The effect of syntrophs on the community structure of Trunk River inoculants

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Abstract

Methanogens play an important syntrophic role in microbial communities in that they keep hydrogen partial pressures low and thus increase the favorability of hydrogen-producing reactions. Here we study how the presence or absence of exogenous methanogens (obtained through an environmental enrichment, hence in impure culture) affects the metabolites produced and the species present in an environmental inoculant, using either maltose or alanine/valine (AV) as electron donors. Inoculations with methanogens produced higher levels of acetate than inoculations without methanogen in both maltose and AV media. The inverse was observed for propionate and butyrate production: inoculations with methanogens present produced less propionate and butyrate than inoculations without methanogens in AV media. These results are consistent with the hypothesis that our methanogens syntrophically lower hydrogen levels and allow the less-favorable acetate-producing reactions to proceed. We found that methane levels were surprisingly higher in non-methanogen-added cultures than in cultures that contained added methanogen, which suggested that another organism (possibly acetogens) may be acting as the syntrophic partner. We used TRFLP and clone library techniques to ask if the observed shift in metabolites in the presence of added syntrophs was due to a change in the composition of the community, or to a shift to alternate metabolic pathways within the same community members. Our data suggest that all maltose cultures contained the same dominant species, whereas AV cultures grown with syntrophs displayed a dominant member that was different than AV cultures grown without syntrophs. The dominant species in syntrophic conditions was a close relative of *Clostridia litorale*, a known glycine fermentative organism.

Introduction

Methanogens play an important syntrophic role in microbial communities in that they keep hydrogen partial pressures low and thus increase the favorability of the hydrogen-producing reactions of their syntrophic partners. Syntrophic relationships with methanogens have been identified in the fermentation of amino acids (alanine, glycine, aspartate, glutamate, threonine, lysine), aromatic compounds (benzoate, gentisate, hydroquinone), other fermentation products (ethanol, butyrate, propionate, acetate), glycolate, and branched-chain fatty acids (1). We have chosen to examine how the presence of methanogens alters maltose fermentation and alanine fermentation of environmental inoculants.

Figure 12 displays a simplified scheme of maltose fermentation. Maltose is fermented to glucose and pyruvate, and pyruvate can undergo several fates. Pyruvate can be oxidize to acetate, resulting in the production of 1 ATP and 1 reduced NADH. Pyruvate can be oxidized to form ethanol, which does not produce any ATP but does serve to maintain the redox balance by oxidizing 2 NADH. Finally, 2 mol of pyruvate can be oxidized to form 1 mol of butyrate, resulting in the production of 1 ATP and oxidation of two NADH (2). Additionally, ethanol and butyrate can be oxidized to product acetate and hydrogen gas (3). An organism seeking the maximal energy yield (i.e. ATP) per mole of glucose

fermented would clearly choose to ferment all pyruvate into acetate. However, if a suitable electron acceptor is not present, NADH molecules will accumulate and prevent further fermentation of pyruvate to acetate. In such a case, glucose will be almost exclusively fermented to ethanol and butyrate. Methanogens (and other hydrogen-consuming species) make protons (H^+) energetically favorable electron acceptor for NADH oxidation by keeping hydrogen gas concentrations low. Hence methanogenic cultures will have higher levels of acetate and lower levels of ethanol and butyrate than cultures devoid of methanogens.

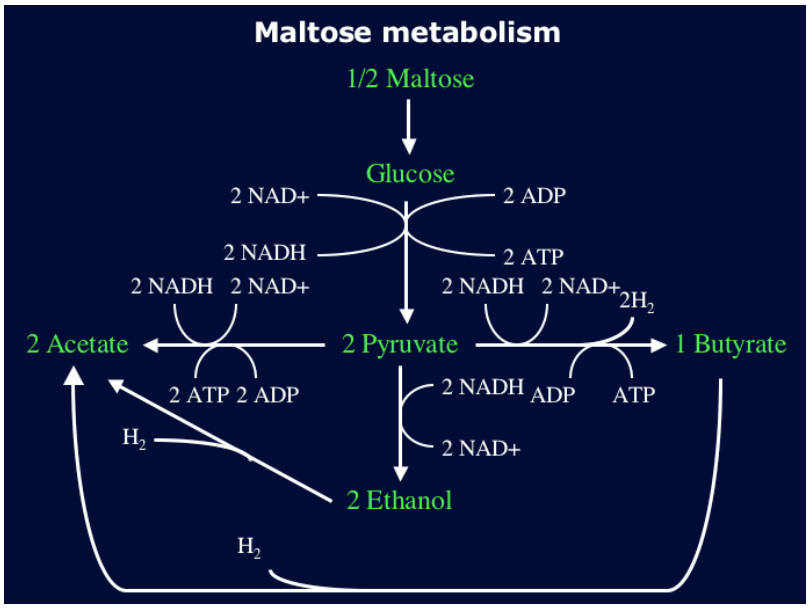

Figure 12: Fermentation of maltose

Figure 13 displays the fermentation of alanine via the acrylate pathway. Alanine is deaminated to form pyruvate, which can then either be converted to acetate in the manner described above, or highly reduced to form propionate and with no accompanying ATP production. Clostridia and other alanine-fermenting organisms ferment three moles of alanine to two moles of propionate and one mole of acetate in order to maintain redox balance (4). However, when hydrogen levels are kept low by hydrogen-consuming microbes such as methanogens, acetate production becomes more favorable (as described above) and the Clostridia produce acetate and propionate at a ratio higher than 1:2.

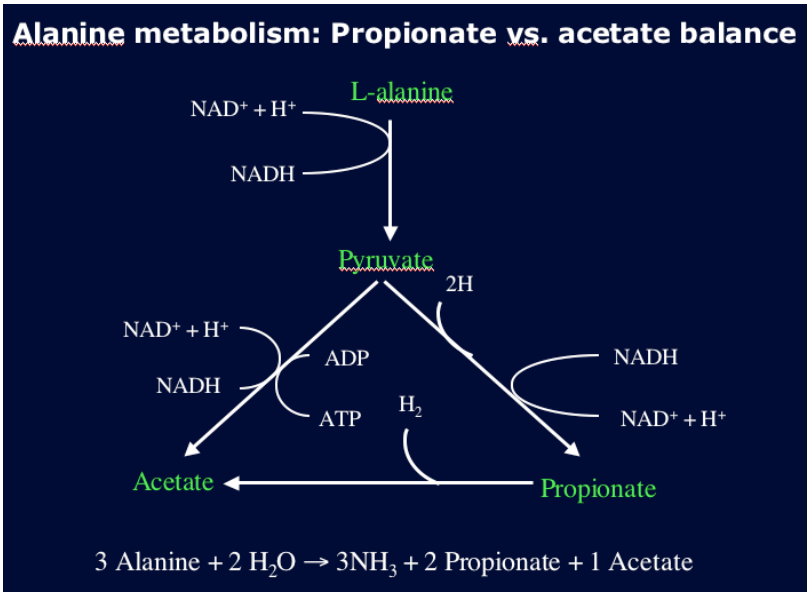

Figure 13: Fermentation of alanine

Here, we show that the addition of methanogen enrichment cultures to environmental inoculations changes the metabolites produced in the fermentation of maltose and alanine/valine, as predicted above. Moreover, we suggest that the resulting dominant maltose fermentor in each culture is the same specie, regardless of the presence or absence of syntrophs. This specie is capable of implementing multiple fermentation pathways. The dominant alanine/valine fermentor in each culture, on the other hand, is different the presence or absence of syntrophs.

Methods

Enrichment

We enriched for methanogens by inoculating "standard" anaerobic media (1L Salt Water base, 0.1% rezasurin, 70 mL 1M NaHCO3, 20 mL 1M MOPS, ph 7.2, 10 mL 0.5 M NH4Cl, 1 mL 1M potassium phosphate, 10 mL trace elements solution, 10 mL vitamin mix, 2 mL 0.2 M cysteine-HCl, 2 mL 0.2 M Na2S) with decaying organic mater from Trunk River. We charged the headspace with H2/CO2 20/80 at 7 psi. We cultured the enrichments serially over four weeks, and inoculated our syntrophic enrichments with the third and fourth cultures of methanogens. We checked the abundance of methanogens using F420 epifluorescence.

We made sets the following four syntrophic enrichments and controls: methanogens only (+ 2 mL of methanogen culture, for a final OD of 1E7 or 1E8 as measured by hemacytometer), inoculant only, inoculant + BES (10 mL of 2.5 M BES), and methanogen with inoculant. We added one of the following three donors to our tubes: maltose (0.7 mM), alanine and valine (10 mM each), or ethanol (10 mM). We grew our tubes at 30°C, and serially transferred the media every 3 – 4 days.

Methane measurements

We measured the methane produced in our cultures daily by injecting 200 ul into a gas chromatograph, and multiplying the resulting number of moles detected by the tube headspace volume.

Fermentation product measurements

We measured the fermentation products of each culture at several points in time by combining 450 uL of culture with 50 uL of 5 N sulfuric acid, centrifuging, and transferring the supernatant to an HPLC vial. We measured organic substances present using a GC, with standards run within the past three weeks.

Clone library analysis

We extracted samples from our cultures at various time points, and harvested DNA using a soil extraction kit. We isolated 16s DNA by running a PCR (Promega PCR mix) on our extracted DNA using 7.5 pmol of 8F and 1492R primers. We cloned the resulting PCR product into cloning vectors (Topo TA), transformed them into competent cells (Top 10) and plated transformants on LB + Kan plates. We sent the resulting white colonies to be sequenced at the Marine Biological Laboratory Bay Paul sequencing center. We built trees using resultant sequence data using the Arb software package.

T-RFLP analysis

We performed PCR using the harvested DNA from our clone library analysis and 7.5 pmol of primers 1492R and 8F (labeled with 6FAM fluorophore). We cleaned up the resultant PCR product using a PCR purification kit (Qiagen) and digested the product overnight using MsgI restriction endonuclease. We submitted the digested fragments, mixed with ladder, to the Bay Paul center for imaging.

Results

Acetate levels are augmented in Methanogen-added cultures

We used organic matter gathered at Trunk River and a methanogen enrichment generated in class to inoculate the following four samples: Methanogen only, Trunk River inoculation (“inoculation”) only, Trunk River inoculation with BES (to inhibit methanogenesis), and Methanogen with Trunk River inoculation. We grew these four samples on 0.7 mM Maltose, 10 mM Alanine and 10 mM Valine, or 10 mM Ethanol. We serially transferred cultures every 3 or 4 days to fresh media. We measured acetate levels using an HPLC machine, and compared measurements at t = 40 hours post-inoculation (maltose cultures) and t = 70 hours post-inoculation (alanine/valine cultures) for all cultures. The results are displayed in Figures 1 and 2. As expected, in acetate levels were highest when methanogen enrichment was added (Methanogen only and Methanogen + Inoculation), and lowest when enrichment was not added (Inoculation only and Inoculation + BES). We believe that the methanogen enrichment-only culture produced a high amount of acetate because, like most enrichments, the methanogen enrichment was not purely comprised of methanogens but other basal-level species as well. The maltose

cultures began displaying the acetate trend with the first inoculation, while the alanine/valine cultures did not display the trend until the first transfer. The fact that acetate levels correlate with added methanogen culture supports the hypothesis that methanogens are shifting metabolic products to acetate.

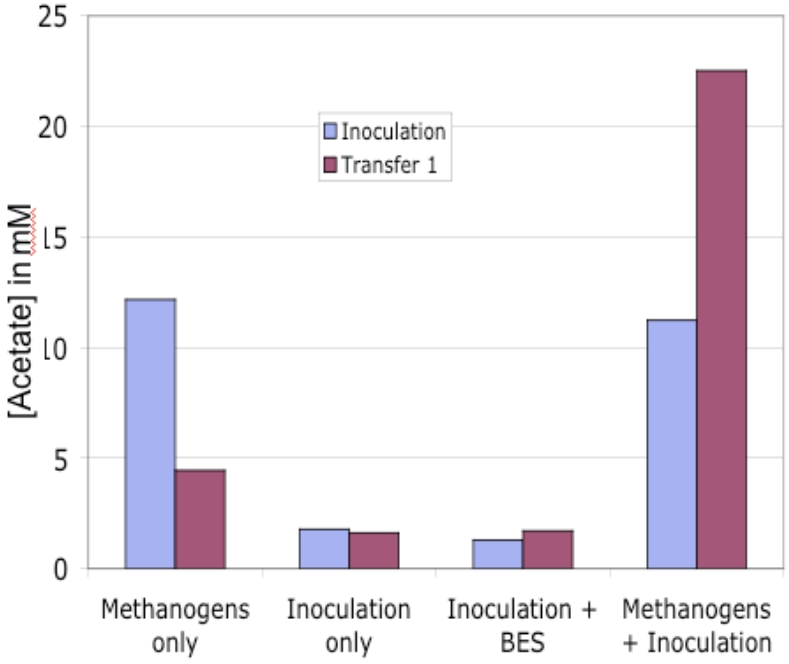

Figure 1: Acetate levels for different maltose cultures, 40 hours post-inoculation.

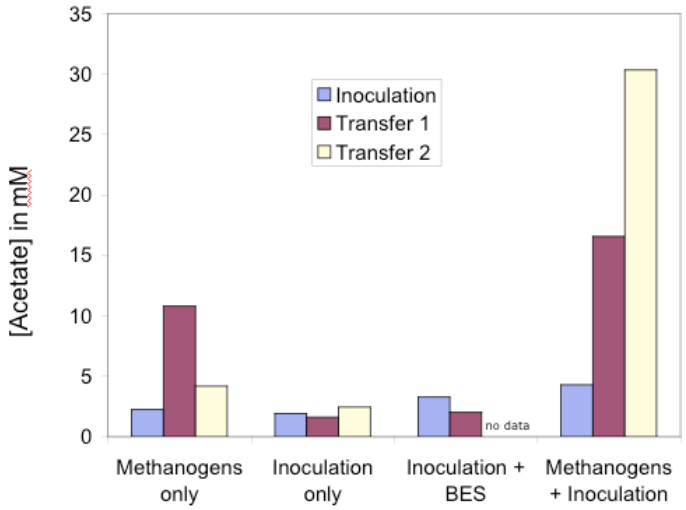

Figure 2: Acetate levels for different alanine/valine cultures, 70 hours post-inoculation.

Propionate and Butyrate levels are decreased in Methanogen-added cultures in Alanine/Valine media

Figures 3 and 4 display the propionate and butyrate levels in alanine/valine cultures at t = 70 hours post inoculation. As expected, propionate and butyrate levels were lower in methanogen-added cultures than in cultures without added methanogen enrichment.

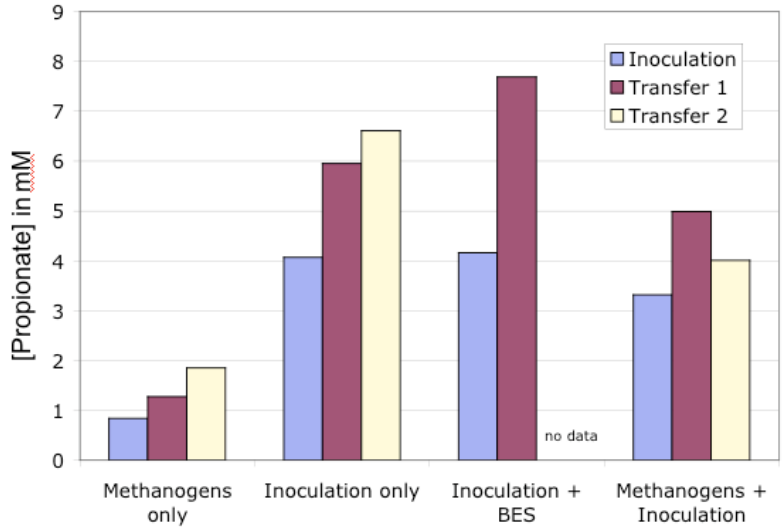

Figure 3: Propionate levels in alanine/valine cultures, 70 hours post-inoculation.

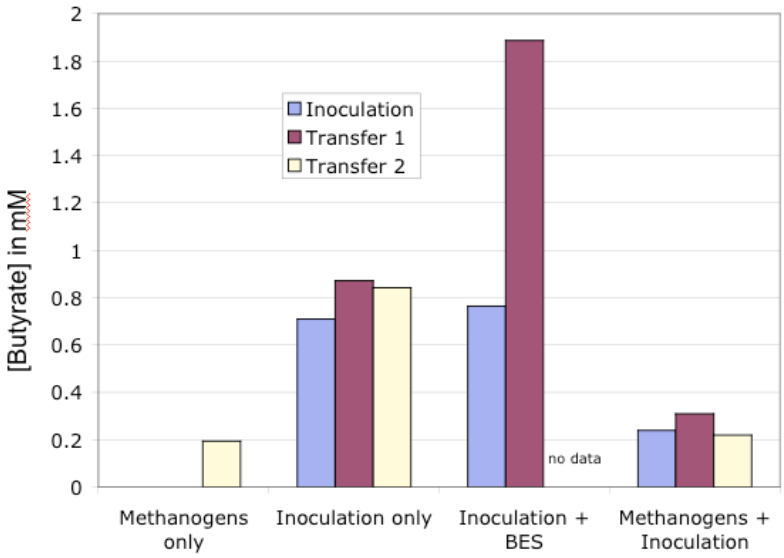

Figure 4: Butyrate levels in alanine/valine cultures, 70 hours post-inoculation.

Methanogen or Acetogen syntrophy?

We measured methane levels at t = 40 and t = 70 hours post-inoculation for the maltose and alanine/valine cultures (Figures 5 and 6). We expected that methane levels should map with acetate levels, i.e. cultures with high acetate levels should have high methane levels as well. However, our data suggested otherwise. Methane levels were typically higher in the inoculation-only culture, whereas acetate levels were low in inoculation-only cultures. Additionally, changes in methane levels did not correlate with changes in acetate levels. The somewhat inverse correlation between acetate levels and methane production suggests that the syntrophic partner being added with the "methanogen enrichment" may be acetogens and not methanogens.

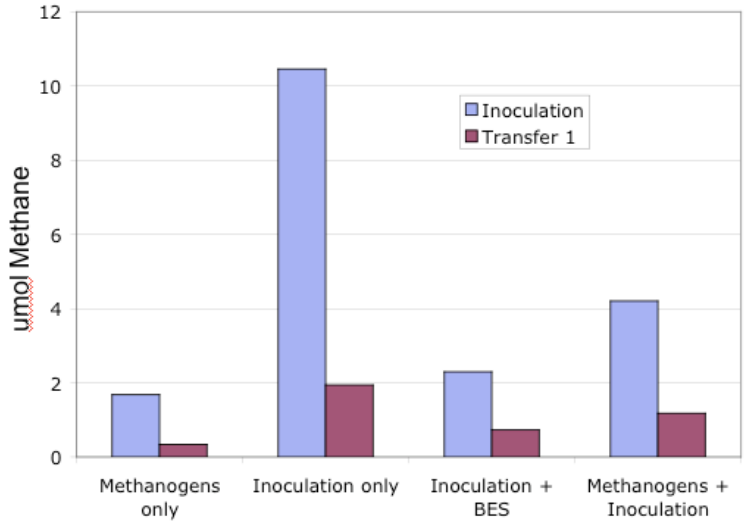

Figure 5: Methane levels in maltose cultures, 40 hours post-inoculation.

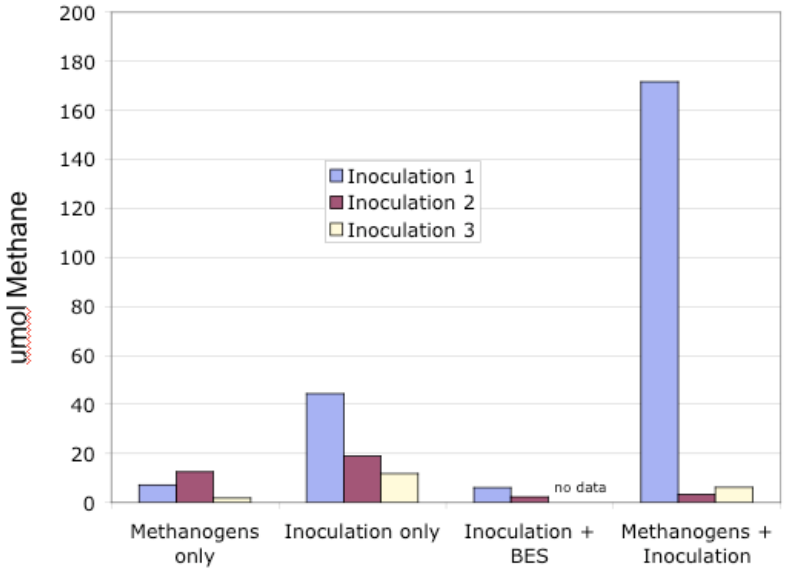

Figure 6: Methane levels in alanine/valine cultures, 70 hours post-inoculation.

Carbon balance caveat

We compared the levels of input carbon (42 umols from maltose and 400 umols from alanine/valine) with the sum of the following measured metabolic products and residual maltose and glucose: citrate, lactate, formate, acetate, propionate, butyrate, and methane. We found that output levels of carbon in the maltose enrichments ranged from 88 to 354 umols. These levels are greatly in excess of the input levels of carbon, and so barring any novel carbon fixation mechanism in our inoculated species, we presume that there was some sort of calibration error in the gas chromatograph.

The same specie may be dominant for all maltose cultures

We have shown that the metabolic products of communities grown with and without our methanogen enrichment are different. We next sought to see if this difference is due to a different dominant species in the community, or alternative metabolic pathways in the same dominant species. We performed a terminal restriction fragment length polymorphism (T-RFLP) analysis on DNA extracted from our maltose culture at different time points. Figure 7 shows T-RFLP electropherograms from transfer1 at time t = 55 hours. The dominant peak for each culture is located at the same position, regardless of the presence or absence of methanogen enrichment. This suggests that the dominant specie in all maltose cultures may be the same across culture conditions; in the future, we will create a clone library of each culture and determine the similarity in their dominant species. If the dominant specie is indeed the same, this implies that different culture conditions favor alternative metabolic pathways in the same dominant specie.

We examined several time points using T-RFLP, and found that the dominant species was established within the first 40 hours post-inoculation (Figure 8).

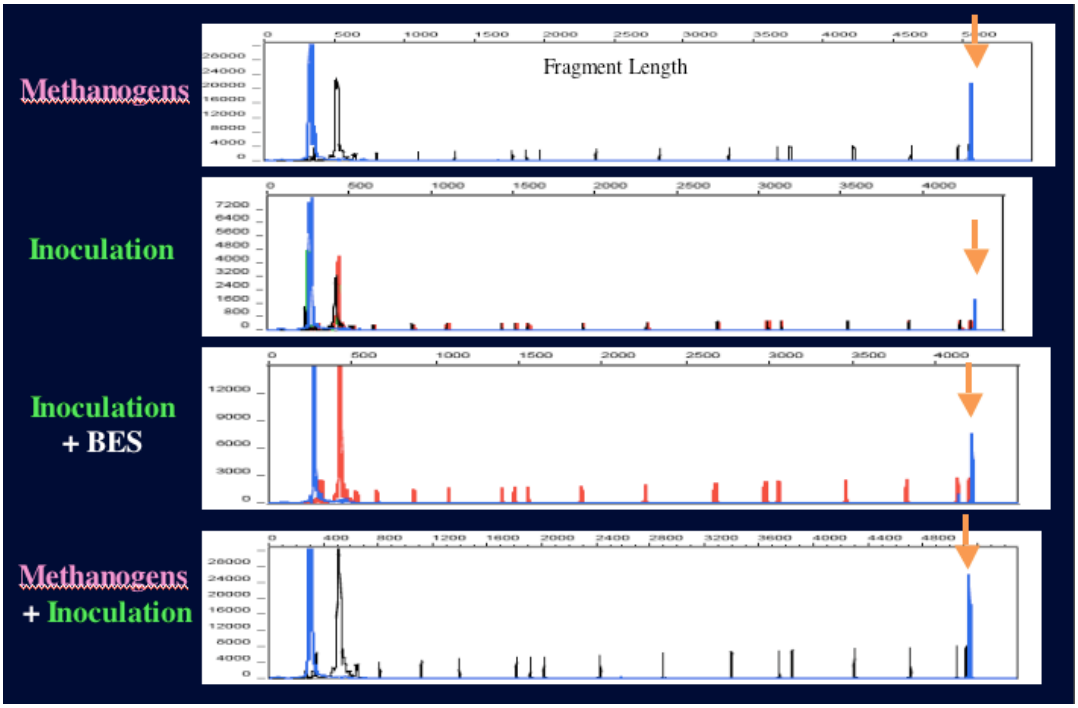

Figure 7: T-RFLP electropherograms from maltose cultures, transfer 1, 55 hours post-inoculation.

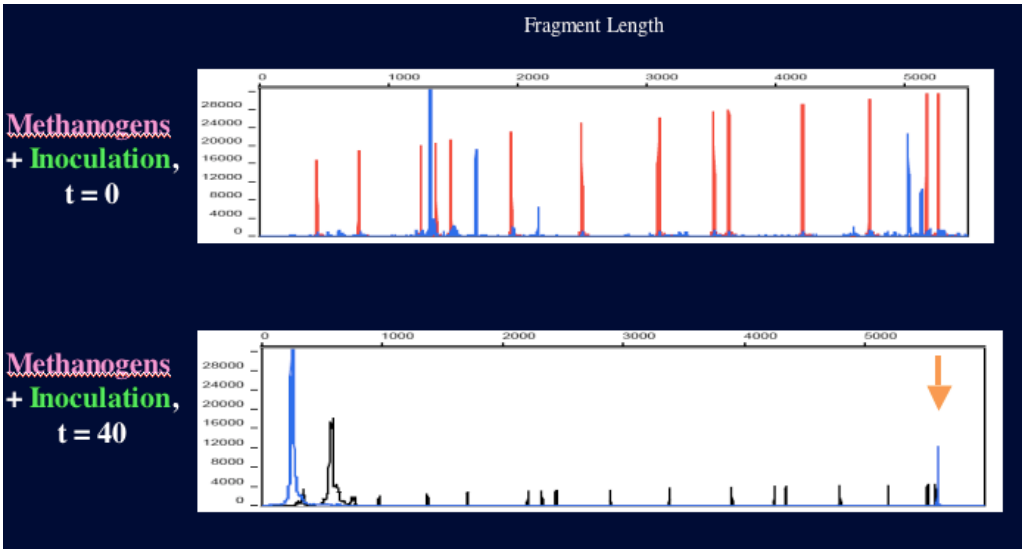

Figure 8: T-RFLP electropherograms from maltose cultures at time 0 and 40 hours post-inoculation.

Analysis of the dominant species in alanine/valine enrichments

We performed a T-RFLP analysis of our alanine/valine enrichments (Figure 9), and found that, unlike the case of the maltose enrichments, the dominant member was different in cultures that contained methanogens and acetogens (inoculation only and methanogen + inoculation cultures), than in cultures without methanogens (inoculation + BES). Again, the dominant members of the inoculation only and methanogen + inoculation cultures have the same peak location, but further analysis is required to ascertain if the dominant species are indeed the same. Unlike the maltose enrichments, the dominant species in the alanine/valine methanogen + inoculation culture does not become prevalent until 41 hours into the first transfer (Figure 10).

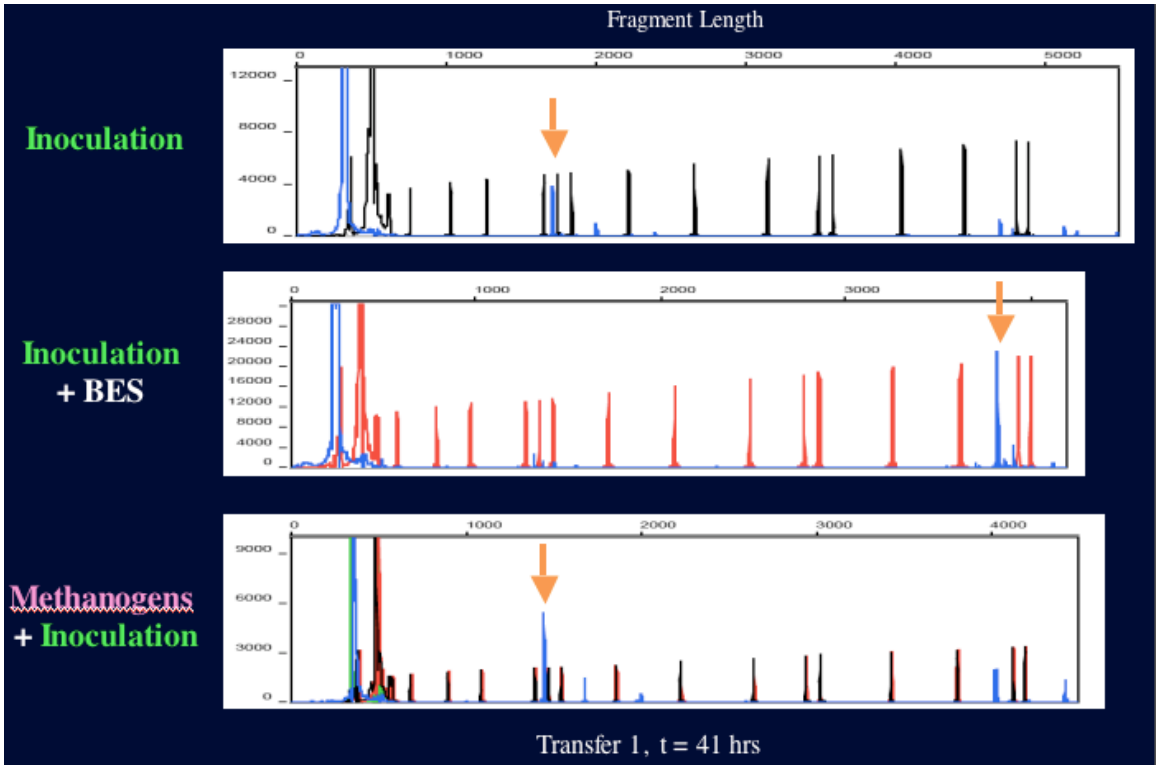

Figure 9: T-RFLP electropherograms from alanine/valine cultures, transfer 1, 41 hours post-inoculation.

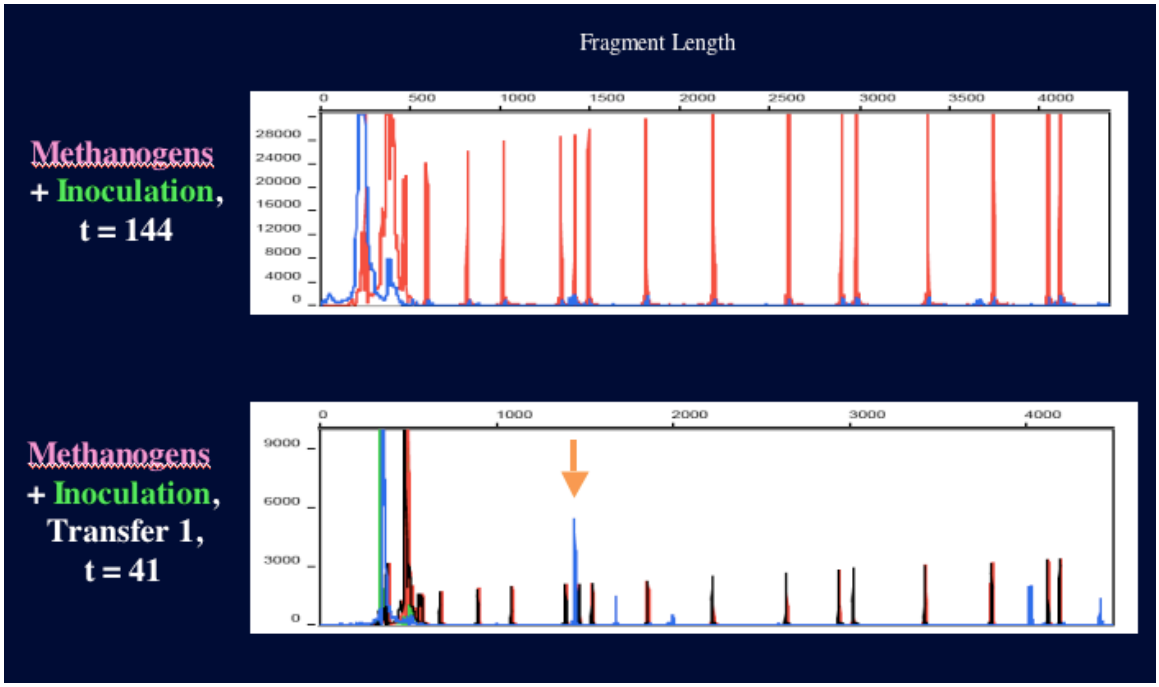

Figure 10: T-RFLP electropherograms from alanine/valine cultures (inoculation and transfer 1) at times 144 and 41 hours post-inoculation, respectively.

We constructed a clone library from the methanogen + inoculation culture (transfer 1 at t = 55 hours), grown on alanine and valine to identify the dominant species there within. A phylogenetic tree of our 42 sequenced clones is shown in Figure 11. Of our clones, 76% fell into one of two groups that are closely related to *Clostridia litorale*. *C. litorale* has been extensively studied for its glycine fermentation, and thus is it not surprising that our alanine/valine fermentative cultures enriched for a similar organism. The T-RFLP position of the dominant specie (restriction fragment length of between 139 and 150 bp) closely correlated with the predicted restriction fragment length of the *C. litorale*-like clones (154 bp), suggesting that the dominant specie seen in T-RFLP is most likely the *C. litorale*-like clone. We attempted to use this comparison technique to ascertain the identity of the second-most dominant peak, but only found distantly related clones with appropriate restriction fragment lengths.

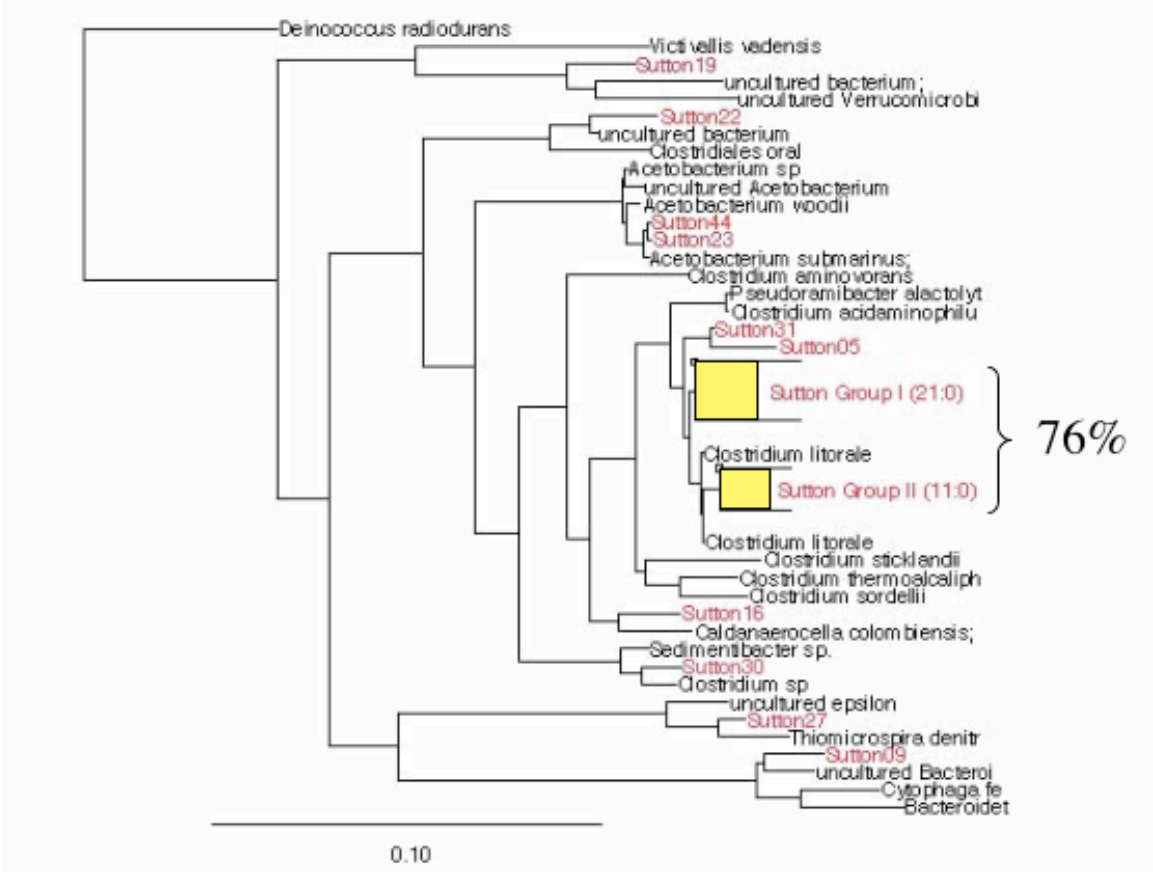

Figure 11: Phylogenetic tree of alanine/valine clone library from methanogen + inoculation enrichment.

Discussion

We have shown that environmental samples grown the presence of a methanogen enrichment display differing levels of acetate, propionate, butyrate, and methane. Cultures grown with maltose and alanine/valine as electron donors display increased levels of acetate in the presence of the methanogen enrichment than in the absence of the methanogen enrichment, suggesting that hydrogen consumption lowers hydrogen levels to a point where pyruvate → acetate fermentation is energetically favorable.

Alanine/valine cultures show the opposite trend with respect to propionate and butyrate levels: in the presence of the methanogen enrichment, cultures display lower amounts of propionate and butyrate than in the absence of the methanogen enrichment. This result suggests that propionate and butyrate are either not as readily produced, or more readily oxidized to acetate in the presence of the methanogen culture.

We initially concluded that methanogens from the in-class methanogen enrichment were forming a syntrophic relationship with our Trunk River inoculations; however, our methane measurements disputed this conclusion. Specifically, we observed that the very cultures with high acetate production exhibited low methane production. Our inoculation only culture exhibited low levels of acetate, and yet high levels of methane produced. We

thus suspect that another organism, such as an acetogen, may be acting syntrophically with our inoculation instead of a methanogen. This explanation is plausible, as the same enrichment conditions that favor methanogens can also favor acetogens. We speculate that the methanogens in the methanogen enrichment are not well suited to interact with the Trunk River inoculation, whereas the acetogens within the methanogen enrichment are. Moreover, in the inoculation-only cultures exist methanogens that are able to interact syntrophically with the rest of the community, and thus play a major role in maltose and alanine/valine fermentation in the inoculation-only cultures.

Our T-RFLP data suggests that the same dominant member is present in all of the maltose cultures, while different dominant members are present in the alanine/valine cultures. The alanine/valine cultures show one particular dominant peak in the inoculation-only and methanogen enrichment + inoculation cultures, and a different dominant peak in the inoculation + BES culture. A possible explanation for this difference is that levels of syntrophs (i.e. methanogens and acetogens) are sufficiently high in the former two cultures to substantially enrich for communities that can interact with the syntrophs. When the inhibitor BES is added to cultures, the loss of methanogenesis could lower the level of syntrophs to the point that the community adopts a non-syntrophic structure.

Finally, based on our clone library and T-RFLP data, we strongly suspect that the dominant specie within our methanogen + inoculation cultures grown on alanine and valine is closely related to Clostridia litorale, a known glycine fermentor. We hope to sequence the rest of the maltose and alanine/valine cultures to determine if our T-RFLP peaks at the same location yield the same dominant species, and which species are present in non-syntrophic communities.

Acknowledgements

I would like to thank all of the amazing people here at the Microbial Diversity course, who made this experience so rich and vibrant. Specifically, I would like to thank:

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