

Biological diversity in prokaryotes: PCR amplification of the 16S rRNA
sequence in a bacterium isolated from seawater

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Introduction

Prokaryotes are everywhere. By conservative estimations, there are between 10,000 and 10,000,000 prokaryote species on the planet today. These organisms constitute a significant portion of world organic carbon. There are approximately $4-6 \times 10^3$ prokaryotic cells in various environments on earth, together containing 350-550 Pg of carbon. This is 60-100% of the carbon found in terrestrial and aquatic plants! Prokaryotes also compose a large fraction of the organic nitrogen and phosphorous on earth. Altogether, the prokaryotes may contain ten times more nitrogen and phosphorous than higher plants. This constitutes the largest pool of nitrogen and phosphorous found in living organisms. Few of these species have been studied (Whitman 1998); only about 5,000 non-eukaryotic species have been described by biologists. The metabolic pathways of these organisms and their ecological organisms are also poorly understood (Pace 1997).

Although they are abundant, prokaryotes were discovered only 300 years ago. Even after their discovery, little progress was made in studying them because the classical methods for studying metazoa could not be used. Original attempts at classification of prokaryotes were done by morphology, but this proved impractical because of their uniformity in shape (bacterium, spirillum, and coccus). In the late 1800s, pure-culture techniques were developed to grow a single species of bacteria. This helped microbiologists study the nutrition of prokaryotes, so that they were then classified by their metabolism. However, both nutrition and morphology proved to be poor bases for classifying prokaryotes because neither created a taxonomy founded on evolutionary relationships (Pace 1997).

Despite their prosaic forms, prokaryotes exhibit a large degree of biological diversity. Because they are asexual and haploid, the major source of genetic diversity in bacteria comes not

from meiosis but from genetic mutations. Although the likelihood of a significant genetic mutation in one individual may seem small, the size and productivity of bacterial communities greatly increases their probability. In marine environments, where productivity is high and cell density averages 5×10^5 cells/ml, significant genetic events may occur with some frequency. It has been calculated that if 4×10^{-7} mutations occur per gene per DNA replication, four simultaneous mutations would occur every 0.4 hours in marine heterotrophs and every 0.5 hours in marine autotrophs (Whitman 1998).

It has only been within the past 25 years that microbiologists have found reliable methods for judging evolutionary relationships in prokaryotes. It was discovered that if common DNA sequences were compared in closely-related prokaryote species, the degree of difference between the strands would give an indication of the amount of change undergone by each species from a shared evolutionary ancestor. However, a sequence that was useful in assessing evolutionary change needed not only to be common, but also to be large enough to measure and have variations occur somewhat randomly. Microbiologists found these characteristics realized in the 16S rRNA sequence. 16S rRNA is useful because it is large with many domains, and has many positions that vary at different rates. Also, this RNA can be sequenced by the reverse transcriptase enzyme, which makes it relatively easy to study (Woese 1987).

Materials and Methods

The first step of the experiment was to set up a dilution series of the bacterial source, salt marsh seawater. Ten test tubes were filled with 9ml sterile seawater, and labeled 10^{-1} through 10^{-10} . The first tube of the series, 10^{-1} , was inoculated with 1ml of salt marsh seawater and then vortexed. From this dilution, 1ml was transferred to the second tube, 10^{-2} , which was then

also vortexed. This pattern was repeated on the remaining test tubes in the series, ending with $1e^{-10}$. A fresh sterile pipette was used in each transfer to prevent contamination.

After the dilution series was set up, ten agar plates were obtained and labeled to match the test tubes. A 0.5ml sample was taken from the last tube, $1e^{-10}$, and spread over the surface of its matching agar plate. This was repeated for each test tube-agar plate pair in the series. Because the transfers were made up the gradient of the series, with bacterial concentration increasing 10x between each test tube, the risk of contamination was low, allowing the same pipette to be used in each transfer. The edges of each plate were then wrapped in paraffin film (to prevent desiccation of the bacteria), and transferred to an incubator set at 25°C.

After three days of incubation, the agar plates were removed from the incubator and examined. Two of these plates (the $1e^{-1}$ and $1e^{-2}$ dilutions) were found to have discernible, well-separated colonies, and were selected for further isolation. A sample from each of the two plates was streaked on a new agar plate according to the method presented in the lab manual. All reasonable measures to reduce contamination of the isolate were performed.

After another four days of incubation, the new plates were examined. Samples from these were then restreaked onto two fresh agar plates using the above method, and allowed to incubate for three days. However, when the second isolates were examined, the cultures they contained did not appear pure, so the culturing process was repeated to yield a third set of isolates.

Allowing three more days of incubation, the third set of isolates were examined and found to be suitable for extraction. A sample of cells was taken from each plate and introduced into a microcentrifuge tube already containing 500µl of sterile water. These two tubes were

vortexed until the cell samples were dispersed, and then placed into a boiling water bath for 10 minutes. Afterwards, the tubes were centrifuged at 13,000 rpm for an additional 10 minutes.

The centrifugation of the samples produced a supernatant, which was then transferred by pipette to two fresh microcentrifuge tubes. The new tubes were labeled A and B, corresponding to the $1e^{-1}$ and $1e^{-2}$ dilutions. These DNA preparations were stored in the lab freezer. Next, three new microcentrifuge tubes were obtained, each already containing a bead of reaction mix. Two of the new reaction tubes were labeled 'a' and 'b.' A third reaction tube, used as a negative control, was labeled '-'. All three of the tubes were inoculated with 23 μ l of distilled water. To 'a,' 1 μ l of supernatant from tube A was added. Similarly, 1 μ l of supernatant from B was added to 'b.' An additional microliter of distilled water was added to the negative control. Before the reaction tubes were put into the thermal cycler, each one was given 1 μ l of forward primer and 1 μ l of reverse primer, and then vortexed. The samples were allowed to cycle 35 times, and then stored in the lab refrigerator.

To prepare the products for gel electrophoresis, 7 μ l from each tube were mixed with 1 μ l of blue loading dye. The mixtures were then loaded in series into the wells of the agarose gel. When all the samples were loaded, a last well was loaded with 5 μ l of DNA marker. Finally, a current was applied across the gel, and the samples were allowed to electrophorese. When electrophoresis was complete, the samples were inspected under UV light, and the product from 'a' was determined to give the best band. In order to obtain the DNA sequence of the sample, the rest of the PCR product from 'a' was submitted to the Molecular Genetics Instrumentation Facility at the University of Georgia.

Discussion and Conclusion

The BLAST search revealed many species that were closely related to our organism. To the 308 bases in our sequence, the most closely related species in GenBank matched 302 bases. This corresponds to a match of 98%. The top five matches were all *Vibrio* spp., suggesting that the organism we isolated is related to members of the *Vibrio* genus. Most of these were described as being marine bacteria. However, the locations where the bacteria were collected varied widely. One species was found in the Adriatic Sea, another was found in both the Northwest Pacific and Otsuchi Bay, Japan. The bacteria also varied in the habitats in which they were found. One species, the closest match, was found associated with leaves of the seagrass *Halophila stipulacea*, while another was found in marine sediment.

Other relatives of our organism also had interesting and varied lifestyles. Many of these were found as pathogens on other marine organisms. A close relative of our organism was found as a pathogen in the cultured flounder *Paralichthys olivaceus*. Another was found as a pathogen on the sea urchin *Strongylocentrotus intermedius*. Sometimes, the pathogenicity was on the larval or immature form of another organism. One close relative, with a 97% match, was found in egg capsule of the squid *Loligo pealei*. A similar species was found on the larvae of the cultured flounder mentioned above. Yet another *Vibrio* species was found associated with early great scallops, *Pecten maximus*, in a relationship that may have been pathogenic or mutualistic.

The phylogenetic tree we constructed also showed how our organism was related to well-studied microbial species. Of the six bacteria offered for comparison, our organism was most closely related to *Escherichia coli*. The next closest match was *Pseudomonas putida*. Following these were *Erythrobacter longus* and *Stappia stellulata* in one branch, and *Bacillus subtilis* and

Prochlorococcus marinus in another. However, the phylogenetic tree showed, as did the BLAST search, that the closest relatives of our organism were members of the *Vibrio* genus.

Our sequence was submitted to GenBank and the Sapelo Island Microbial Observatory. The accession number for GenBank was 462603. The isolate identification number assigned by SIMO was IS-S75-245.

The absence of an exact match of our sequence with one at GenBank suggests many possibilities. The first possibility, considering that related matches were high, is that we isolated a species that has already been submitted. In this case, an exact match might not have occurred because of corruption of the sequence, or because of the relatively short size of the fragment (~300 bases). Another possibility is that we isolated a new species that is very similar to species previously described. This is likely, considering the extreme diversity of prokaryotic species. As mentioned before, some areas of the 16S rRNA sequence are known to vary more rapidly than others. It is possible that we isolated a section in which 6-9 bases of sequence were altered in the evolution of our organism from an ancestor shared with other *Vibrio*. However, the evidence here is not substantial enough to make a decision one way or the other. If the experiment could be reconducted to analyze a larger portion of the 16S rRNA sequence, then perhaps a more satisfactory conclusion could be drawn.

Literature Cited

Pace, N. R. 1997. A molecular view of microbial diversity and the biosphere. *Science*. **276**: 734-740.

Whitman, W. B., D. C. Coleman, and W. J. Wiebe. 1998. Prokaryotes: the unseen majority. *Proceedings of the National Academy of Sciences*. *Proceedings of the National Academy of Sciences (USA)*. **95**: 6578-6583.

Woese, C. R. Bacterial evolution. 1987. *Microbiological Reviews*. **51**: 221-271.