Isolation and identification of *Bdellovibrio* and like organisms (BALOs) from various saltwater sites in the southern Cape Cod area, and analysis of their prey range specificity.

Microbial Diversity 2005
Marine Biological Laboratory

Laura Hobley
University of Nottingham, UK

1. Abstract

Bdellovibrio is a genus of the delta-proteobacteria, which consists of bacteria that replicate within the periplasm of other 'Gram-negative' cells. They have been isolated from many different environments, both aquatic and terrestrial, yet very little is known about their true diversity and abundance in the environment. This aims of this project were:-

- Isolate and identify several marine bacteria from each environment into pure culture to be used as potential prey for isolation of *Bdellovibrio* spp.
- Create *Bdellovibrio* specific clone libraries from four enrichments, from two different environments enriched with two different prey.
- Isolate *Bdellovibrio* spp. From each environment and compare prey range of each to test for specific prey preferences.

Isolates and enrichments were made using samples from the following six sites in the Woods Hole area:-

- Eel pond
- Garbage Beach
- Trunk River
- Lesser Sippewissett – empty mussel shells and water
- Greater Sippewissett – live mussels and water
- Decomposing clams from the sea table in lab.

This study shows that *Bdellovibrio* can be isolated from all of these environments along with a variety of potential prey species, raising the question as to the effects *Bdellovibrio* have on the bacterial populations in marine environments

2. Introduction

Bdellovibrio bacteriovorus is a small highly motile, predatory bacterium, which has an unusual intra-periplasmic growth phase. They are members of the delta group of Proteobacteria, and as such they are related to the *Myxobacteria*, which includes a range of other predatory bacterial species (Woese, 1987).

The genus *Bdellovibrio* consists of 5 main species: *Bdellovibrio bacteriovorus* (Stolp & Starr, 1963), *Bacteriovorax marinus*, *Bacteriovorax litoralis* (Baer et al, 2004), Bacteriovorax *stolpii* (Baer et al, 2000) and *Peredibacter starrii* (Davidov and Jurkevitch, 2004). Figure 1 (Page 6) shows a neighbour-joining tree constructed using all sequences originally present within the ARB database, the type strains for each species are marked by the red arrows. The database shows large areas of the tree contain only sequences from uncultured strains, which suggests that the genus may in fact include more than the reported five, and suggests that new isolation techniques need to be developed to isolate strains that fall within the uncultured groups.

Bdellovibrio bacteriovorus has a bi-phasic life cycle including free-swimming flagellate attack-phase cells, and an intra-periplasmic non-flagellate growth phase. *Bdellovibrio* have been shown to only be able to predate on other Gram-negative bacteria. The life cycle 'begins' with free-swimming *Bdellovibrio* and prey. (Stage 1, Figure 2, page 7 below). Upon collision, attachment occurs between predator and prey (Stage 2), at this point the attachment is still reversible. After irreversible attachment has been achieved between the *Bdellovibrio* and the prey cell, the *Bdellovibrio* penetrates into the host cell (Stage 3), achieved by secreting enzymes that form a pore in the outer membrane of the prey, this pore is then resealed once the *Bdellovibrio* has entered the periplasm. After penetration by the *Bdellovibrio*, the combined cell forms a "bdelloplast", a spherical structure

containing the dead host cell, with the *Bdellovibrio* growing within the periplasm (Stage 4).

After the formation of the bdelloplast, the *Bdellovibrio* elongates into a filamentous cell (Stage 5), remaining in the periplasm of the host cell. When the nutrient supply from the cytoplasm has been depleted, the filament fragments into unit size cells, the number of which depends upon the size of the host cell (Stage 6). After fragmenting the cells become flagellate, and then synthesise a lytic enzyme to lyse the cell, releasing the flagellate attack phase cells (Stage 7). The life cycle then restarts, with free-swimming attack phase *Bdellovibrio* and free-swimming host cells.

3. Prey isolation.

To isolate potential prey for *Bdellovibrio* enrichment and isolation I required species that grew quickly and had basic media requirements. Therefore I chose to isolate bacteria that grew overnight at 30ºC on sea water complete agar, and were identified as non-agar-eaters.
The six sampling sites were:
1) Decomposing clams from the sea table in the lab
2) Eel Pond
3) Garbage Beach
4) Trunk River
5) Lesser Sippewissett – Mussel shell and water
6) Greater Sippewissett – Live Mussels and water

Dilutions of each sample (100ul of sample, 10^{-1}, and 10^{-2}) were spread-plated on 1.5% agar plates, and single colonies were then picked and re-streaked at least 4 times to get pure isolates for PCR. Five colonies for each sampling site were picked, such that a range of colony shapes and colours was chosen for each sample. An additional prey species was isolated later, it first formed a colony on an overlay plate being used for *Bdellovibrio* isolation, but its unusual red pigment was intriguing, hence it was also purified and used in the prey range analysis, although it was not used in the initial enrichments and isolation.

To identify the species isolated colony PCRs were done to amplify the 16S rRNA gene using the 8F and 1492-rev universal bacterial primers. This worked for 12 of the isolates, but did not work for the others after several attempts, hence genomic DNA was prepared for the remaining strains using liquid cultures in the MoBio Soil DNA extraction kit. DNA extracted this way resulted in PCR products for all but 2 of the isolates. These PCR products were then cleaned and sent for sequencing. The identification of these species is shown in Figure 4 (page 8).

The identification of some of the isolates as *Bacillus* provided a nice negative control, as these species are not susceptible to predation by *Bdellovibrio*.

Isolate 6.6, the red-pigmented isolate from Greater Sippewissett was found to be *Vibrio gazogenes*, and a TEM picture of the isolate, showing the capsule surrounding the cells, is shown in Figure 3 below (page 7).

4. *Bdellovibrio* Clone Libraries.

Four enrichments (described in section 5 below) were used in the preparation of *Bdellovibrio* clone libraries. The enrichments used were 1.4, 1.5, 6.2 and 6.3. These were chosen to allow pairwise comparisons between *Bdellovibrio* spp from clams and those from mussels along with comparisons between the prey used in the enrichments. The primers used were universal bacterial primers 8F and 1492-rev, along with the *Bdellovibrio* specific primers described in Snyder et al (Snyder et al, 2002). PCR products are shown in Table 1 (page 9) with the primers used. The products containing at least one of the *Bdellovibrio* primers were combined and used in the preparation of the clone libraries. 24 sequences were obtained for each sample, and the resulting neighbour joining trees are shown in Figures 5 and 6 (pages 9 and 10).

The addition of the PCR products obtained with the *Bdellovibrio* specific forward primer and the 1492-rev universal primer to the cloning reaction resulted in no *Bdellovibrio* sequences being obtained. This work would therefore need to be repeated using only the PCR products from the reactions containing both *Bdellovibrio*-specific primers.

5. *Bdellovibrio* enrichment and isolation.

Two methods of *Bdellovibrio* enrichment were used: double layer agar plating and liquid enrichments.

Double layer agar plates were made using a method similar to that used for plating phage. Sea water complete (SWC) agar plates were poured with an agar concentration of either 1 or 1.5%. A top layer was added to each containing 5ml of 0.6% SWC agar, 200ul of an overnight prey culture and 100ul of sample. This layer was allowed to set for between 5 and 10 minutes before the plate was turned over and placed in the 30ºC incubator, where they were incubatd for between 2 and 4 days, until zones of clearing appeared. Usually when trying to enrich for freshwater *Bdellovibrio* strains that predate on lab strains of *E. coli* or *Salmonella*, it takes a minimum of 7 days and an average of 10 days before any clearing can be observed. During these experiments I first noticed *Bdellovibrio* plaques appearing after 2 days of incubation and most appeared within 4 days. This suggests that enrichment of *Bdellovibrio* on prey isolated from the same environment results in more efficient isolation and enrichment than using lab strains, the majority of which would not be found in their natural environment.

Liquid enrichments contained 10ml of sterile sea water, 1ml of overnight prey culture, and 750ul of sample. These were incubated at 30ºC with shaking and analysed microscopically after a couple of days.

Liquid enrichments that showed obvious signs of *Bdellovibrio* (small, highly motile cells and bdelloplasts) were diluted and plated on overlay plates with the corresponding prey species.

Any zones of clearing that appeared were put into a 2ml "mini-lysate" containing 2ml of sterile sea water, 200ul of prey, and 100ul of sample. These were then monitored by microscopy for signs of *Bdellovibrio* activity.

By the end of the three weeks I had 40 such "mini-lysates" most of which had definite signs of *Bdellovibrio* activity. Unfortunately the time-constraints of the project meant that these could not be purified any further, however after shipping them to my home lab, I intend to purify them into pure cultures.

These 40 "mini-lysates" were from a mixture of all six samples, with several representatives on different hosts from each. Once pure, the isolates will be identified using 16S rRNA sequencing to allow some analysis of *Bdellovibrio* diversity to be made. I attempted to PCR some of the plaques and "mini-lysates" where I could be fairly sure that they only contained one type of *Bdellovibrio* by boiling some of the plaque or lysate for five minutes, and then adding 2ul of this to the PCR reaction. However this did not yield any PCR products, which was probably due to the high salt concentration of the sea water and SWC media in which the cells were suspended.

6. Prey range analysis.

Prey range analysis was done on 9 nearly pure *Bdellovibrio* isolates that had been grown originally on plates, then the plaques picked into "mini-lysates", which although were not pure cultures should only contain one strain of *Bdellovibrio*. 50ul of each lysate was spot-plated onto an overlays of different hosts and the results are summarized in Figure 7 (page 11). Many of the lysates still contained other bacterial species which formed large colonies on the plates, making it difficult to analyse whether there was any predation occurring. Some plates however had obvious clearing of the prey lawn, therefore it can be concluded that the *Bdellovibrio* are able to predate on these species.

This experiment showed that the *Vibrio gazogenes* isolated from Greater Sippewissett was easily predated upon by the *Bdellovibrio* species isolated both from the same environment and from other marine environments. Thus the capsule observed with the TEM does not inhibit predation by *Bdellovibrio*. The proportionally greater number of *Bdellovibrio* isolates predating on this strain suggests that it would be an ideal prey to use in enriching for *Bdellovibrio* from marine environments in future experiments.

None of the enrichments on the *Bacillus* spp. yielded any positive signs of *Bdellovibrio*. Plaques appeared on one or two plates, but there were no *Bdellovibrio* when observed

microscopically, suggesting that the plaques were made as a result of phage infection. This reinforces the data that says that *Bdellovibrio* are unable to predate on cells with a Gram-positive type of cell wall.

From the limited data collected so far, there does not seem to be any obvious correlation between sampling site and prey preference. However, increased purity of the *Bdellovibrio* cultures and repetition of the prey range experiment may still determine a link between sampling site and prey preference.

7. Conclusions and Future Work.

Many different bacterial species can be isolated from a variety of marine environments using sea water complete agar plates. Many of these are susceptible to predation by *Bdellovibrio*, and using them to enrich and isolate *Bdellovibrio* is 3-5× quicker than using lab strains of *E. coli* and *Salmonella*.

Bdellovibrio belonging to different groups can be identified by using different sets of *Bdellovibrio* specific primers, giving an indication of the range of the diversity in the sample. However the combination of a *Bdellovibrio*-specific forward primer with a universal bacterial reverse primer results in PCR products that do not contain *Bdellovibrio* sequences. Therefore any clone libraries made that target *Bdellovibrio* must use both the forward and reverse specific primers.

Bdellovibrio can be enriched for and partially purified in a three-week project, but it is not possible to completely purify them due to the delay in growth on plates. However they can be partially purified to the state where they can be identified and (incomplete) prey range analysis performed.

No correlation between prey range and sample site can be seen from the limited data collected - need pure cultures to retest in order to get accurate analysis. However the data so far suggests that *Vibrio gazogenes* is predated upon by the majority of the isolates tested so far, and that the capsules surrounding the cell do not prohibit infection by *Bdellovibrio*.

Future work to complete this project will include:-

- purification of the *Bdellovibrio* isolates and characterisation, both phylogenetically and by their morphology using Electron Microscopy, predation efficiency, including repetition of the prey-range experiment, and whole cell protein fingerprinting.

- continuing the isolation of *Bdellovibrio* from different environmental samples, using prey also isolated from the same environments, as a quicker and more efficient method of enrichment that conventional enrichments on lab strains.

- analysis of the predatory role of *Bdellovibrio* in the control of natural bacterial populations in varying environments including both soil and water, using both microbiological, genetical and mathematical techniques.

Acknowledgements

I would like to thank both the directors Bill Metcalf and Tom Schmidt; the faculty Jared Leadbetter and Jeanne Pointdexter; course coordinator Dion, and the TAs: Amaya, Mark, Laurie, and Adam for all the help and advice I have received throughout the summer.

Also I want to thank all the students of “Team MD 2005” for making the course so much fun, and for keeping me entertained through all the joint late nights in the lab – I couldn’t have done it without you!

I also need to thank all the organizations who have funded me to attend this course, including the Society for General Microbiology, The University of Nottingham, the Horace W. Stunkard Scholarship Fund, the S.O.Mast Memorial Fund and the Bernard Davis Fund.

Finally I want to thank Liz Sockett, my PhD supervisor, both for advising me to apply to the course, and helping me to raise the funds necessary to attend.

References.

Baer, M. L., Ravel, J., Chun, J., Hill, R. T., Williams, H. N. (2000). A proposal for the reclassification of *Bdellovibrio stolpii* and *Bdellovibrio starrii* into a new genus,

Bacteriovorax gen. nov. as *Bacteriovorax stolpii* comb. nov. and *Bacteriovorax starrii* comb. nov., respectively. *International Journal of Systematic And Evolutionary Microbiology* **50**, 219-224.

Baer, M. L., Ravel, J., Pineiro, S.A., Guether-Borg, D., Williams, H. N. (2004). Reclassification of salt-water bdellovibrio sp. As Bacteriovorax marinus sp. nov. and Bacteriovorax litoralis sp. nov. *International Journal of Systematic And Evolutionary Microbiology* **54**, 1011-1106.

Davidov, Y. & Jurkevitch, E. (2004). Diversity and evolution of Bdellovibrio-and-like-organisms (BALOs), reclassification of bacteriovorax starrii as Peredibacter starrii gen. Nov., comb. nov., and description of the Bacteriovorax-Peredibacter clade as Bacteriovoracaceae fam. nov. *International Journal of Systematic And Evolutionary Microbiology* **54**, 1439-1452.

Snyder, A.R., Williams, H. N., Baer, M. L., Walker, K.E., Stine, O.C. (2002). 16S rDNA sequence analysis of environmental bdellovibrio-and-like organisms (BALO) reveals extensive diversity. *International Journal of Systematic And Evolutionary Microbiology* **52**, 2089-2094.

Sockett, R.E. & Lambert, C. (2004). Bdellovibrio as therapeutic agents: a predatory renaissance? *Nature Reviews Microbiology* **2**(8),669-75

Stolp, H. & Starr, M. P. (1963). *Bdellovibrio bacteriovorus* gen. Et sp. N., a predatory, ectoparasitic, and bacteriolytic microorganism. *Antonie van Leeuwenhoek Journal of Microbiology and Seriology* **29**, 217-248.

Woese, C. R. (1987). Bacterial Evolution. *Microbiological Reviews* **51**, 221-271.

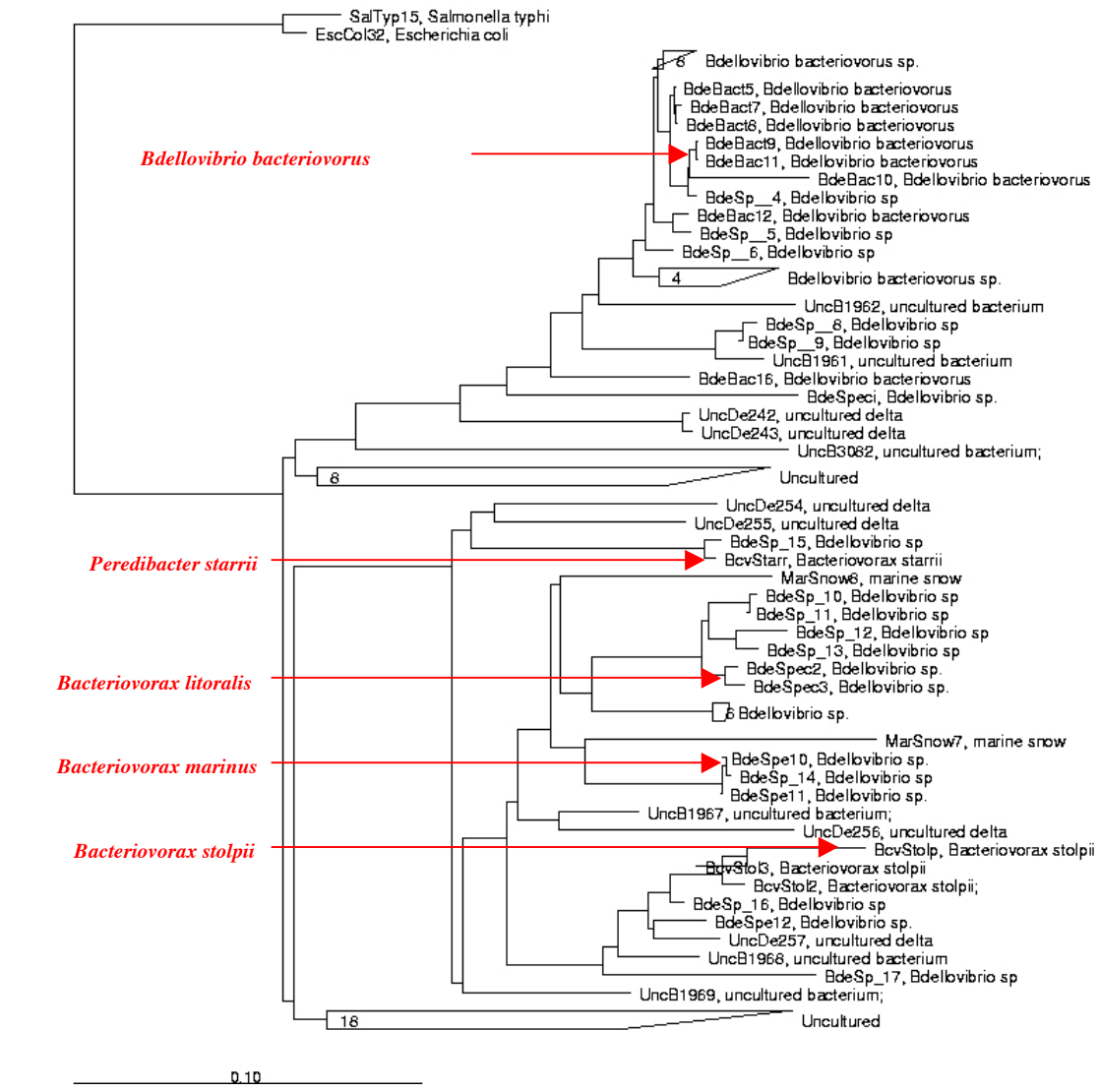

Figure 1: Neighbour joining tree of *Bdellovibrio* sequences present in the current ARB database (as at August 2005), produced using *E. coli* and *Salmonella typhi* as an outgroup.

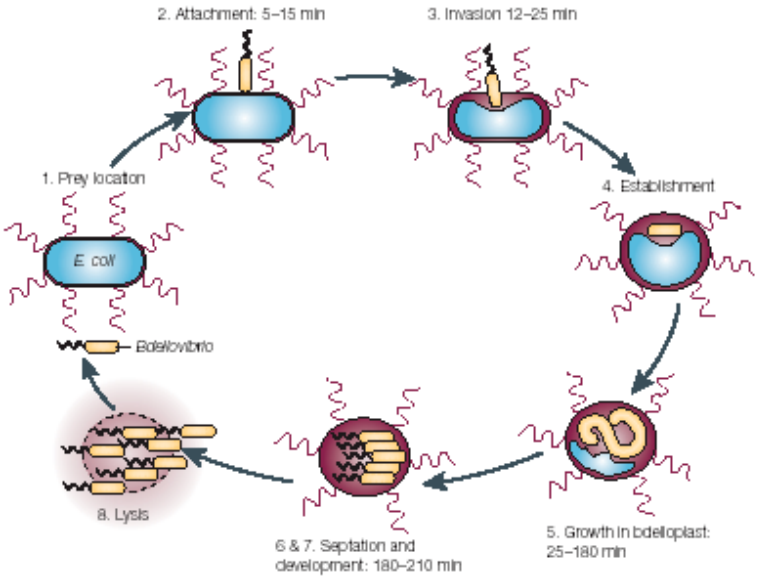

Figure 2: Life cycle of *Bdellovibrio bacteriovorus*. (Reproduced from Sockett & Lambert, 2004)

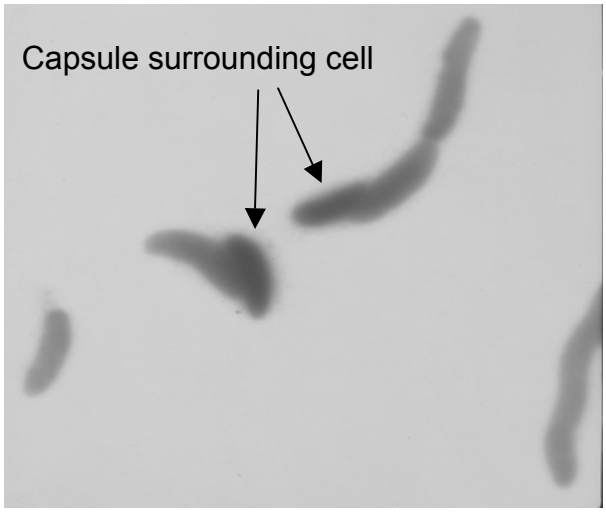

Figure 3: TEM of isolate 6.6, related to *Vibrio gazogenes*.

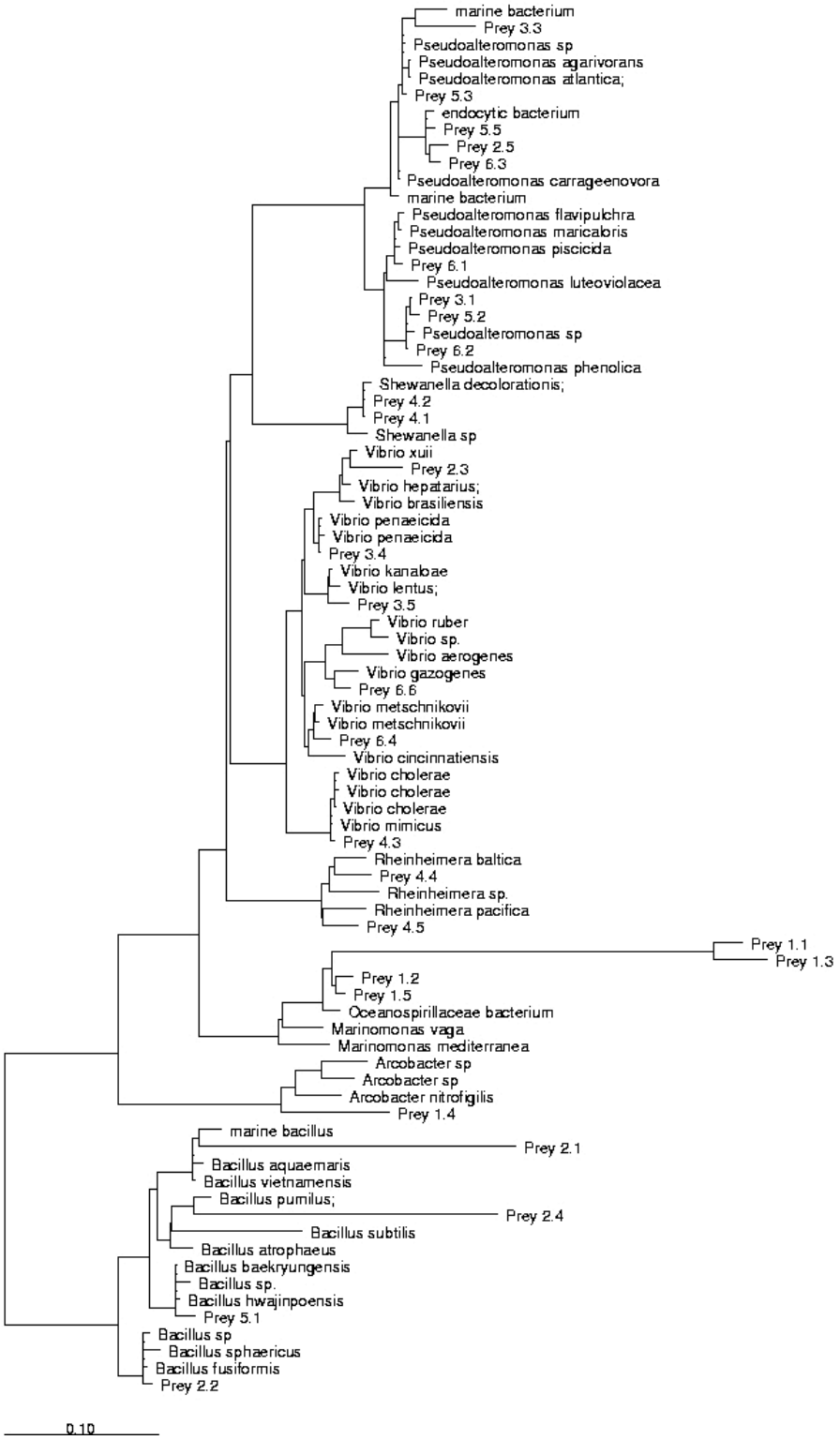

Figure 4: Phylogenetic tree of the partial 16S rRNA sequences of the isolated prey used in this study. (Produced using neighbour joining method in Arb.)

Forward primer	Reverse primer	Clams Prey1.4	Clams Prey1.5	G.Sipp Prey 6.2	G.Sipp Prey6.3
8F	1492rev	✔	✔	✔	✔
Bdello	1492rev	✔	✔	✔	✔
Bdello	bact	✘	✘	✘	✔
Bdello	starrii	✔	✔	✔	✔
Bdello	stolpii	✔	✔	✔	✘
Bdello	saltwater	✘	✘	✘	✘

Table 1: *Bdellovibrio* PCR primer combinations and positive PCR results from liquid enrichments from Clams and Mussels.

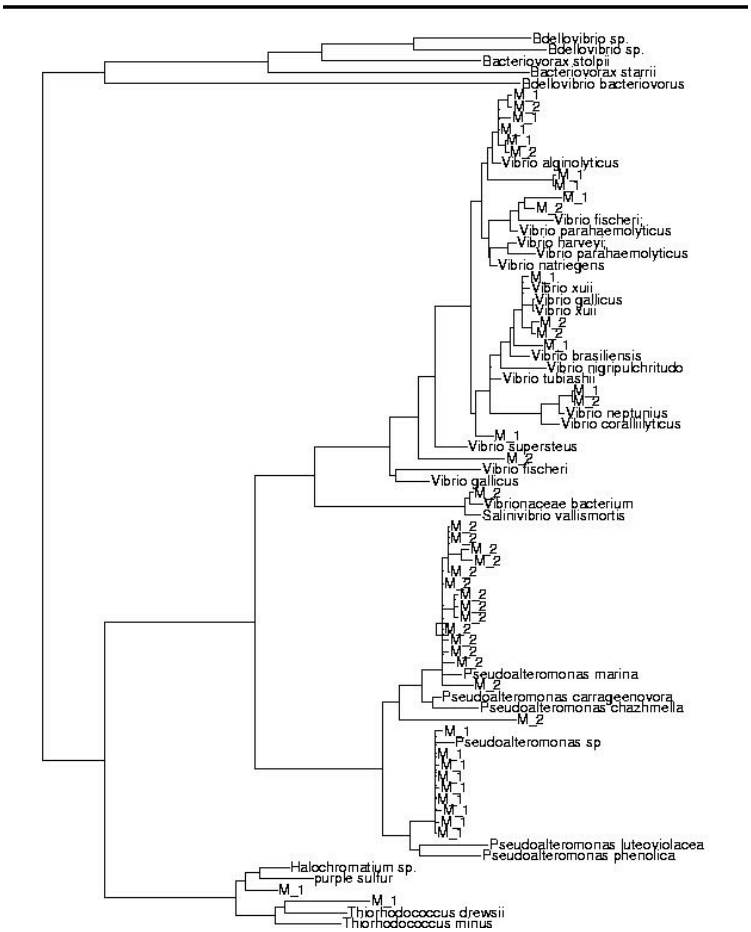

Figure 5: Clone Library obtained from the liquid enrichments from the mussels from Greater Sippewissett. M_1 are clones from the enrichment with prey 6.2, M_2 are clones from enrichment containing prey 6.3.

Figure 6: Clone Library obtained from the liquid enrichments from the decomposing clams on the sea table. C_1 are clones from the enrichment with prey 1.4, C_2 are clones from enrichment containing prey 1.5.

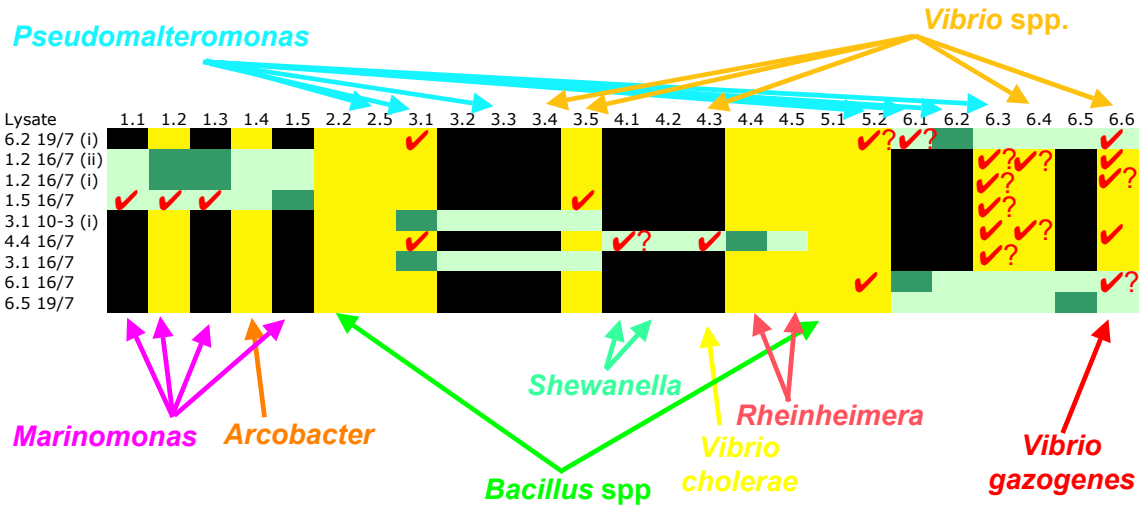

Figure 7: Prey range of nine potentially different *Bdellovibrio* isolates. Pale green cells indicate prey from same environment as *Bdellovibrio* isolate, dark green is prey *Bdellovibrio* originally isolated on, yellow are others tested. Ticks indicate obvious clearing of the prey lawn, ticks with question mark indicate that some clearing could be seen, but whether or not this can be attributed to the *Bdellovibrio* was unclear. The lack of ticks in most boxes does not indicate that no predation occurred, just that there were too many other bacterial species in the isolate for clearing to be observed. The absence of any clearing of the lawn of the *Bacillus* isolates 2.2 and 5.1 was as expected, as *Bdellovibrio* are unable to predate on *Bacillus*.