more common in aged kefir grains than in younger cultures, and may be indicative of some level of ecological succession occurring in the grains. Taken as a whole, this project can be considered a partial success, with the FISH and microscopy components yielding valuable data, and the molecular component yielding interesting hints, but few hard facts.
References:


Appendix 1, Phylogenetic Tree of 16s Clonal Libraries

[Diagram of phylogenetic tree with species names and numbers]

Lactobacillus plantarum, 1329
Lactobacillus sp. CR 7AS, 1250

Lactobacillus kefiranofaciens, 1516
Lactobacillus kefiranofaciens subs. kefiranofaciens, 1516

AnaFlos4, Anaebaena flos aquae, 1389

Uncat576, uncultured gamma proteobacterium, 1266
Appendix 2: Complete Phenol:Chloroform Extraction Protocol


Solutions:
Phenol:Chloroform:Isoamyl Alcohol pH 8-saturated with Tris Buffer

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<th>Component</th>
<th>For 1L</th>
<th>For 100mL</th>
<th>For 50mL</th>
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<td>4.5g</td>
<td>0.45g</td>
<td>0.225g</td>
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<td>60mM K₂HPO₄</td>
<td>10.5g</td>
<td>1.05g</td>
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<td>7.6mM Ammonium Sulfate</td>
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<td>0.05g</td>
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<tr>
<td>3.3mM Sodium Citrate</td>
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<td>0.097g</td>
<td>0.0485g</td>
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<tr>
<td>4mM MgSO₄·7H₂O</td>
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<td>0.1g</td>
<td>0.05g</td>
</tr>
<tr>
<td>MilliQ Water</td>
<td>1L</td>
<td>100mL</td>
<td>50mL</td>
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</table>

Lysis Buffer

<table>
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</tr>
</thead>
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<td>1mL</td>
<td>0.5mL</td>
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<tr>
<td>100mM EDTA</td>
<td>200mL</td>
<td>20mL</td>
<td>10mL</td>
</tr>
<tr>
<td>10mM NaCl</td>
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<td>0.2mL</td>
<td>0.1mL</td>
</tr>
<tr>
<td>0.5% SDS</td>
<td>50mL</td>
<td>5mL</td>
<td>2.5mL</td>
</tr>
<tr>
<td>MilliQ Water</td>
<td>738mL</td>
<td>73.8mL</td>
<td>36.9mL</td>
</tr>
</tbody>
</table>

Protocol:
1. To a Bead Beating Tube, add:
   500uL liquid sample, or ~0.5g Sample
   250uL Minimal Salt Solution
   500uL Lysis Buffer
2. Bead Beat 1 minute or as necessary
3. Spin 5 minutes at max speed
4. Transfer Lysate to 750uL Phenol:Chloroform:Isoamyl Alcohol
   Invert several times.
   Spin 3 minutes at max speed.
5. Transfer Aqueous Layer (top) to fresh Phenol:Chloroform:Isoamyl Alcohol
   Invert several times.
   Spin 3 minutes at max speed.
6. Transfer aqueous phase to sterile microfuge tube.
   Add 150uL Sodium Acetate and 1.5mL absolute ethanol
7. Spin 10 minutes at max speed
8. Decant Supernatant
   Dry if speedvac available,
9. Resuspend in water or 0.1M Tris pH 8.0
Acknowledgements:

I would like to sincerely thank Dagmar Woebken for her endless patience with FISH, Tracey Teal and Kristen DeAngelis for their help with the molecular side of things, Louie Kerr and the Central Microscopy Facility for assistance in cryosectioning, the Microbial Diversity TA’s and Faculty for making all of this possible, and the entire Microbial Diversity course for making this an incredible summer.