

Microbiological & Chemical Analysis of Bottles from the SS *Republic*

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Between October 2003 and November 2004, Odyssey Marine Exploration surveyed and excavated the shipwreck of the sidewheel steamer SS *Republic*, lost at a depth of approximately 500m in the Atlantic Ocean and over 150km off the southeastern coast of the United States. The *Republic* was traveling from New York to New Orleans with passengers and a composite commercial and monetary cargo when she foundered during a hurricane on 25 October 1865.

Particularly conspicuous within the diverse cargo were 8,429 glass and stoneware bottles once stored in the ship's aft and forward cargo holds (59% of all artifacts recovered from the *Republic*). These included the largest collection of medicinal 'cures', ink bottles and inkstands, food products, beauty products and alcoholic beverages found on an American shipwreck. The majority of bottles no longer contain their original contents, yet scientific analyses of a sample of bottles with intact contents has revealed bacterial and archaeal structural genes. Previously unknown phylogenies within the bacterial branch of the tree of life found in one bottle containing probable currants.

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1. Introduction

In late 2005, Odyssey Marine Exploration submitted a series of organic samples to the Department of Biomedical Sciences at Florida State University's College of Medicine, Tallahassee, Florida, for scientific evaluation. The samples consisted of organic remains preserved in six bottles recovered from the wreck of the sidewheel steamship *Republic*, lost during a hurricane in 1865 at a depth of about 500m, approximately 150km off the coast of Georgia. Each glass bottle still featured its original seal and stoppers intact and/or held residue suggestive of its original content.

The objective of the analysis of the bottles was to attempt to identify the contents and characterize the effects of low temperature, high pressure, and low light on those contents during 140 years of submersion in a deep-ocean environment. One simple question in the analysis was whether the original contents, both chemical formulations as well as preserved fruits, might have survived substantially in unchanged form from the time the ship sank.

The current report describes in scientific terms the types of bacteria found in sample bottles, indications of original contents, and an approximation of the physical processes affecting the samples within the various bottles. None of the original contents proved to be preserved intact, either due to seawater intrusion in at least two of the samples or consequent to microbial activity in the better-sealed samples.

However, the research found that in several samples, unidentified microbes (both bacteria and archaea) had developed. In one well-sealed container, apparently holding preserved currants (Sample E, R-03-00357-BE), most of the microorganisms present proved to be of an unknown, unidentified species, most likely even constituting new genera, families, or orders within the bacterial branch of the tree of life. Further study must confirm these results, but if they are sustained, a new group of previously undiscovered bacteria has been identified with the lost artifacts of the SS *Republic*.

2. Scientific Summary

All cells on earth are divided into two categories: prokaryotic cells and eukaryotic cells. Higher organisms are composed of eukaryotic cell types. Microorganisms, like bacteria and archaea, are prokaryotes. Hence, the tree of life as currently presented by science has three main domains: eukaryotes, bacteria, and archaea. Eukaryotes include plants, animals, fungi and protists.

This research focuses on the prokaryotes – the bacterial and archaeal domains. The eukaryotic domain in the samples presented here (mostly food items) is primarily responsible for providing a source of carbon to the bacteria and archaea present. For the purposes of following this report sample by sample, it is important to note that

archaea are subdivided into three main lineages: *Crenarchaeota*, *Euryarchaeota* and the more newly defined *Korarchaeota*, for which little information is currently available.

The following data were obtained using a 16S rRNA method for analysis of bacterial and archaeal structural genes (Wiesburg *et al.*, 1991). Phylogenetic trees were constructed to present a clear visual relationship between the clone sequences identified in these samples and known prokaryotic groups. Some chemical analyses were also performed for each bottle in an attempt to better characterize the prokaryotic communities in these samples, and whenever possible, their sources.

All six bottles were analyzed for the presence of both bacteria and archaea. Only two bottles were positively identified as containing archaea, while all six bottles were found to contain various types of bacterial cells. Bacteria are fairly ubiquitous and very adaptable to their surroundings. Therefore, it was expected that all samples would contain several different types of bacteria.

While some mesophilic archaea do exist, archaea are typically thought of as existing and thriving primarily in extreme environments. Cold temperatures like those surrounding the *Republic* shipwreck are considered extreme, so it was believed that archaea would be present. Although the contents of the bottles have not been scientifically confirmed, it is likely that the chemical composition of the food items (or other) present has been providing resident microorganisms with a continued energy supply for the past 140 years at the bottom of the ocean.

3. Scientific Results

1. Sample A (R-04-00245-BE; Fig. 1).

Deep brown glass bottle used for beer, lager or ale; its dark and dense glass, often referred to as 'black glass', served to strengthen the container and reduce breakage. H. 20.3cm, base diam 7.1cm, outer mouth diam. 2.7cm.¹

Both bacteria and archaea were found in sample A. Many bacteria found in sample A were most closely related to a common marine bacterium, *Halomonas*, that is an extreme halophile (salt-loving) and often psychrophilic (cold-loving). Chemical analysis concluded that sample A contains extremely high concentrations of chloride (salinity ~87 ppt; seawater ~35 ppt), making this the perfect environment for a metabolically-versatile halophilic bacterium like *Halomonas*. A few clone sequences were found to be closely related to *Rhodospirillaceae*, which is also known to be metabolically versatile. Typically, members of this group are capable of growing photosynthetically in light and aerobically in the dark (by oxidative phosphorylation).

However, other methods of growth are known, and increasing knowledge of mutants broadly expands our understanding of their pool of versatile metabolic capabilities.

There were two other less well-defined bacterial groups from sample A, an unknown *Bacteroidetes* and an unknown member of the Gamma *Proteobacteria*. In marine waters, *Bacteroidetes* are only second in abundance to *Proteobacteria*. Some classes of *Bacteroidetes* are known to be psychrophilic. During the past decade, members of *Bacteroidetes* have also been found in hypersaline environments, such as the media found in sample A. Both *Bacteroidetes* and Gamma *Proteobacteria* groups contain numerous enteric bacteria; so it was difficult to determine whether these clone sequences derive from human contamination or seawater intrusion. Because of the results from chemical analyses, it is unlikely that a great deal of seawater has intruded on sample A, although it is possible that enough seawater seeped in to allow certain adaptable bacteria to grow in this rather harsh environment.

Archaea from sample A were an extremely interesting group in that they all derived from the kingdom *Crenarchaeota* and clustered tightly together with no known close relatives. This means that all of the clone sequences were closely related to each other, but they were not closely related to any other previously identified organisms. Previously, *Crenarchaeota* were known only as thermophilic microbes, meaning they were able to grow in extremely hot environments. Recently, psychrophilic *Crenarchaeota* have been identified using 16S rRNA methods, as in this study (Karr *et al.*, 2006; Murray and Grzymski, 2007; Perrault *et al.*, 2007). However, none of these microbes have been cultured to date.

2. Sample B (R-03-00432-BE; Figs. 3-4).

An aqua colored glass bottle embossed with the product name 'Phalon and Son's Chemical Hair Invigorator' manufactured by New York City's Phalon & Son's Perfumery, a 19th-century firm that produced a line of toilet goods, including perfumes, hair restoratives and hair dyes. H. 17.3cm, base diam. 7.0cm, outer mouth diam. 2.8cm.

The clear liquid inside had an extremely potent smell reminiscent of a petroleum byproduct and may have contained methanol, as many species from the bacterial genus *Paracoccus* are capable of using methanol as a carbon source. The only other group of bacteria in this sample was *Clostridium*, a gram-positive bacterium with versatile metabolic capabilities. This poses some confusion over the chemistry of the sample in the bottle, being that *Paracoccus* is obligatorily aerobic, while *Clostridium* is obligatorily anaerobic. Part of this sample was very oily, while the remaining liquid was more hydrophilic in nature, so it is



Fig. 1. A deep brown glass bottle (R-0400245-BE) intended for beer, lager or ale. H. 20.3cm.



Fig. 2. A small, barrel-shaped clear glass 'mustard barrel' jar (R-04-01417-BE). H. 12.5cm.



Fig. 3. An aqua-colored glass bottle (R-03-00432-BE) embossed with the product name Phalon & Son's Chemical Hair Invigorator. H. 17.3cm.



Fig. 4. A Phalon and Son's Chemical Hair Invigorator bottle being recovered from the wreck of the Republic using a limpet suction device.

possible that aerobic and anaerobic zones exist within the same sample. There were no archaea in sample B.

3. Sample C (R-04-01417-BE; Fig. 2).

A squat and round, barrel-shaped clear glass jar, referred to as a mustard barrel, its distinctive shape used to store both dry and prepared mustard. H. 12.5cm, base diam. 5.1cm, outer mouth diam. 5.0cm.

When the analyses were initiated the cork was found to have fallen in on the sample. The preservative state of the jar at the time of recovery is unknown, so it was difficult to determine whether the contents of this jar were little more than marine sediment and seawater. The latter is very likely the case since the clone sequences present were primarily from the same group of common marine bacteria, *Halomonas* (as in Sample A). The only other bacterial genus present was *Idiomarina*.

Both genera are known for being halophilic, and chemical analysis determined that sample C had the second highest salinity of all six bottles (approximately 47 ppt). There were archaea present in this sample, but it should be noted that archaea can be easily found in cold marine sediments. Archaea from sample C are found primarily (all but one) in the *Euryarchaeota* kingdom, which is known for its members being extreme halophiles. If this sample once contained either dry or prepared mustard, its high salt content may have accounted for the presence of extreme halophiles in this sample.

The three groups of archaea present included organisms distantly related to the *Picrophilus* genus, the *Methanoculleus* genus, and the same unknown group of archaea as seen in Sample A. *Picrophilus* is known for growing at extremely low pHs, while *Methanoculleus* is a methanogen known for producing methane as a byproduct while using carbon dioxide and hydrogen gas for growth. Because sample C had a pH of approximately 8.0 (near seawater), it is not likely that the clones from this sample have much in common with *Picrophilus*. However, it would not have been surprising to find methanogens in this sample if the cork had remained intact and all of the oxygen in the bottle had been used during the 140-year period post-dating the act of wreckage (because methanogens are strictly anaerobic microorganisms).

4. Sample D (R-03-00241-BE; Fig. 5).

A tall, aqua colored glass preserve bottle with a distinctive long cylindrical neck and rounded shoulders. Its cork is fairly intact and the bottle appears to contain a foodstuff resembling chunks of rhubarb, possibly intended as pie filling. There is a dark black precipitate in the bottle and a smell that indicates the presence of sulfides. H. 29.9cm,

base diam. 7.5cm, outer mouth diam. 5.0cm.

The bacterial classes represented in this bottle included *acteroidetes*, *Spirochaetes*, *Clostridia*, and Delta *Proteobacteria* that were most likely of the *Desulfuromusa* genus. This grouping of microbes was very interesting because it indicated that this sample was most likely anaerobic (little to no oxygen present in the bottle). While only a few *Bacteroidetes* are known to function anaerobically, *Clostridia* obligatorily rely on anaerobic energy metabolism, and have been known to enter into co-cultures with anaerobic *Spirochaetes* during the degradation of cellulose to cellobiose (Pohlschroeder *et al.*, 1994).

It has even been reported that the presence of the *Spirochaetes* increases the depolymerization rate of cellulose by some species of *Clostridia*. Some *Spirochaetes* are also known to reduce thiosulfate and elemental sulfur to sulfide (H₂S), which may have accounted for the dark black precipitate in this bottle. *Desulfuromusa* is also an obligate anaerobe, requiring conditions with little to no oxygen leading to a low redox potential (highly reducing environment), with the ability to reduce elemental sulfur to sulfide.

Within the class *Clostridia*, a genus with similar characteristics to the genus *Clostridia* exists – *Acetobacterium*. The difference between these two genera for the most part is their ability to form spores. Several clone sequences were closely related to *Acetobacterium bakkii* with high similarity ranks, a psychrophilic acetogenic bacterium with the ability to produce acetate from H₂ and CO₂, which are often produced during respiratory reactions of other bacterial groups.

5. Sample E (R-03-00357-BE; Figs. 6, 8).

A tall, aqua colored glass bottle with a distinctive long-neck and rounded shoulders known today as a preserve bottle, containing a fruit resembling currants, possibly intended as pie filling. The cork is intact and appears fairly stable. H. 30.2cm, base diam. 7.5cm, outer mouth diam. 4.8cm.

Chemical analysis identified the liquid inside as having a pH of 4.0, which is extremely low, making the contents of the bottle highly acidic. It seemed most likely that little or no seawater had infiltrated this bottle, as the pH of seawater is approximately 8.0. Salinity in this sample was approximately 30 ppt, just slightly lower than seawater. Low similarity ranks for nearly all clone sequences from this sample indicated that most of the microorganisms present were unknown, unidentified species, most likely even constituting new genera, families, or orders within the bacterial branch of the tree of life.

The majority of the clone sequences had some distant relationship to *Sulfospirillum*, which is known to be pH-tolerant, and reduces elemental sulfur to H₂S,



Fig. 5. An aqua-colored glass preserve bottle (R-0300241-BE) containing chunks of rhubarb; cork partly intact (and covered with a modern plastic cap for preservation). H. 29.9cm.



Fig. 6. An aqua-colored glass preserve bottle (R-03-00357-BE) containing fruit resembling currants, with its original cork intact (and secured with a modern plastic cap). H. 30.2cm.



Fig. 7. An aqua-colored glass preserve bottle with intact cork (R-03-00066-BE) and contents that appear to be gooseberries. H. 29.8cm.



Fig. 8. Currant bottle R-03-00357-BE being recovered from the wreck of the Republic using a limpet suction device. A curious marine growth is attached to the mouth.



Fig. 9. Gooseberry bottle R-03-00066-BE being recovered from the wreck of the Republic using a limpet suction device.

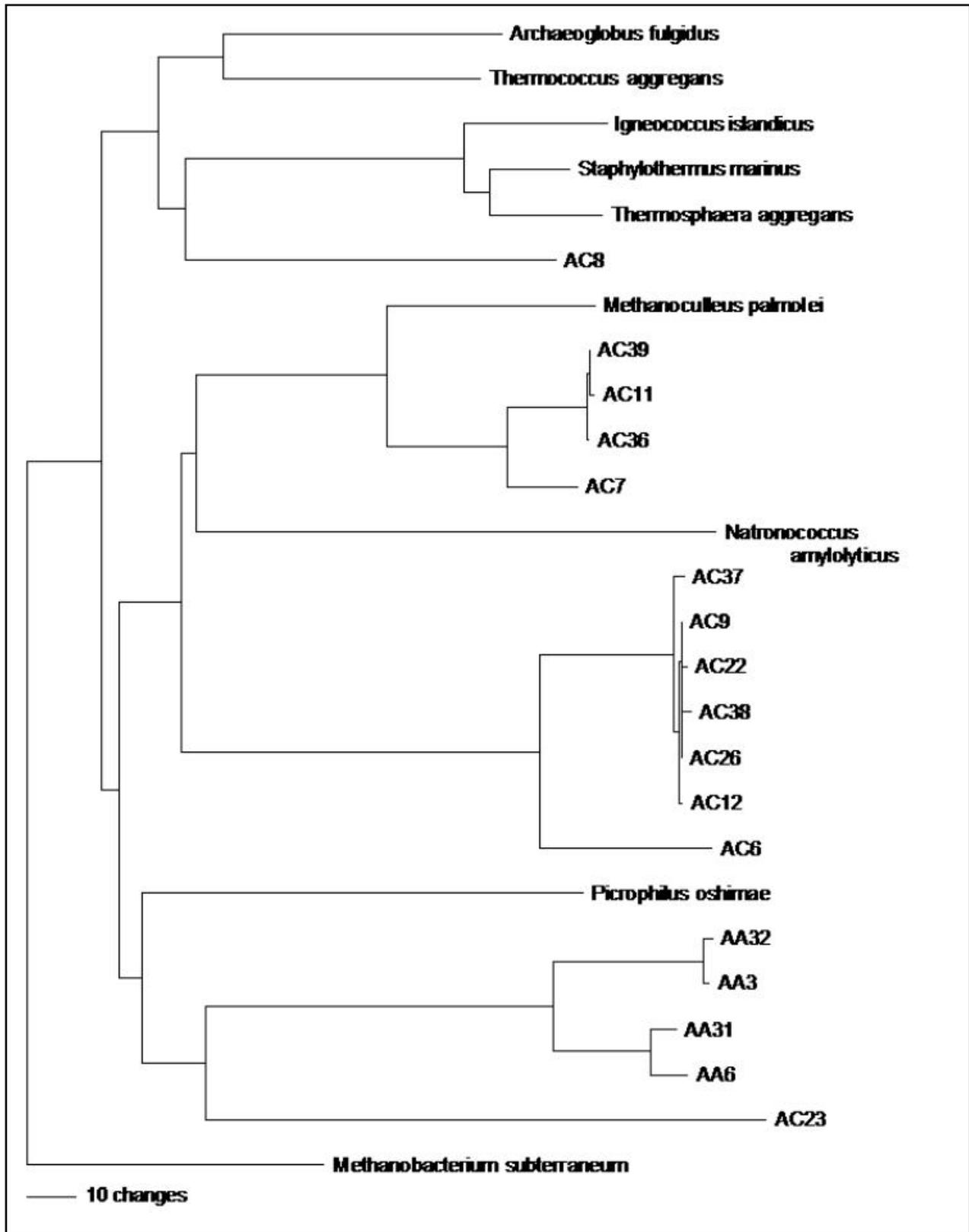


Fig. 10. Phylogenetic tree showing the relationships between archaeal clone sequences found in the six bottles described and known archaeal strains. Clone sequences are designated with two letters: A for Archaea, while the second letter is the sample bottle identification number used in this report. Letters are followed by a number representing the clone sequence for identification purposes.

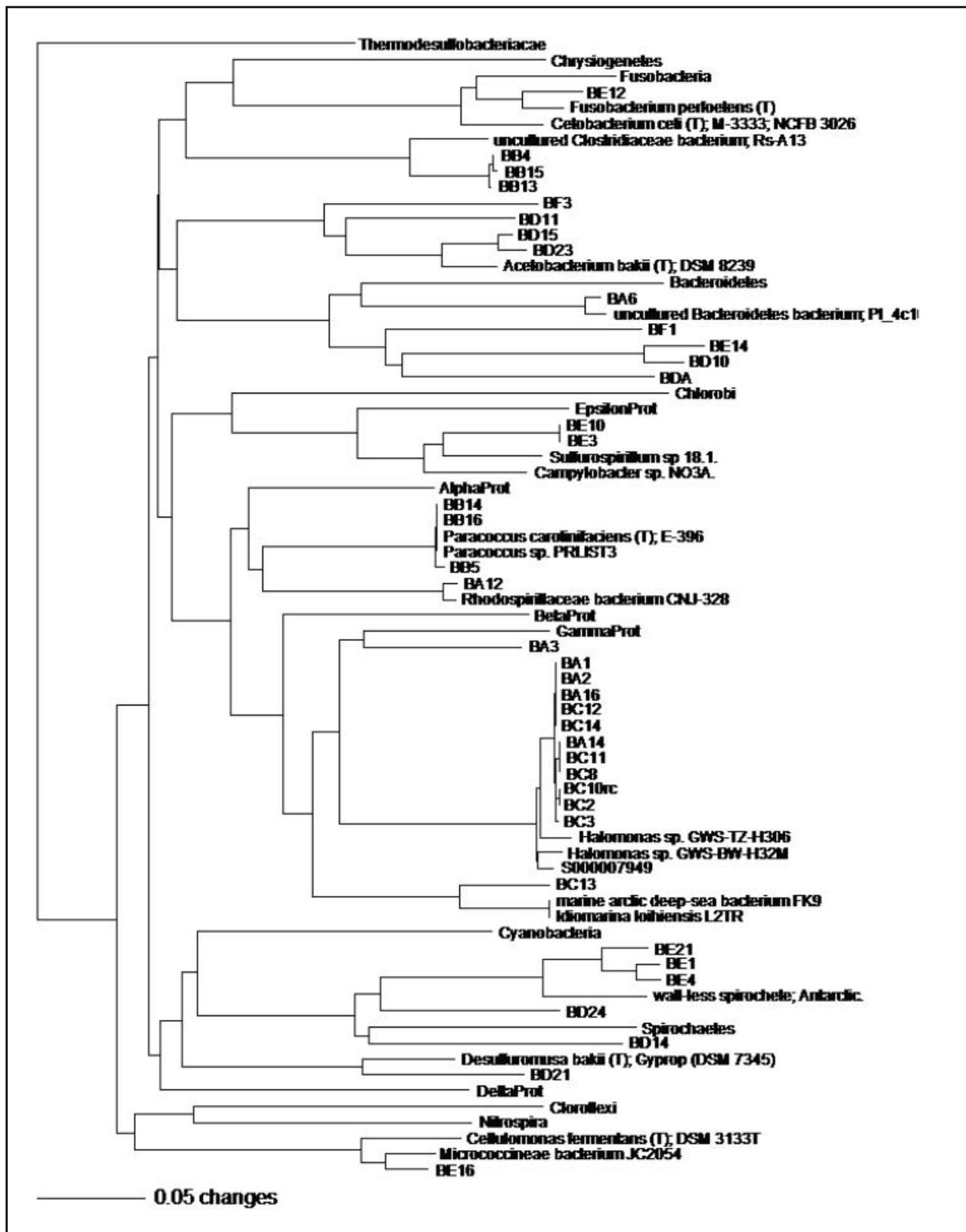


Fig. 11. Phylogenetic tree showing the relationships between bacterial clone sequences found in the six bottles and known bacterial strains. Clone sequences are designated with two letters: B for Bacteria, while the second letter is the sample bottle identification number used in this report. Letters are followed by a number representing the clone sequence for identification purposes.

while incompletely oxidizing an organic substrate (some form of carbon from the currants) to acetate. There was no black precipitate in the bottle and very little smell of sulfide, but this may be due to the low pH. Five clone sequences were located within the *Spirochaete* group, as previously discussed, and these organisms were likely reducing elemental sulfur as well at this low pH.

Two clone sequences were related to *Fusobacterium perfoetens*, which is known for being particularly sensitive to the presence of oxygen. The low pH in this sample was a good indication that some fermentation of glucose has occurred by *Fusobacterium* species. All *Fusobacterium* species are parasites of humans and animals, so it is difficult to determine from where these microbes have derived. In any case, the microbial community composition within this bottle was indicative of an anaerobic environment with a moderately low redox potential.

6. Sample F (R-03-00066-BE; Figs. 7,9).

A tall, aqua colored glass preserve bottle with a long cylindrical neck and rounded shoulders. The cork was well-secured and the bottle contained what appeared to be gooseberries, possibly intended as pie filling. H. 29.8cm, base diam. 7.4cm, outer mouth diam. 4.3cm.²

Clone sequences from this sample were only distantly related to currently known classifications. The highest similarity rank found was 0.6 for *Acetobacterium* (with 0 being the lowest, and 1.0 being the highest rank) again indicating the possibility that some acetogenesis had occurred ($H_2 + CO_2 \rightarrow \text{acetate}$). There were also Spirochaete-like organisms in this sample, but the similarity ranks were so low they could not be included in this tree.

The final group identified from this sample derived from the *Bacteroidetes* group, as previously seen in samples A, D and E. Chemistry in this sample showed a slightly higher chloride content than seawater, but there was no sulfate present. Seawater is usually high in sulfate content, so it was likely that little or no seawater intrusion occurred in Sample F either.

4. Conclusion

In conclusion, at least four out of the six bottles analyzed were surprisingly well-sealed after 140 years at the bottom of the ocean, with little to no seawater intrusion. Growth temperatures of 4°C allowed for the growth of some interesting bacteria and archaea, many of which are adapted to living in extreme environments.

It is possible that some of the clone sequences retrieved from these samples were true psychrophiles (preferring cold temperatures), while others were mesophilic (preferring

warmer temperatures, but not hot), yet capable of growing at colder temperatures. There was a variety of metabolic capabilities observed from these samples, but, as expected, the microbial diversity was limited due to temperature and the presence of limited electron donors/acceptors within these bottles.

Acknowledgements

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Notes

1. Bottle descriptions in this report are by Ellen Gerth, and dimensions and photographs of the bottles taken on land are by Chad Morris, both of Odyssey Marine Exploration.
2. The dimensions of Sample F (R-03-00066-BE) and photographs of this gooseberry bottle are actually derived from a different, though typologically identical, bottle, R-04-02918-BE-001.

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