The Isolation and Identification of Chemolithoautotrophic, Thiosulfate-Oxidizing Bacteria from the Deep-Sea Hydrothermal Vents of 9°N, East Pacific Rise

Adam Bohnert[1], Melitza Crespo-Medina[2], Costantino Vetriani[2]

[1] Rhodes College, 2000 N. Pkwy, Memphis, TN 38112

[2] Institute of Marine and Coastal Sciences, Rutgers University, 71 Dudley Rd., New Brunswick, NJ 08901

ABSTRACT

At deep-sea hydrothermal vents, chemolithoautotrophic bacteria play a central role in the primary production of organic carbon. In performing such a function in this extreme environment, these bacteria are thus largely responsible for sustaining deep-sea communities. Nevertheless, much pertaining to their diversity is still unknown. Therefore, given the significance of these deep-sea microorganisms as well as the mystery which still surrounds them, this study aimed to isolate, characterize, and identify actual chemolithoautotrophic bacteria from deep-sea hydrothermal vents located at 9°N, East Pacific Rise. By enriching for thiosulfate-oxidizers which can grow aerobically at 30°C, ten pure cultures were initially established. Ultimately, this set of pure cultures was shown to include three distinct species: *Halothiobacillus hydrothermalis*, *Thiomicrospira thermophila*, and *Thiomicrospira crunogena*.

INTRODUCTION

In the past, the deep-sea was commonly looked upon as a nutrient-poor, energy-lacking environment (Jeanthon, 2000). After all, at depths exceeding 2500 meters, deep-sea habitats are quite removed from the primary production which occurs via photosynthesis near surface waters (Ruby et al., 1981). However, with the recent discovery of invertebrate communities inhabiting areas alongside deep-sea hydrothermal vents, many prior notions concerning the deep-sea have been called into question and subsequently modified (Karl, 1995).

A vital element in this revised understanding came with the discovery of chemolithoautotrophic bacteria at these deep-sea vents. Instead of harnessing light energy, these bacteria utilize chemical energy obtained through the oxidation of inorganic compounds emitted in vent flumes to reduce CO_2 to organic carbon (Jannasch, 1995) (Fig. 1). Such living organic carbon, in turn, supports the macrofaunal communities adjacent to these vents and forms the base of vent food webs (Karl, 1995). Thus, in studying these bacteria, one not only has the potential to improve the current understanding of deep-sea microbial diversity, but one also has the potential to heighten knowledge regarding these deep-sea environments as a whole.

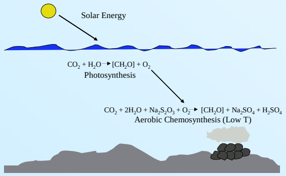

Figure 1: Pictured to the left is a visual representation of microbial chemosynthetic activity at a diffuse flow vent. In this case, the oxidation of thiosulfate produces energy which is in turn used to reduce CO_2 to organic carbon.

METHODS

Samples from 9°N, East Pacific Rise were initially obtained by the DSV *ALVIN*. Once pure cultures of thiosulfate-oxidizers were isolated from these samples and grown aerobically at 30°C on 142-A media, DNA was extracted and the 16S rRNA gene was amplified via PCR. RFLP screening was then utilized to determine the variety of species under study, and cloning subsequently was used to concentrate bulk quantities of DNA. Finally, sequencing was performed to identify distinct species.

RESULTS

1. Isolation of Pure Cultures

Ten pure cultures were isolated from the initial samples and assigned unique names (Fig. 2). All of these isolated cultures were acid-producing. Such a characterization was made clear by the fact that the media used in this study turned yellow when inoculated (Fig. 3). Furthermore, acridine orange staining (Fig. 4) of the isolated cultures indicated that all of them possessed rod-shaped cells (Fig. 2).

NAME	DIVE #	MORPHOLOGY
EPR85	4103B	Rod
EPR86	4103A	Rod
EPR94	4102	Rod
EPR95	4104A	Rod
EPR96	4104B	Rod
EPR97	4107A	Rod
EPR98	4107B	Rod
EPR99	4109A	Rod
EPR100	4109B	Rod
EPR101	4112	Rod

Figure 2: The table to the left reveals from which *ALVIN* dive each culture was obtained. For example, EPR94 was isolated from a sample taken on Dive 4102. Likewise, EPR85 and EPR86 were isolated from samples associated with Dive 4103. The "A" or "B" following the dive number is included in order to clarify that the relevant samples are from the same dive yet from different transfer dates (A=earliest transfer date; B=latest transfer date). Furthermore, one can also see from this table that all of the cultures exhibited rod-shaped cells.

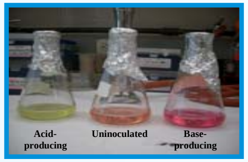

Figure 3: Unlike uninoculated 142-A media (which is orange) and media inoculated with base-producing bacteria (which is pink), the media inoculated in this study turned yellow and thus indicated that the cultures are acid-producing.

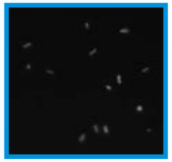

Figure 4: Above is an example of cells stained with acridine orange. The culture under examination is EPR98.

2. DNA Extraction and PCR

Sufficient amounts of DNA and PCR product were eventually obtained for all ten isolates. However, the strongest bands were typically seen for EPR85, EPR86, EPR96, EPR99, and EPR100 (Figs. 5 & 6). This is presumably because these cultures grew the best under the outlined conditions and thus provided the most significant amounts of biomass.

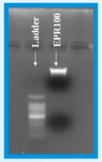

Figure 5: Above is an example of a strong band of DNA.

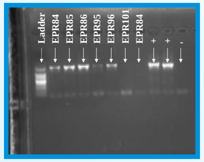

Figure 6: Above is an example of PCR products in a gel. In this case, the best bands belong to EPR85 and EPR86.

3. RFLP Screening

All ten cultures and two controls (EPR65 and EPR75) were subjected to RFLP screening. The ten unknown cultures showed five different band patterns, suggesting that they could then be divided into five groups (Fig. 7). Though Groups 3 and 4 were nearly identical in band pattern, they nevertheless were kept separate because the bands of Group 4 appeared to be slightly lower than those of Group 3.

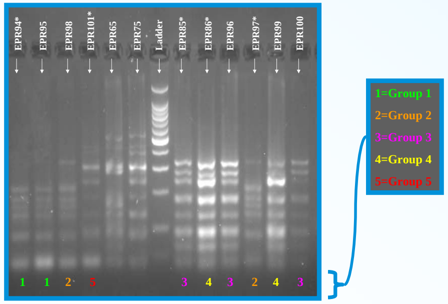

Figure 7: The ten cultures were separated into five groups based on the band patterns produced by RFLP. Asterisks next to culture names indicate which isolates were chosen as representatives for their respective groups.

4. Cloning and Sequencing

Cloning was successful for all five of the representative cultures. Once *E. coli* competent cells were transformed with appropriate ligation reactions and plated on LB agar, white colonies containing the inserted 16S rRNA gene were observed in each case (Fig. 8).

Figure 8: White colonies on these plates were selected because they possessed the inserted gene. Blue colonies represented background clones that did not contain the insert.

BLAST searches of the 16S rRNA gene sequences ultimately produced the following results (Fig. 9):

NAME	RFLP GROUP #	IDENTITY OF THE CLOSEST RELATED SPECIES	PERCENTAGE OF SIMILARITY
EPR94	1	*Halothiobacillus hydrothermalis*	99%
EPR97	2	*Halothiobacillus hydrothermalis*	99%
EPR85	3	*Thiomicrospira thermophila*	100%
EPR86	4	*Thiomicrospira thermophila*	99%
EPR101	5	*Thiomicrospira crunogena*	100%

Figure 9: BLAST searches specified that all of the sequences were >98% similar to the next closest species, thereby indicating that each culture could be considered the same species as its respective closest relative.

CONCLUSIONS & DISCUSSION

As the sequencing results clearly indicate (Fig. 9), the set of cultures investigated in this study includes the following three species: *Halothiobacillus hydrothermalis*, *Thiomicrospira thermophila*, and *Thiomicrospira crunogena*.

Though RFLP Groups 1 and 2 were initially separated following analysis of their band patterns, closer observations reveal that their patterns are identical except for the top band shown for Group 2 (Fig. 7). Various factors, including inconsistencies in the gel, might have contributed to this discrepancy. However, as sequencing later clarified, both of these groups nevertheless belong to the same species, *Halothiobacillus hydrothermalis*.

Likewise, though RFLP Groups 3 and 4 were also treated separately, sequencing later indicated that they both are in fact representative of the same species, *Thiomicrospira thermophila*. Yet, given that their percentages of similarity to the closest relative differ (Fig. 9), they might be two different strains of this particular species.

Furthermore, it makes sense that EPR101 (which comprises its own group, Group 5) turned out to be *Thiomicrospira crunogena*. After all, its RFLP band pattern matched up well with that of EPR75 (Fig. 7), which previously had been identified as *Thiomicrospira crunogena*. Thus, finding EPR101 to be a member of this species was not unanticipated.

Nevertheless, is it surprising that these particular thiosulfate-oxidizers were found in this study? Not only have members of the genus *Halothiobacillus* been shown to be involved in CO_2 fixation at hydrothermal vents (Sievert et al., 2000), but members of the genus *Thiomicrospira* have also been shown to be ecologically invaluable to deep-sea communities (Muyzer et al., 1995). Thus, their presence in these samples is quite expected.

ACKNOWLEDGEMENTS

Much thanks is given to the crew of the *R/V Atlantis* as well as to the crew and the pilots of the DSV *ALVIN*. James Voordeckers and Ronald Wong are also thanked for their assistance. This project was sponsored by an NSF-REU grant, by Rutgers' Institute of Marine and Coastal Sciences, and by NSF grants MCB 04-56676 and OCE 03-27353 to C.V.

REFERENCES

Jannasch, HW (1995) Microbial interactions with hydrothermal fluid. *In* Humphris, SE, Zierenberg, RA, Mullineaux, LS, Thomson, RE (Eds.), *Seafloor Hydrothermal Systems: Physical, Chemical, Biological, and Geological Interactions*. Washington, D.C.: American Geophysical Union, pp. 273-296.

Jeanthon, C (2000) Molecular ecology of hydrothermal vent microbial communities. Antonie van Leeuwenhoek 77: 117-133.

Karl, DM (1995) Ecology of free-living, hydrothermal vent microbial communities. *In* Karl, DM (Ed.), *The Microbiology of Deep-Sea Hydrothermal Vents*. Boca Raton: CRC Press, pp. 35-124.

Muyzer, G, Teske, A, Wirsen, CO, Jannasch, HW (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch Microbiol 164: 165-172.

Ruby, EG, Wirsen, CO, Jannasch, HW (1981) Chemolithoautotrophic sulfur-oxidizing bacteria from the Galapagos Rift hydrothermal vents. Appl Environ Microbiol 42: 317-324.

Sievert, SM, Heidorn, T, Kuever, J (2000) *Halothiobacillus kellyi* sp. nov., a mesophilic, obligately chemolithoautotrophic, sulfur-oxidizing bacterium isolated from a shallow-water hydrothermal vent in the Aegean Sea, and emended description of the genus *Halothiobacillus*. Int J Syst Evol Microbiol 50: 1229-1237.