

Individual project reports
Microbial Diversity Summer Course, 2007

Are there any “true” archaeal photo-
trophs lurking in Sippewissett Salt
Marsh Mats?

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Characterization of Kefir grains : fast
growers vs. Kefiran producers!

K Kritee
Rutgers University, New Jersey, United States.

Are there any “true” archaeal phototrophs lurking in Sippewissett Salt Marsh Mats?

Abstract

During the past decade *Archaea* have been recognized as a widespread and significant component of marine picoplankton assemblages and the presence of novel archaeal phylogenetic lineages continues to get detected in coastal marine benthic and deep sea environments. But inspite of diversity of novel sequences being found in natural environments, many monophyletic groups have no cultured representatives and hence their metabolic potential and ecological role is not understood. This project aimed at enriching archaea in growth medium originally designed for enriching anaerobic bacterial phototrophs with addition of bacteriostatic/bacteriocidal antibiotics. After two weeks on enrichment, 2 out of 20 enrichment bottles showed intense visible turbidity and bacterial and archaeal 16S rDNA clone libraries were constructed to assess the diversity of microbial population in the enrichments. Phylogenetic analysis of the sequences amplified from these primary enrichments show enrichment of archaea from Marine Benthic group D within Euryarcheota in both the bottles. Bacterial clone libraries donot show enrichment of any specific group of bacteria including anaerobic bacterial phototrophs probably indicating that the antibiotic treatment was effective. Although there was no clear pigment development in these enrichments suggesting absence of “true phototrophs”, these results are promising and could lead to successful culturing of representatives from groups whose ecological role is presently unknown.

Introduction

Many archaea are extremophiles. They thrive at high temperatures, at high salt, at high pressure and in concentrated acidic environments. Nevertheless, the largest proportion and greatest diversity of archaea exist in cold environments. (1). During the past decade *Archaea* have been recognized as a widespread and significant component of marine picoplankton assemblages and the presence of novel archaeal phylogenetic lineages continues to get detected in coastal marine benthic and deep sea environments (2-4). Detailed studies on the distribution of the planktonic *Crenarchaeota* and *Euryarchaeota* illustrated that the *Euryarchaeota* were most abundant in surface waters, whereas the *Crenarchaeota* dominated at depth (2). But inspite of diversity of novel sequences being found in natural environments, many monophyletic groups within *Crenarchaeota* and *Euryarchaeota* have no cultured representatives and hence their metabolic potential and ecological role is not understood.

The author was interested in phototrophic metabolism and realized that there no known phototrophic archaea. Phototrophs are traditionally known to occur within Cyanobacteria, Proteobacteria, Green sulfur and non-sulfur lineages of bacteria. But recently Bryant et al (5) have demonstrated presence of an phototroph within Acidobacteria. It is plausible that phototrophs exist within other bacterial clades and archaea as well. , even hetrotroths like

Goal

This project aimed at enriching archaea in growth medium originally designed for enriching anaerobic bacterial phototrophs with addition of bacteriostatic/bacteriocidal antibiotics.

Methods

Enrichments conditions and media: The details of variety of inocula used for enrichments, light regime and electron donors used is summarized on the next page. The medium used was exactly similar to what was described in lab manual for the course (2007) except for addition of 50mM BES in bottles with acetate medium. The inoculum used for enrichments set up on 7th July was not a totally defined mat layer but a mixture of different types of samples collected from the Little Sippewissett marsh and Trunk river areas. The bottles were incubated in dark for 2-3 hours after inoculation to let anaerobes stabilize in the new environment after exposure to oxygen during sampling and to prevent photochemical reactions immediately after exposure to oxygen.

Enrichment bottles 13-20 were set up after 3 days because it seemed that the antibiotic mixture used earlier was not effective and slight turbidity was observed in the bottles within 2 days. Microscopic observation of a few bottles at that time indicated presence of bacteria which seemed to have sulfur granules in them. But the turbidity did not increase later on!

Molecular analysis: Genomic DNA was extracted from bottles 14 and 15 after 2 weeks of incubation using MOBIO ultraclean soil DNA isolation kit. Insipte of high visual turbidity, microscopic observation indicated low cell abundance in the enrichments and 6 ml of the sample yielded only ~6 ng/ul DNA (Nanodrop).

Summary of Enrichment conditions

Bottle no:	Start date	Inoculum	e- donor	Light condition	Antibiotics	Status on 07.31.07
1	07.07.07	Sippewissett	Thiosulfate	660 nm	A, V, Cl, Cf	No turbidity
2	07.07.07	Sippewissett	acetate	660 nm	A, V, Cl, Cf	No turbidity
3	07.07.07	Sippewissett	Thiosulfate	880 nm	A, V, Cl, Cf	No turbidity
4	07.07.07	Sippewissett	acetate	880 nm	A, V, Cl, Cf	No turbidity
5	07.08.07	Trunk River	Thiosulfate	Incandescent	A, V, Cl, Cf, R	Little turbidity
6	07.08.07	Trunk River	acetate	Incandescent	A, V, Cl, Cf, R	High Turbidity
7	07.08.07	Trunk River	Thiosulfate	660 nm	A, V, Cl, Cf	No turbidity
8	07.08.07	Trunk River	acetate	660 nm	A, V, Cl, Cf	No turbidity
9	07.07.07	Sippewissett	Thiosulfate	880 nm	A, V, Cl, Cf	No turbidity
10	07.07.07	Sippewissett	acetate	880 nm	A, V, Cl, Cf	No turbidity
11	07.09.07	Marine sponge	Thiosulfate	Incandescent	A, V, Cl, Cf, R	Little turbidity
12	07.09.07	Marine sponge	acetate	Incandescent	A, V, Cl, Cf, R	Little turbidity
13	07.11.07	SP: "Iron bog"	acetate	880 nm	Am, R, Cl*	Transferred after 10 days
14	07.11.07	SP: "Algal"	acetate	880 nm	Am, R, Cl*	Extremely Dense, Clone library and transfer
15	07.11.07	SP: "Algal"	acetate	660 nm	Am, R, Cl*	High Turbidity, Clone library and transfer
16	07.11.07	SP: "Iron bog"	acetate	660 nm	Am, R, Cl*	High Turbidity
17	07.11.07	SP: "Iron bog"	Thiosulfate	880 nm	Am, R, Cl*	High Turbidity
18	07.11.07	SP: "Algal"	Thiosulfate	880 nm	Am, R, Cl*	High Turbidity
19	07.11.07	SP: "Algal"	Thiosulfate	660 nm	Am, R, Cl*	High Turbidity
20	07.11.07	SP: "Iron bog"	Thiosulfate	660 nm	Am, R, Cl*	High Turbidity

All the bottles containing acetate as e- donor were supplemented with 50mM BES to inhibit growth of methanogens

A = Amoxicillin 500 ug/ml

R = Rifamycin SV 300 ug/ml

V = Vancomycin 500 ug/ml

Am = Ampicillin 300 ug/ml

Cl = chloreracyclin 25 ug/ml

Cl* = Clortetracyclin 300 ug/ml

Cf = Cefotaxime 100 ug/ml

A, V, Cl and Cf are known to be light stable

PCR conditions: Bacterial 16S rDNA fragments were amplified using universal 8F and 1492R primers. Archaeal 16S rDNA fragments were amplified using universal 4F and 1392R primers. Primer set 571F and 1204R for archaea failed to amplify any fragments from the genomic DNA samples. A temperature gradient PCR amplification study was done to find a range of temperatures at which only single length of DNA was amplified using 4F and 1392R primer set. [Temperature gradient : 54, 54.2, 54.8, 55.5, 56.6, 57.9, 59.4, 60.7, 61.7, 62.3, 62.8 and 63]

Cloning was done using Invitrogen's TOPO cloning kit. 4.5 ul DNA was used instead of 2 ul (Page 12.8, Lab manual). A total of 48 sequences were submitted for sequencing [24 sequences from each bottle (12 from archaeal library, 12 from bacterial library)].

Phylogenetic analyses: An alignment of approximately 10,000 homologous full and partial primary sequences available in public databases (ARB Project, Ludwig and Strunk 1997) was used. The new bacterial and archaeal 16S rRNA gene sequences (approximately 500-800) were fitted in the 16S rRNA alignment by using the respective automated tools of the ARB software package. Maximum-likelihood (fastDNAmI) methods were applied for tree reconstruction as implemented in the ARB software package.

Results

The combination of high amounts of antibiotics Rifamycin SV and chlortetracycline gave the bottles an orange color at time zero. During the next few days the turbidity in a few bottles starting increasing but there was no clear pigment development in these enrichments suggesting absence of "true phototrophs". Microscopic observations suggested that most of the visual turbidity was due to suspended inorganic aggregates possibly formed by dead bacteria and organic matter. Within a week of starting the enrichment, 3 out of 20 enrichment bottles showed dense visible turbidity. Bottles 14 and 15 were chosen for further microscopic and molecular analysis (Fig. 1). Bacterial and archaeal 16S rDNA clone libraries were constructed to assess the diversity of microbial population in the enrichments.

Bacterial clone libraries do not show enrichment of any specific group of bacteria including anaerobic bacterial phototrophs (Green sulfur or purple sulfur) indicating that the antibiotic treatment was effective (Figures 2 and 3). There was no repetition of sequences in the bacterial sequences obtained indicating that 12 samples were not enough to represent the diversity of bacterial population in the enrichments. It was assumed that because of antibiotic treatment, the diversity of bacterial sequences recovered from the bottles would be very low.

As shown in Fig 4, Archaeal sequences fell into 4 different groups. The following sequences were found to be identical after alignment b14.1 = b14.11 = b14.6 = b14.5 = b14.8 (Total 5); b14.2 = b14.3 = b15.6 (Total 3); b15.1 = b15.9, b15.11 = b15.2

B15.4, B15.5 and B15.7 and B14.4 and B14.12 were Eukaryotic sequences (data not shown).

Discussion

Three out of the 4 groups found by sequencing archaeal 16S rDNA clone libraries have been described previously as Marine benthic groups B,C and D (2). Phylogenetic analysis of the sequences amplified from these primary enrichments show enrichment of archaea from Marine Benthic group D and a related group within Euryarcheota, especially in bottle 14. Marine benthic group D first proposed by Vetriani et al, has been found at many locations including liquid CO₂-hydrate hydrothermal system (6) but the function of members of this group remains unknown. Marine benthic group C found by Madigan and co-workers in Lake fryxell (7) and deep sea mud volcanoes (8). No sequences were found in Marine group 1 to which *Crenarcheota symbiosum* and many other non-thermophilic crenarcheota belong.

It is noteworthy that almost all the archaeal sequences amplified in this study are closely related to sequences obtained from deep sea hydrothermal vents (archaeon VC2.1 (9) and others (10)), deep sea benthic sediments (CRA sequences (2)) and highly saline brine sediments in red sea (KTK sequences from (11)). None of the closely related sequences in the ARB database except 2MT series and 2C series (12) have been isolated from salt marshes.

A few heterotrophic extremophilic archaea have been isolated in past few years (13) but no mesophilic non-methanogenic heterotrophic archaea utilizing acetate are known to this date. A study in the recent past of demonstrated activity of heterotrophic archaea (including some from Marine benthic group B) in sedimentary ecosystems (14).

These likely enrichment of acetate consuming archaea are promising and could lead to successful culturing of representatives from groups whose ecological role is presently unknown.

Characterization of Kefir grains : fast growers vs. delicious Kefiran producers!

Abstract

Kefir is a refreshing probiotic beverage which originated many centuries ago. The starter culture for making kefir is a self enclosed microbial community called 'Kefir grain'. The grains are formed through the effort of a symbiotic relationship shared among the complex microflora consisting of lactic acid bacteria, acetic acid bacteria and yeast. The microflora render an irregularly sheathed 3-dimensional structure, composed of protein, polysaccharide and lipid complex. Kefir grains are considered to be self regulating their microbial community and spatial organization but culturing conditions can change the relative microbial activities in the grain and hence affect the quality of Kefir made. The goal of this study was to elucidate reasons of production of bad quality kefir by grains which were left in refrigerator for 3 weeks by comparing their degree of respiration, oxygen depth profiles and metabolic activities with freshly procured 'new round healthy' kefir grains. The results show that the old grains had lost their three dimensional structure, respired oxygen much faster than the new grains and did not have the anaerobic cavity needed to maintain production of ethanol by yeast and Kefiran by bacteria. This study discusses the reasons of shift in structure and function of kefir grains and underscores the importance of maintaining appropriate culturing conditions for optimum quality Kefir.

Introduction

Kefir is a refreshing probiotic beverage, which originated many centuries ago, in the Northern Caucasus Mountains. The word kefir is said to be derived from the Turkish word *keif*, which loosely translates to good feeling or feeling good. The starter culture for making kefir is a self enclosed microbial community called 'Kefir grain'. The grain is formed through the effort of a symbiotic relationship shared among the complex microflora [bacteria and yeast], which render an irregular sheath, composed of protein, polysaccharide and lipid complex. The irregular fashioned sheaths usually form multiple irregular lobules covering the surface of each grain. The irregular lobules have a natural tendency to form as self-enclosed 3-dimensional bio-structures (Fig. 5). Some people describe the grains as little cauliflowers. These structures increase in weight and size after successive transfers in milk.

The smaller bodies, or baby-grains which develop during self propagation, eventually propagate into mother-grains, usually by increasing in overall size,

with multiple lobules forming over the entire grain. This growth-cycle simply repeats, to continue the ongoing process in a similar fashion.

Research suggests internal structure of the grains show a predominance of *Lactobacilli* with few yeasts; cells are not bound to one another but encapsulated within a muco-polysaccharide believed to be produced by the encapsulated microorganisms. Other research suggests stained sections of grains studied under a microscope, showed that yeasts were mainly located on the edge of the internal cavities, and occasionally along the peripheral channels of the matrix, while the exterior is mainly occupied by bacteria (15). The yeast produce ethanol under anaerobic conditions in the centre of the grain. *Lactobacilli* can produce Kefiran only in the presence of ethanol.

Kefir grains and kefir have been described to have numerous health benefits (16). They have been studied for diversity of microbes (17). The effect of culturing condition such as temperature and milk fat concentration have been reported (18) but no one has studied the effect of degree of anaerobicity of the grain on quality of kefir produced.

This study describes various methods used to infer the reasons of production of inferior quality of kefir by kefir grains left in refrigerator for 3 weeks. These grains were very rigid, robust, grew very rapidly but did not make viscous kefir. They produced highly acidic “yoghurt” and did not seem to show any improvement in the quality of kefir even after 20 daily transfers. They are being referred to as “old grains”. The old grains were further divided into two categories based on their three dimensional structure. Some grains were completely flat and had lost three dimensionality and are being called ‘old flat’ grains. Some old grains were not completely flat but retained some degree of lobular structure and are called “old round” grains. Fresh grains were obtained from a “kefir grain breeder” during this study and they produced viscous and delicious kefir and these will be referred to as “new round” grains.

Goals

1. Determine degree of anaerobicity in the centre of the old vs. new grains using oxygen microelectrode.
2. Determine respiration rate of old vs. new grains in milk using micro-respirometer
3. Grow old vs. new grains in a defined medium and measure the metabolic exudates of the grains using liquid chromatography

Methods

Oxygen depth profile: Unisense's Oxygen microelectrode OX50 with automated profiling set-up was used to measure the oxygen concentration profile of the old and new round grains. The system for automated profiling measurements consists of a motorized micromanipulator stage, MMS, and a motor A controller, MC-232. All sensor movements were controlled by the software and measurements were automated. The grain samples were always kept on the surface of 1% LE agarose with or without milk poured over the top. A fresh grain straight out of the milk after 24 hours of fermentation was used for oxygen concentration profile first and then it was washed in water multiple times to get rid of all adhering milk particles. The grain was later "killed" by exposing it to dilute solution of bleach (1%) for 5 seconds.

Respiration rates: Unisense's micro-respirometer was used for measuring rates of oxygen consumption by old flat, old round and new round grains with Stonyfield 2% organic milk as the growth medium in ~4 ml chambers. The exact weights of the grain samples and volumes of the chambers were:

- In Chamber 1 (Volume 4.25 ml) Old flat Grain weight : 0.227
- In Chamber 2 (Volume 4.26 ml) Old round Grain weight : 0.225
- In Chamber 3 (Volume 4.24 ml) New round Grain weight : 0.230

A minimum of 50 consecutive readings were taken for each chamber. It was ensured that no bubbles remain in the chamber and the mini-stir bars actively stirred the sample throughout the course of the measurements.

Difference in metabolic potential of old vs. new grains.

Similar weights of old flat and new round grains (in duplicate) were placed in sterile test tubes and overlaid with 5 ml of growth medium containing 5% lactose and 1% acetate in addition to trace minerals and vitamins. The media was buffered with MOPS and the starting pH of the media was 6.1. The tubes were incubated in 30°C shaker at 120 rpm for 24 hours and samples were taken at Time = 0h, time = 6h and time = 24h and concentrations of lactose, acetate, lactate and other products for which standards were available were measured using HPLC.

HPLC measurements were taken as described in the handout given to the class. 100 μ l 5N H₂SO₄ was added to 900 μ l of the sample to acidify the sample and the larger solids were removed by centrifugation for 5 minutes. The supernatant was transferred to the HPLC sample vials and analyzed by Shimadzu HPLC with a temperature controlled autosampler. A UV monitor detected organic acids (acetate, lactate, formate, succinate and citrate) and in-line refractive index

monitor detected alcohol (ethanol) and carbohydrate (lactose). The column used for separation was Aminex HPX-87H.

Salinity/viscosity measurements were done using Refractometer.

Results & Discussion

As shown in figures 5-9, following conclusions can be made about the old flat grains:

- They do not have the degree of anaerobicity which exists in the centre of round grains because of loss of three dimensional structure (Fig. 5). In spite of being big in size (length and width), they are very thin (maximum 2 mm). The old round grains did have anaerobic centre (Fig. 6) but their rates of respiration were much higher than for new round grains.
- They respire oxygen at a much faster rate than old round and specially new round grains (Figure 7). This might explain why the old grains propagate at a much faster rate as compared to new grains. It is plausible that during the initial few hours of 24 hour fermentation cycle, presence of high abundance of yeasts (The wet mounts of exudates (Fig. 9) clearly show that the old flat grains had much more abundance of yeast) in the old grains leads to their faster growth.
- They do not produce enough concentration of ethanol that is needed for kefir production. (Fig. 8)
- They can aerobically utilize acetate (see the relative loss of acetate from the two conditions after 24 hours - Fig 8). Yeasts can aerobically utilize acetate to produce CO₂ but lactic acid bacteria can't! this indicated that the old grains contained more yeast per unit mass of the grain as compared with the new grains.

The refractometer indicated that the salinity of the kefir produced by old grains was 30% ($^{20}d_{20} = 1022$) and the salinity of the kefir produced by fresh newly procured grains was 70% ($^{20}d_{20} = 1052$). In the absence of a good viscometer, these measurements indicate the quality of kefir produced.

It is plausible that the kefir grains that were left in fresh unfermented milk at 4°C for three weeks remained aerobic for an extended period of time and therefore selected for growth of yeast over lactic acid bacteria. This might have led to change in microbial community composition such that even after the daily transfers were resumed, the balance of yeasts vs. bacteria was not regained. It is known that even the healthy grains enter a "flat" stage periodically but the difference for the old grains studied here was that these seemed to have lost

their capability to revert back to round conformation and hence lost their degree of anaerobicity needed to make ethanol and kefiran.

This study clearly points out the importance of culturing conditions and storage of kefir grains in maintaining production of flavorful kefir!

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Figure 1. Microscopic analysis of bottles 14 (incubated at 880 nm) and 15 (660 nm) at 1000X magnification indicated growing presence of *Thiocapsa* like morphologies. No sequences similar to *Thiocapsa* were recovered by the clone libraries.

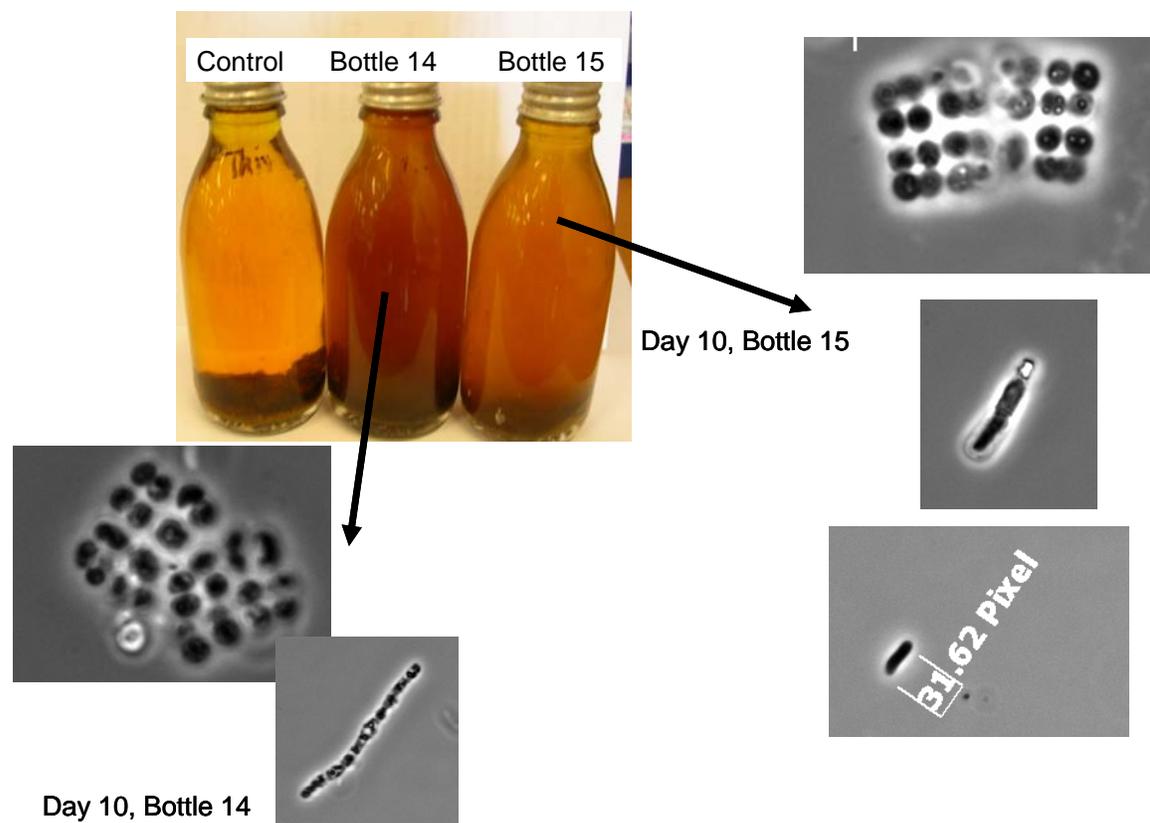


Figure 2. Phylogenetic tree of bacteria from within Proteobacteria

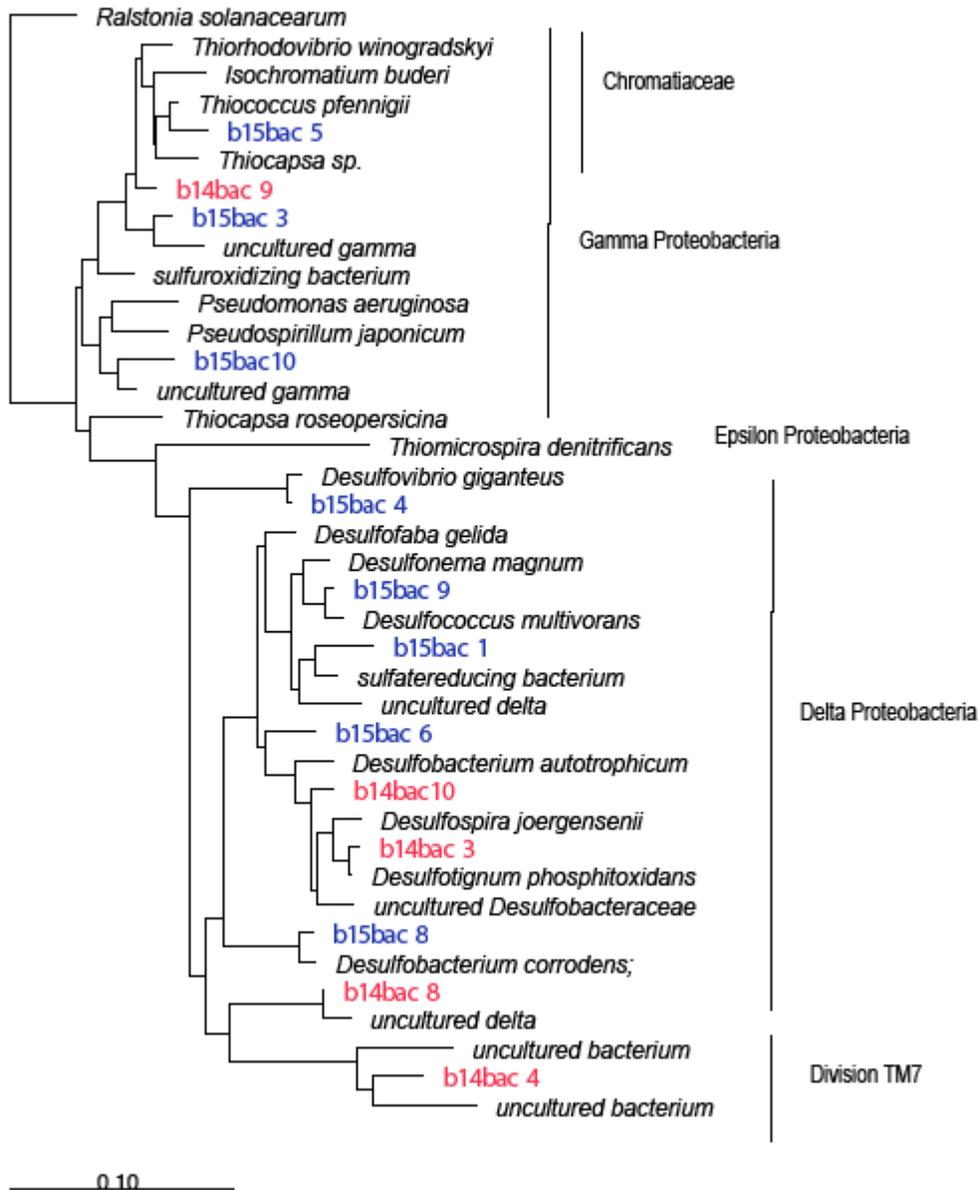


Figure 3. Phylogenetic tree of bacteria from the Bacteroides, Cytophaga, Flavobacterium and Verrucomicrobia groups.

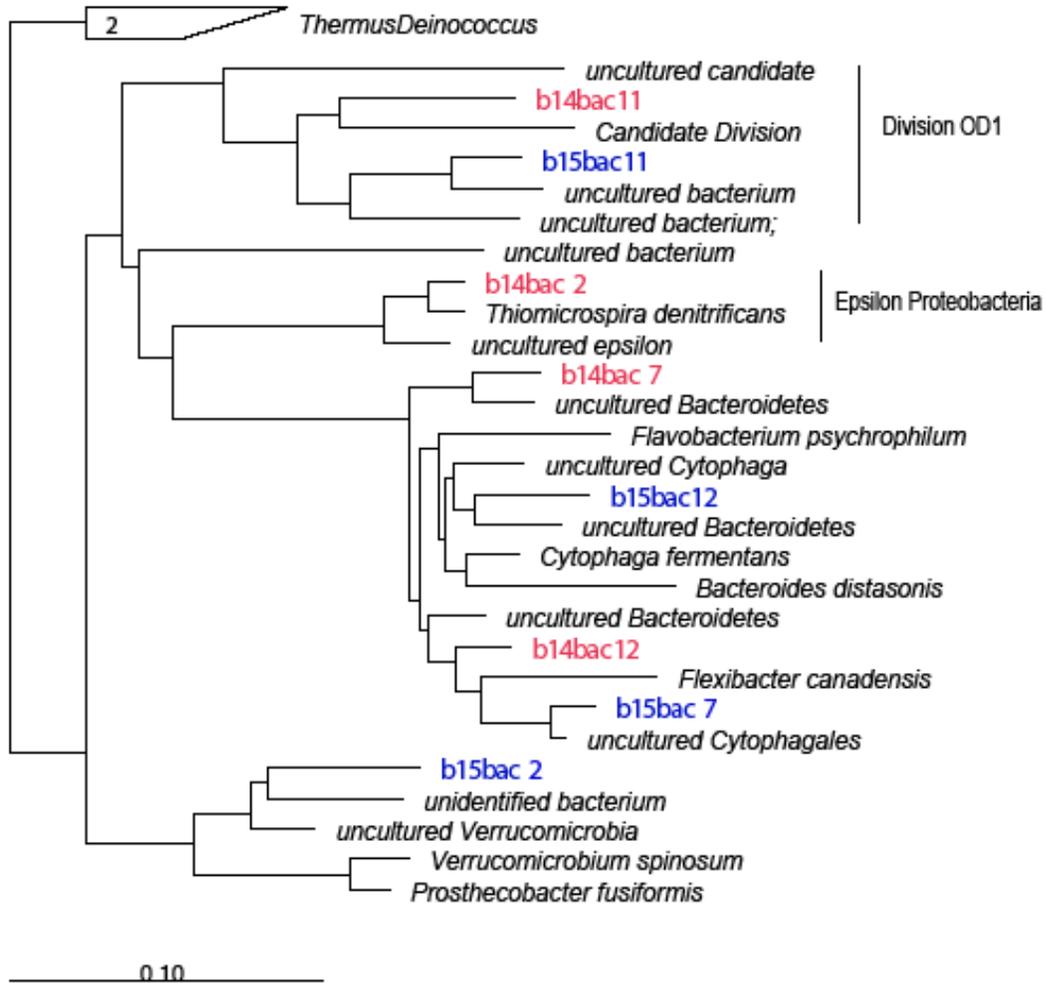
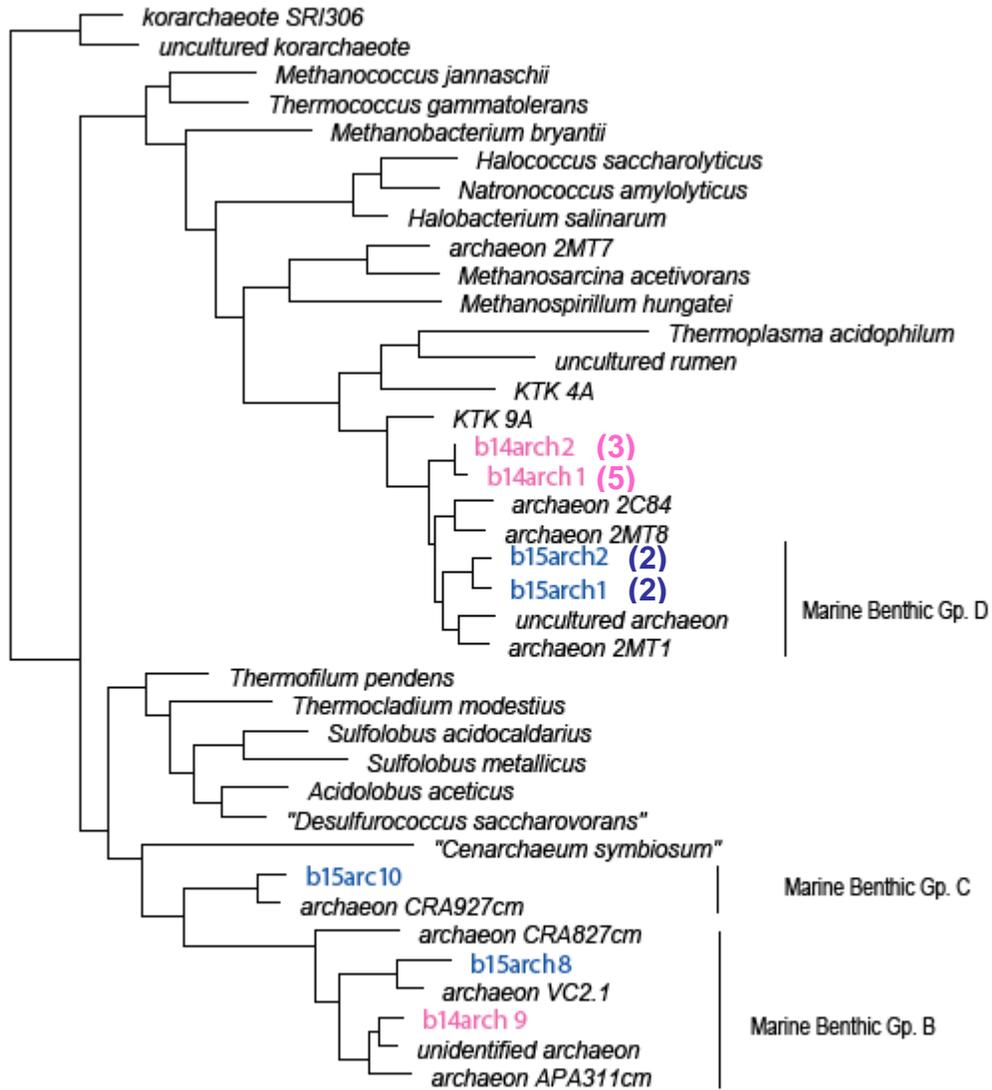


Figure 4. Phylogenetic tree of archaeal sequences recovered from clone library from bottles 14 and 15.



0.30

Figure. 5 Schematic showing the arrangements of bacteria and yeast in a healthy kefir grain(A) and macroscopic views of new round healthy grain(B), old flat grain(C) and old round grain(D)

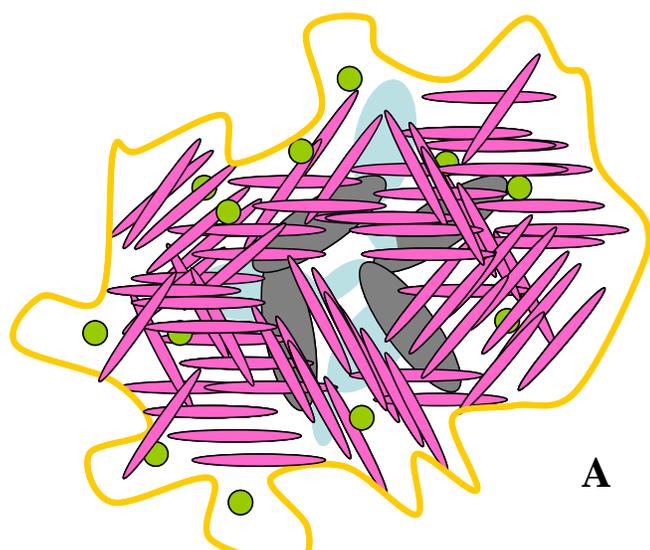
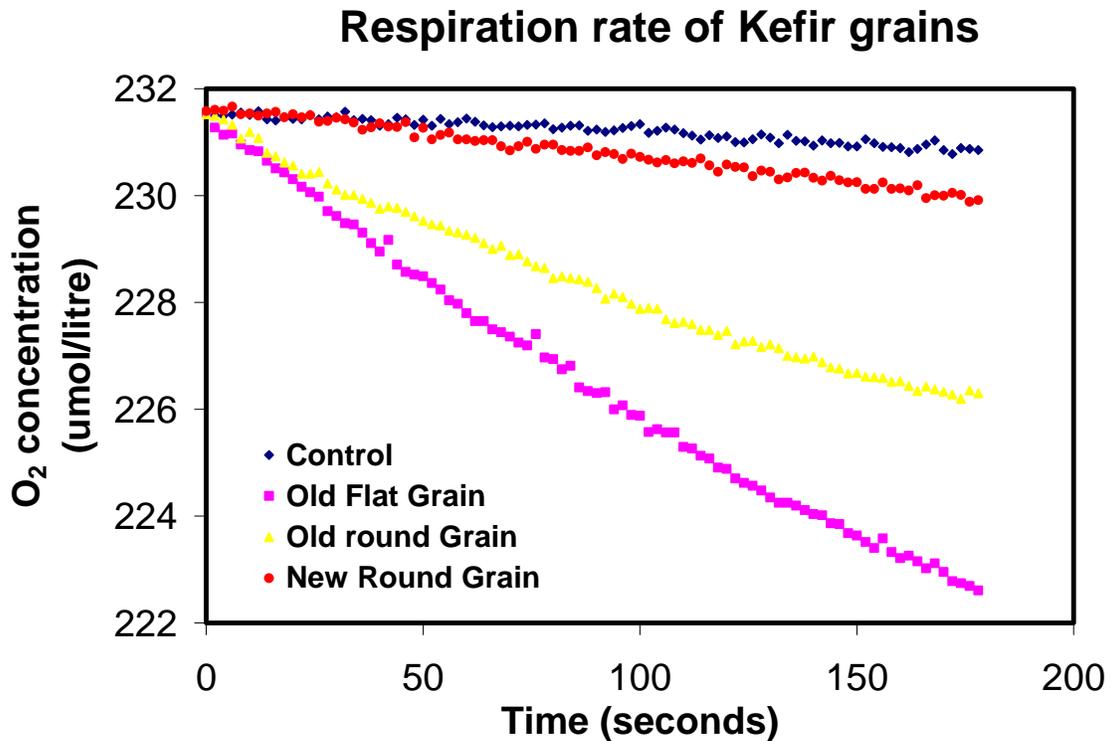


Figure 6. Respiration rates of old flat and old round fast growers vs. fresh new round grains in 2% organic milk as measured by micro-respirometer



Rates of respiration from slopes

3377 nmol/hr per g Old flat

2026 nmol/hr per g Old Round

637 nmol/hr per g New Round

Fig. 7 Oxygen concentration profile of new round and old round grains

Oxygen concentration profile of grains

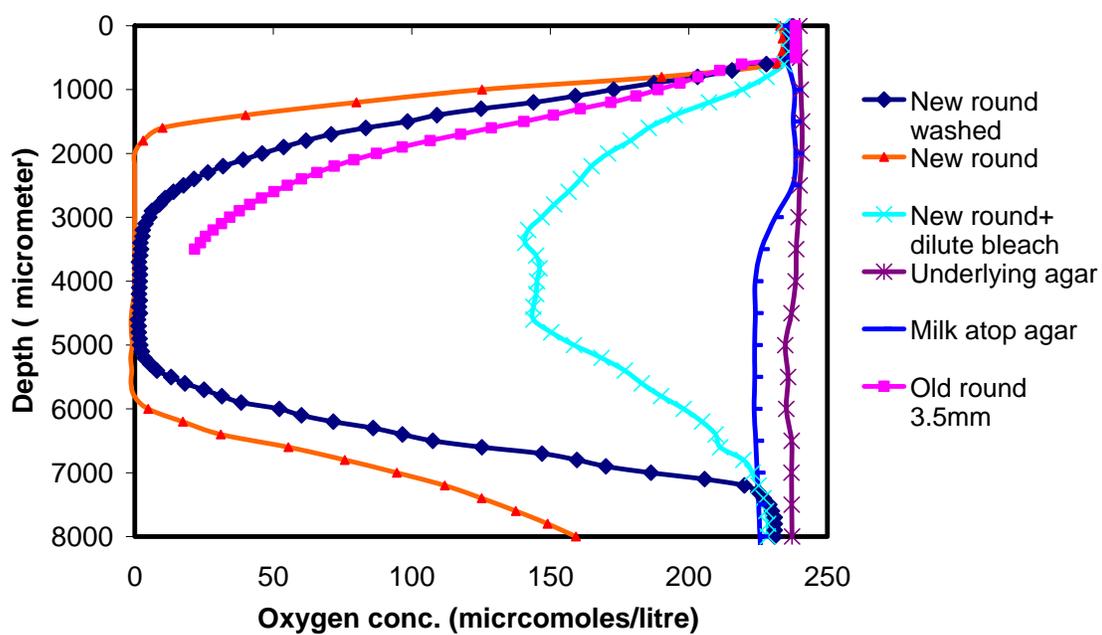


Figure 8. Metabolic activities of old flat vs. new round grains (HPLC analysis)

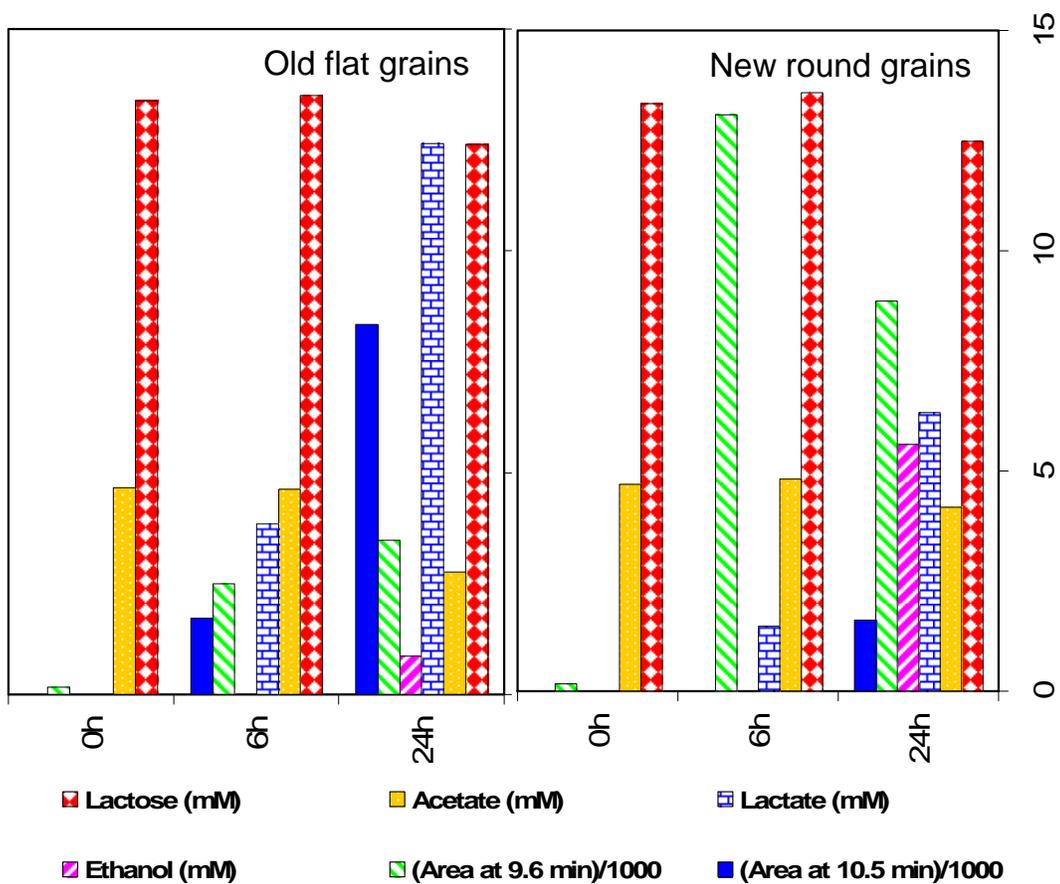


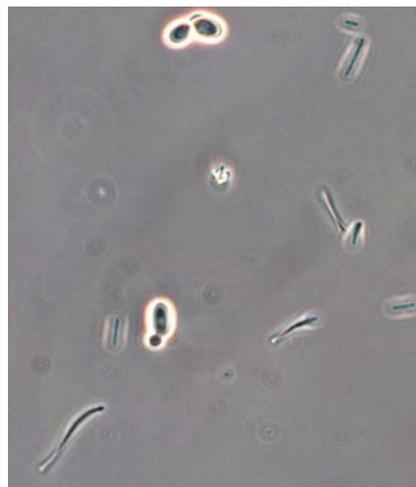
Figure 9 Microscopic analysis of kefir grain exudates

Microscopic evidence of increased activity and presence of yeasts
(After 24 h in media containing initial conc. of 5% lactose, 1% acetate, pH 6.1)



Old flat grains – 24 h exudate

• Av. pH after 24 h = 3.55



New round grains – 24 h exudate

• Av. pH after 24 h = 4.12

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