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Evidence of sub-vent biosphere: enzymatic activities below 308 ºC deep-sea hydrothermal systems at Suiyo seamount, Izu-Bonin Arc, Western Pacific Ocean.

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Abstract

A high-temperature deep-sea hydrothermal system related to dacitic arc-volcanism was drilled using a tethered, submarine rock-drill system as a part of the Archaean Park Project. The benthic multi-coring system (BMS) employed allowed for direct sampling of microorganisms, rocks and fluids beneath hydrothermal vents. The samples examined in this study were from sites APSK 05 and APSK 07 on the Suiyo Seamount of the Izu-Bonin Arc in the Pacific Ocean. Based on the vertical distribution of samples derived from this vigorous sub-vent environment, a model of deep-sea subterranean chemistry and biology was determined detailing optimal microbial activities. Deep-sea hydrothermal sub-vent core samples of dacitic arc-volcanism obtained at the Suiyo Seamount, Izu-Bonin Arc, Western Pacific ocean were analyzed for alkaline and acid phosphatase enzymatic activities. Useful biomarkers of acid phosphatase (ACP) and alkaline phosphatase (ALP) enzymatic activities were positively correlated against each other and was greatest at the partial middle core sequences; ACP and ALP activities determined were as high as 5.10 nmol/min/g-rock and 6.80 nmol/min/g-rock, respectively. Biochemical indicators of ACP and ALP were consistent with the behavior of total hydrolyzed amino acids (THAA) and the chiral ratio of D- and L-amino acid forms. The significant enzymatic activities demonstrated in this study provides crucial evidence that sub-vent regions represent part of the previously unknown extreme-environment biosphere, extending the known subterranean habitable spaces of, for example, extremophilic microbes.

Key word: Enzymatic activity, deep-sea hydrothermal systems, habitable zone.

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1. Introduction

Acid and alkali phosphatases (orthophosphate monoester phosphohydrolases) are considered crucial enzymes in catalytic reactions involving phospho-monoesterases [1]. Many phosphatases have been characterized since the 1960s including the *Escherichia coli* alkaline phosphatases, which have been widely studied in terms of biosynthesis [2,3,4], structure and catalytic properties [5]. The fact that acid phosphatase (ACP) and alkaline phosphatase (ALP) have been widely found in nature in taxonomic groups ranging from bacteria to mammals, suggests their importance in fundamental biochemical processes [6]. Enzymatic activity is also generally recognized as playing a key role in the degradation and utilization of organic polymers by bacteria, as only compounds with molecular masses lower than 600 Da can pass through cell pores [7,8,9]. The cycling of nitrogen compounds is largely influenced by the C/N ratio of organic matter in sediments [10], and the bacterial carbon conversion efficiency is inversely related to age of the detritus [11]. Temperature has also been identified as a factor that controls enzymatic activity [12], but with a few notable exceptions [13]. Investigating thermostable enzymes can, in addition to increasing our knowledge and understanding of life in extreme environments, provide the basis for the industrial application of ALPs exhibiting thermostable characteristics [14,15,16].

Recently, interest in the limitations of life in high-temperature environments has been growing. Since the discovery of hyperthermophilic microbial activity in hydrothermal fluids recovered from "smoker" vents on the East Pacific Rise, the widely accepted upper temperature limit for life has risen from below 113ºC [17], while many microbiologists seem willing to speculate that the maximum may be closer to 150ºC [18]. The recent discovery of a microbe living at 121ºC has broken the established temperature limit and extended the zone of microbial habitable temperature [19]. It would be of interest to examine microbial activities within thermal gradient zones of sub-surfaces.

Deep-sea hydrothermal systems represent natural laboratories for the study of organic geochemistry regarding vigorous microbial habitats in extreme environments. The historic discovery of the Galapagos deep-sea hydrothermal systems [20] has lead many researchers to consider that deep-sea hydrothermal systems are suitable environments for chemical evolution, with possible implications for the origins of life on the Earth [21]. The extremophilic characteristics of these environments has attracted interest from many scientific perspectives including geology, oceanography, biology, chemistry and physics [21]. In fact, a number of submarine ecological colonies have been identified near black or clear smokers and the associated organic-rich seafloor mats [22,23,24]. While the existence of a deep bacterial biosphere in oceanic sediments has been reported and is a remarkable discovery, sub-vents, or areas subjacent to seafloor hydrothermal vents, are only recently being explored through deep-sea floor drilling experiments at submarine hydrothermal vents.

Here we present the first determination of enzymatic activities below deep-sea hydrothermal systems through the use of coring investigations. In an effort to construct a consolidated model of the extreme environments in submarine hydrothermal vents and the interactions, we present the first report detailing crucial evidence of a sub-vent biosphere.

2. Geological location and hydrothermal fluid discharge

The Izu-Bonin Arc lies on the eastern rim of the Philippine Sea plate. This arc is about 1,200 km long, extending from the Izu Peninsula (35 ºN, 139 ºE) to Minami-Iwojima Island (24 ºN, 141 ºE). The arc belongs to the circum-Pacific island-arc system and is adjacent to the Northeast Japan Arc to the north and the Mariana Arc to the south. Many volcanic islands and submarine volcanoes run parallel to the Izu-Bonin trench and form the volcanic front of this intra-oceanic island-arc system. The southern Izu-Bonin Arc, which is divided by the Sofugan tectonic line from the northern Izu-Bonin Arc [25] (Fig. 1-a), is thought to have become active at around 42 Ma [26]. The Shichiyo seamount chain forms a volcanic front (Fig. 1-b) around which the arc crust is thought to be thinner than that in the northern part [27]. The Suiyo Seamount, one of the volcanoes in the Shichiyo chain, has two major peaks, located on the eastern and western sides of the seamount. The Suiyo Seamount is an active submarine volcano, where vigorous hydrothermal activity has occurred on the caldera floor atop the west peak [28,29]. Dacitic rocks of a calc-alkaline rock series and low-potassium andesites have been recovered from this area [30], and

preliminary reports of seafloor hydrothermal alteration at Suiyo have been documented with respect to geochemical and mineralogical characteristics [31,32,33]. Numerous short black smokers and clear smokers were observed on the sandy floor. Hydrothermal circulation reaches the region adjacent to the magma source, and volatile constituents are extracted by water-rock interactions [34,35].

3. Materials and Methods

3.1. Sampling

The deep-sea hydrothermal sub-vent core samples were collected as part of the Archaean Park Project during a cruise over the Suiyo Seamount (28° 33 ’N, 140° 39 ’E) in the Pacific Ocean in July, 2001. The deep-sea subterranean biosphere and geochemical interactions were examined by taking core samples using a fixed seafloor benthic multi-coring system (BMS) for pinpoint drilling in the caldera [34,35] (Fig. 1-c). The maximum depth of coring at sites APSK 05 and 07 were 6,650 mm and 2,690 mm below the sea floor, respectively. The hydrothermal fluid temperature measured using a custer-type thermometer [36] at the APSK 05 and APSK 07 sites was 304ºC and 156ºC, respectively. After one month, hydrothermal water from those bore holes was measured using a submersible *Hakuyo 2000* from a mother-ship *Shinsei-Maru* cruise and the temperature determined for the APSK 05 and APSK 07 sites was 308.3ºC and 272.0ºC, respectively.

3.2. Preparation of stock solutions and pre-treatment of samples

Rock core samples were carefully obtained and quickly sealed with a pack of dehydrating and de-oxygenation reagent (AGELESS, Mitsubishi Gas Chemicals Co.). Interior portions of the sample were freeze-dried and powdered. De-ionized water was passed through a Millipore Milli-Q LaboSystem™ and Millipore Simpli Lab-UV™ (Japan Millipore Ltd., Tokyo, Japan) to remove both inorganic ions and organic contaminants (hereafter, Milli-Q water). All glassware used in the sampling and analysis was soaked overnight in 7 M HNO_3 and rinsed with Milli-Q water. Glassware was heated for 2 hours at 500°C in a high-temperature oven (Yamato DR-22) prior to use to eliminate any possible organic contaminants.

Modified universal buffer (MUB) stock solution was prepared by dissolving 12.1 g of tris-hydrochloric aminomethane, 11.6 g of maleic acid, 14.0 g of citric acid and 6.3 g of boric acid in Milli-Q water, adding 488 ml of 0.1 M NaOH, and adjusting the final volume to 1,000 ml (MUB stock solution).

3.3 Determination of Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP) activity

Determination of ALP activity was performed according to previously published methods [37,38]. Briefly, MUB stock solution (200 ml) was adjusted to pH 11 by 0.1 M NaOH and diluted to 1,000 ml with Milli-Q water (MUB working solution). Finally, 0.93 g of *p*-nitrophenyl phosphate was dissolved in 100 ml of prepared working solution (MUB substrate solution, pH 11).

Rock core samples were placed in sample vials, sealed with MILLI WRAP

filters (Millipore Co.), freeze-dried and then gently pulverized. Powdered sample (0.25 g) was incubated in 50 μl of toluene (ultra-pure grade), 1 ml of MUB working solution and 250 μl of MUB substrate solution for one hour at 37°C in a water bath (ADVANTEC LS-180 series). The reaction was terminated by the addition of 250 μl of 0.5 M $CaCl_2$ and 1 ml of 0.5 M NaOH. The solution was then filtered through a 0.20-μm membrane filter (ADVANTEC PTFE) and the absorbance at 410 nm of the reaction product (*p*-nitrophenol) at was measured using a JASCO V-550 UV-VIS spectrometer.

ALP and ACP activities (Z) were calculated as

$$Z = \Delta C \times V / 1000 / \Delta t \quad [\mu mol/min] \qquad (1)$$

where ΔC represents the increase in PNP concentration (μmol), V is the total volume of substrate solution (ml) and Δt is the incubation time (min). ALP activity in the sediment sample was expressed as nmol/min/g-rock. MUB stock solution (200 ml) was adjusted to pH 6.5 by 0.1 M NaOH and diluted to 1,000 ml with Milli-Q (MUB working solution, pH 6.5). Finally, 0.93 g of *p*-nitrophenyl phosphate was dissolved in 100 ml of MUB working solution (MUB substrate solution, pH 6.5). The aforementioned experimental procedure was performed for the ALP analysis.

Results and Discussion

Vertical distribution of Enzymatic Activities

The enzymatic activity of ACP and ALP in the sub-vent core samples at the two sites are summarized in Tables 1 and 2. Figs. 3 and 4 depicts the vertical

distribution of the ACP and ALP profiles. The values determined for ACP and ALP ranged from 0.53 to 5.10 nmol/min/g-rock and 0.32 to 6.80 nmol/min/g-rock, respectively, in the APSK 05 series. While in the APSK 07 series, the ACP and ALP values ranged from 0.19 to 2.40 nmol/min/g-rock and 0.19 to 2.40 nmol/min/g-rock, respectively. Anomalous ACP and ALP enzymatic activities present in some areas of the middle core samples were thought to be along a pyrite-rich hydrothermal vein. The positive correlation (r) between ACP and ALP was shown to be 0.69 as seen in Fig 5. These activity values were plotted yield a straight line defined by a least-squares method and expressed by the following equation:

$$ACP = 0.91\ ALP + 0.42\ (R=0.69) \qquad (2)$$

The slope of the straight line is 0.91 which is close to unity, differing only 0.09.

We had previously reported a large enantiomeric excess of proteinous L-form amino acids (chiral amino acids) in the same core sequences. Furthermore, the lack of evidence supporting abiotically synthesized amino acids of ω-amino acid specimens and the abiotic tendency of products confirmed the biological origin of amino acids and the existence of a vigorous sub-vent microbial oasis [39,40]. Consequently, biochemical indicators of ACP and ALP were consistent with the behavior of total hydrolyzed amino acids (THAA) and the chiral ratio of D- and L-amino acid forms. Preliminary investigation of the caldera revealed a high-temperature hydrothermal pool covered with several meters of volcanic sediment containing sulfate and boundary cap rock [34,35]. Therefore, the significant enzymatic activities present in the middle column sediment may indicate that the prevailing thermal gradient in that area gives

rise to a habitable temperature that is optimal for subterranean microbes.

Terrestrial origin and sinking organic matter

As to the terrestrial origin of organics on the Suiyo Seamount, it was reported that the total fatty acid compositions in the surface sediments obtained from the Suiyo hydrothermal system, Izu-Bonin Arc did not represent significant components of sedimentary organic matter [41,42]. Analytical examination of the surface sediments indicated very low contributions by terrestrial sediments. Furthermore, an age determination of the unaltered dacite by the Ar-Ar method showed 9,000 ± 8,000 yrBP, suggesting zero age [32,33]. The caldera floor is predominantly covered with sandy sediment and hydrothermal precipitations, and lacks any evidence of muddy pelagic sediments. The vertical variations in the mineral assemblages of these cores are presented in Figure 2. The core profile is characterized by dacitic lava and/or pyroclastic rocks at the surface underlying unconsolidated volcanic sands and pumice fragments; a sheath of clay minerals and anhydrite cement with minor pyrite and other sulfide minerals that acts as a cap rock of the geothermal system; and end-member fluid ponding beneath the sheath. Extensive hydrothermal alteration was observed in the sedimentary unit and the upper fraction of the volcanic rocks.

In general sedimentation environments, our previous studies showed that the enzymatic activities of alkaline phosphatase (ALP) and acid phosphatase (ACP) were greatest at the surface and decreased with the depth in the early stage of diagenesis [43]. Enzymes form one part of a group of sensitive labile organic compounds in the extreme

environment that includes nucleic acid bases and phospholipids. Generally, labile components proceed to a semi-labile component by thermal alteration and decomposition during sedimentation on a geological time scale. These then proceed continuously as semi-labile components to biologically inactive refractory-labile components in the next step. In the present study, however, the middle area of the sub-vent zone possessed highly active labile enzymes. This is evidence of vigorous microbial activity in the core sequence correlated with labile enzymes. In our previous study, significant positive correlations provided good evidence for the relationship between the population of subterranean microorganisms and the extant enzymatic activities [44].

Hence, the vertical distribution of ACP and ALP presented here is essentially independent from the nature of the surface, with energy derived from chemical sources in the form of fluids migrating upward from deeper levels in the present study. It was demonstrated in the room experiment that enzymatic activities were not stable under simulated hydrothermal conditions. In the actual hydrothermal systems, however, large amounts of organic matter derived from microbial activities might be greater than that associated with thermal degradation in the sediment. Consequently, the apparent enzymatic activities of ACP and ALP would be determined on the base of biogenic organic matter and microbial activity in the sub-vent region.

Thermal limit of microbial activity and labile enzymatic activity

The industrial importance of thermostable enzymes is increasing. Therefore, it

comes as no surprise that the isolation, characterization, and engineering of thermostable enzymes, as well as the search for the determinants of thermostability, represent hot spots of current research [45,46,47]. Thermostable ALP has been investigated from thermophilic bacterial sources including *Thermotoganeapolitana* [48], *Thermus caldophilus* [15], *Thermus thermophilus* [14] and *Bacillus stearothermophilus* [49]. *Pyrococcus abyssi* is a heterotrophic hyperthermophilic euryarchaeon isolated from a deep-sea hydrothermal vent with an optimal growth temperature of 100°C [50].

The currently accepted thermal limit of life has been estimated between 113°C [17] to 121°C [19]. Actually, following phylogenetic analysis of more than 120 clones, several novel phylotypes were detected within Proteobacteria, photosynthetic bacteria (PSB)-related K-Proteobacteria and Euryarchaeota clusters [51]. A number of archaeal clones were also detected from the borehole samples. These clones formed a novel monophyletic clade, SSSV-AE1 (Suiyo Seamount sub-vent origin, Archaea domain, Euryarchaeota, group 1), approximately between methanogenic hyperthermophilic members of Methanococcales and environmental clone members of DHVE Group II [51]. Further isolation trials of ACP and ALP are necessary in an effort to delineate the enzymatic functions of these thermostable enzymatic entities.

Concerning the end-member hydrothermal fluid, it is interesting to note that approximately 10^4–10^5 cells/ml of microbes were found in 308°C hydrothermal fluid from the drill hole in the Suiyo hydrothermal area when the drill hole was cased with metal to block the infiltration of fluid [52]. The hydrothermal gradient zone may be such that optimum fluid temperatures for microbial life occur in the sub-vent habitable

regions. Using an *in situ* growth chamber called a vent cap that is placed atop of the venting chimney, a few successful trial experiments have managed to expand our knowledge of microbial diversity in hydrothermal vent fields [51] at the Suiyo Seamount. Microbial diversity in natural vent chimney has been also revealed at the Suiyo Seamount [53,54].

The microbial diversity and populations in a hydrothermal plume that were also present inside the caldera of the Suiyo Seamount were investigated by performing a phylogenetic analysis of the 16S *r*RNA gene with fluorescence *in situ* hybridization (FISH) [56]. An indicator of turbidity, the vertical total cell count varied from 5.6 x 10^4 to 1.1 x 10^5 cells/ml. In addition to the sub-vent environment, the hydrothermal plume also represents a habitable space for microbes. Thus, the Suiyo Seamount caldera has functioned as a natural continuous incubator for microbes in the deep-sea environment [56,57]. Further isolation trials of ACP and ALP will be necessary to ascertain the precise enzymatic functions of these thermostable enzymes.

5. Conclusions

It is noteworthy that some of the findings presented here were made possible by the application of enzymatic activity, especially ACP and ALP, as that these can be used as useful biomarkers of subterranean microbial activity and organic matter for extreme environments. Significant enzymatic activities are consistent with the biological origins of amino acids and low chiral ratios (Takano et al., 2004ab), which is crucial evidence of a sub-vent biosphere. We presented the sub-surface biosphere

based on an interdisciplinary approach employing microbial and geochemical analyses of deep-sea volcanisms of the Suiyo Seamount, a case in keeping with rapidly evolving geophysical understanding of the stability of labile enzymes under deep-sea hydrothermal conditions.

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Figure 1 (a) Geological location of the Izu-Bonin Arc on the eastern edge of the Philippine Sea plate, western Pacific ocean. (b) Topographic map of the Suiyo seamount in the Shichiyo seamount chain (Cited from the Ref. 25). *Si* = Suiyo Seamount; *OR* = Ogasawara Ridge; *OT* = Ogasawara Trough; S = Sofugan Island; N = Nichiyo Seamount; G = Getsuyo Smt.; K = Kayo Smt.; M = Mokuyo Smt.; Kn = Kinyo Smt.; D = Doyo Smt.; *Ns* = Nishinoshima Island. (c) Distribution of chimneys, mounds, and BMS drilling sites in the bottom of the caldera at Suiyo Seamount.

Figure 2 The vertical variations in the mineral assemblages of the core samples of APSK 05 and APSK07.

Figure 3 Vertical distribution of acid phospahatase (ACP) and alkaline phosphatase (ALP) at APSK 05 site, Izu-Bonin Arc, Western Pacific Ocean.

Figure 4 Vertical distribution of acid phospahatase (ACP) and alkaline phosphatase (ALP) at APSK 07 site, Izu-Bonin Arc, Western Pacific Ocean.

Figure 5 The correlation between acid phosphatase (ACP) and alkaline phosphatase (ALP)) in deep-sea hydrothermal system core samples of APSK 05& 07 site, Izu-Bonin Arc, Western Pacific Ocean.

Table 1 Vertical concentration of enzymatic activities of acid phosphatase (ACP) and alkali phosphatase (ALP) in deep-sea hydrothermal system core samples of APSK 05 at Suiyo seamount, Izu-Bonin Arc, Pacific Ocean. *Core samples were collected by a seafloor fixed type Benthic Multi-coring System (BMS). Maximum depth was 6,650 mm below sea floor. The recovery of core samples were 53.9 %. The fluid temperature of end-member hydrothermal water was up to 308.3˚C. Each value stands for the unit of nmol/min/g-rock.

Table 2 Vertical concentration of enzymatic activities of acid phosphatase (ACP) and alkali phosphatase (ALP) in deep-sea hydrothermal system core samples of APSK 05 at Suiyo seamount, Izu-Bonin Arc, Pacific Ocean. *Core samples were collected by a seafloor fixed type Benthic Multi-coring System (BMS). Maximum depth was 2,690 mm below sea floor. The recovery of core samples were 59.5 %. The fluid temperature of end-member hydrothermal water was up to 272.0 ˚C. Each value stands for the unit of nmol/min/g-rock.

Figure 1

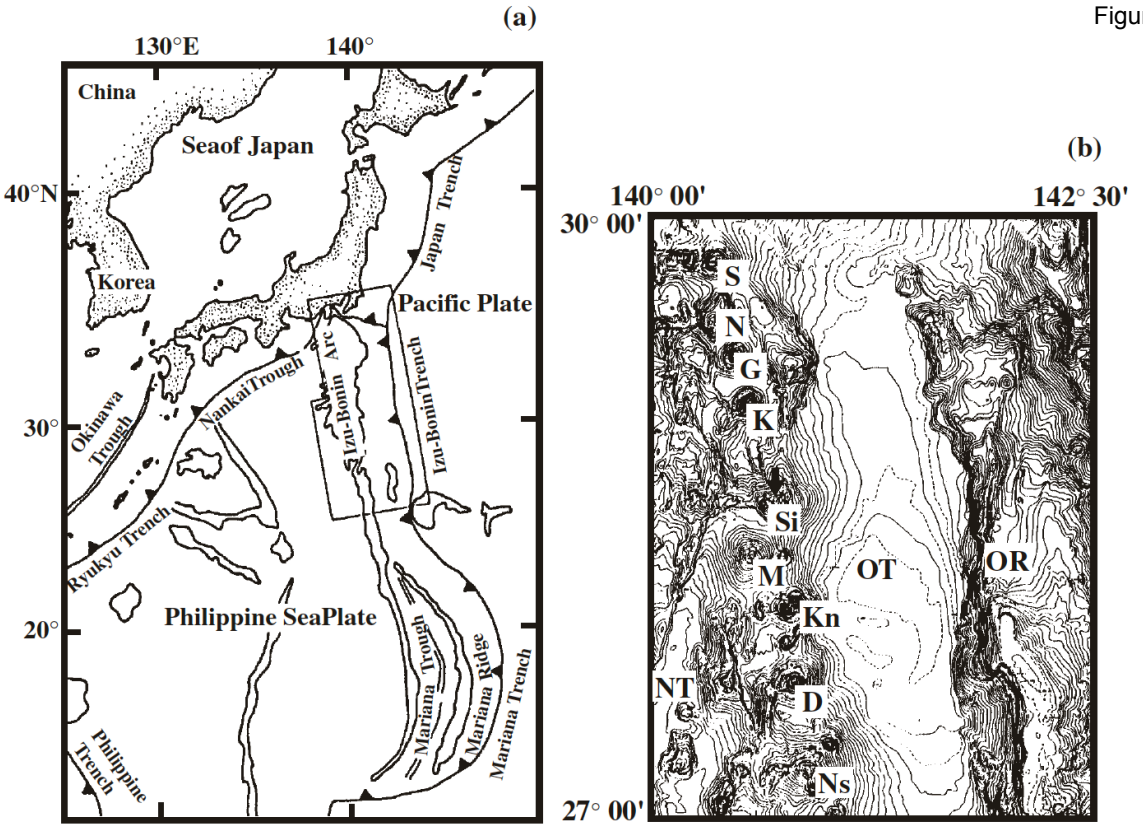
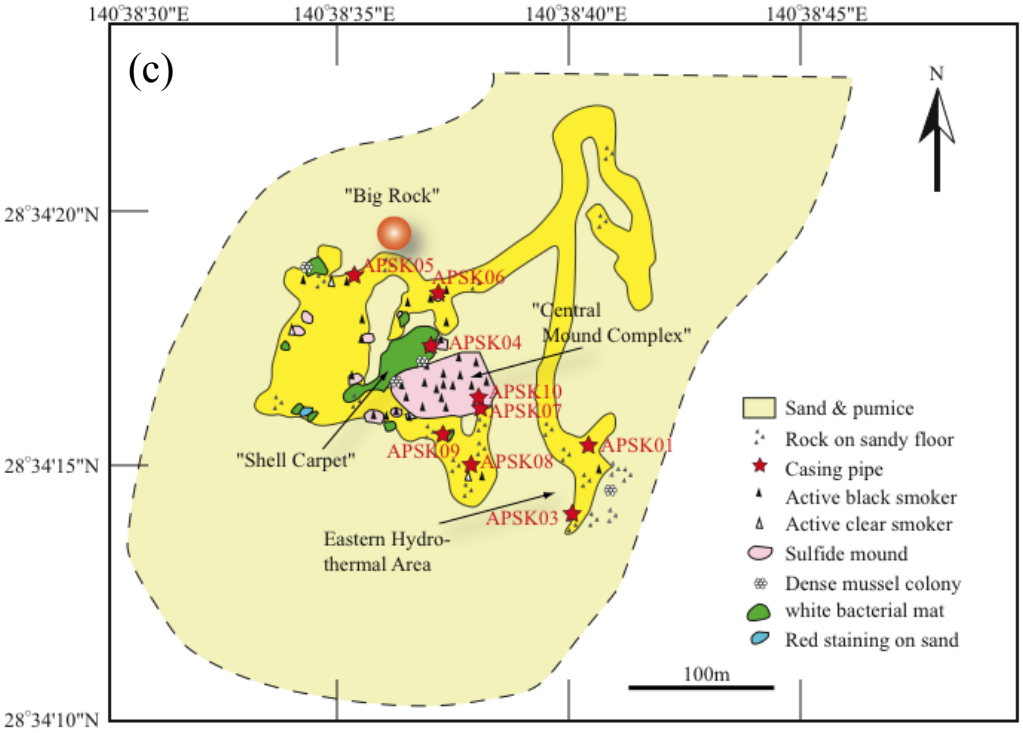


Figure 1



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Figure 2

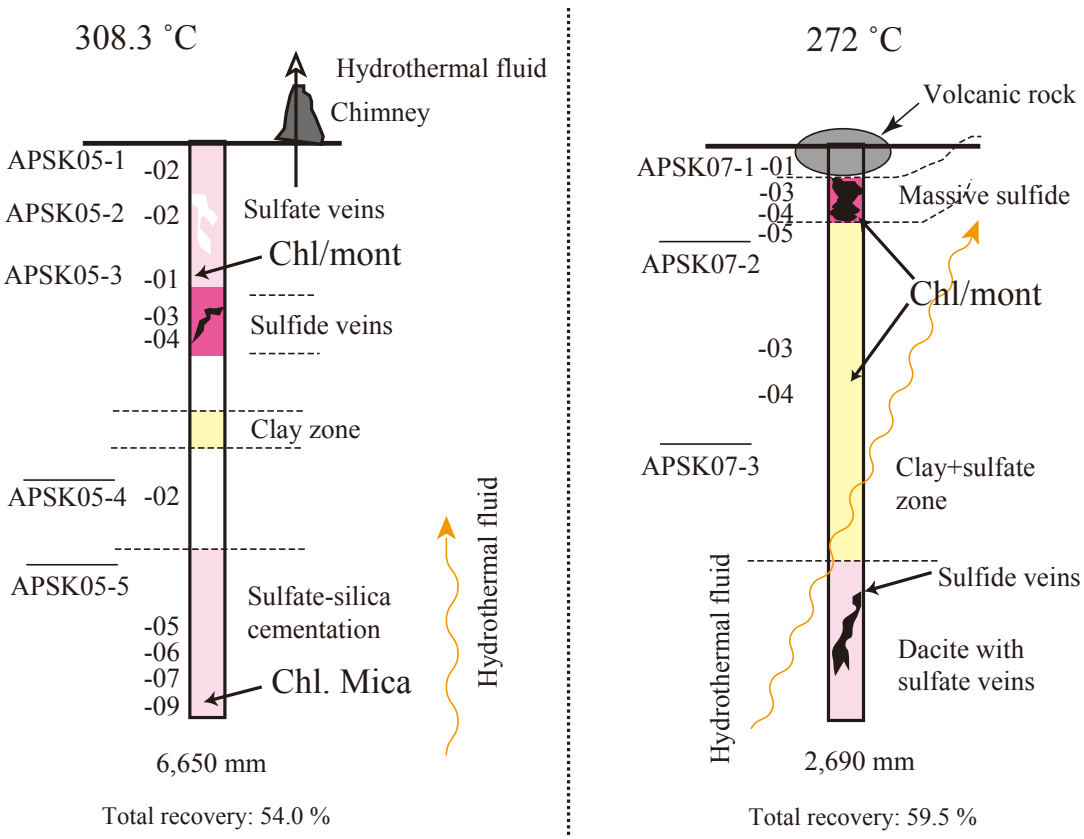
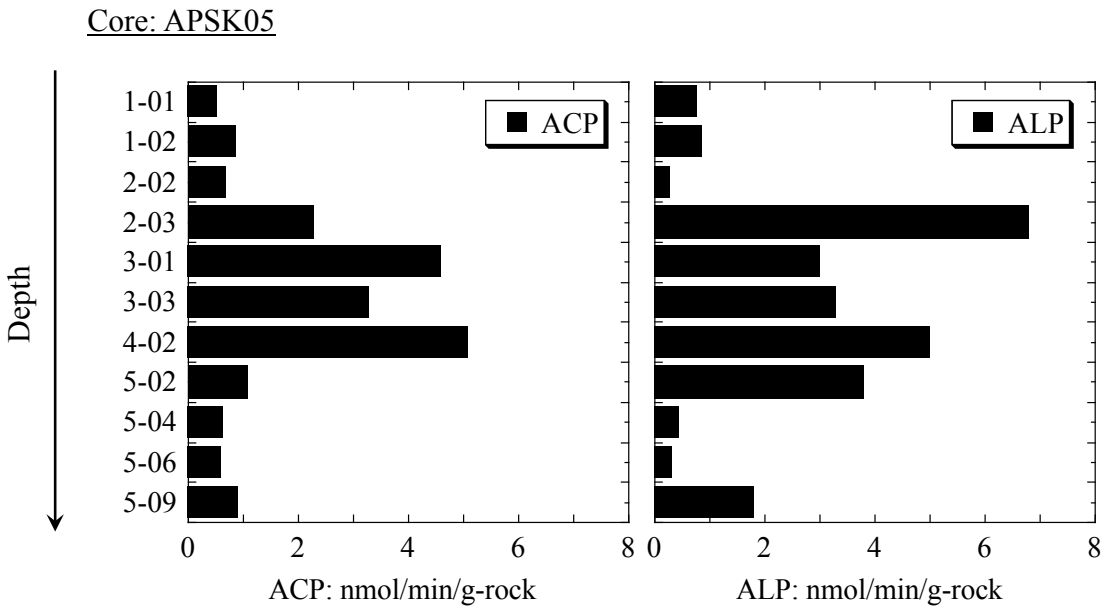


Figure 3



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Figure 4

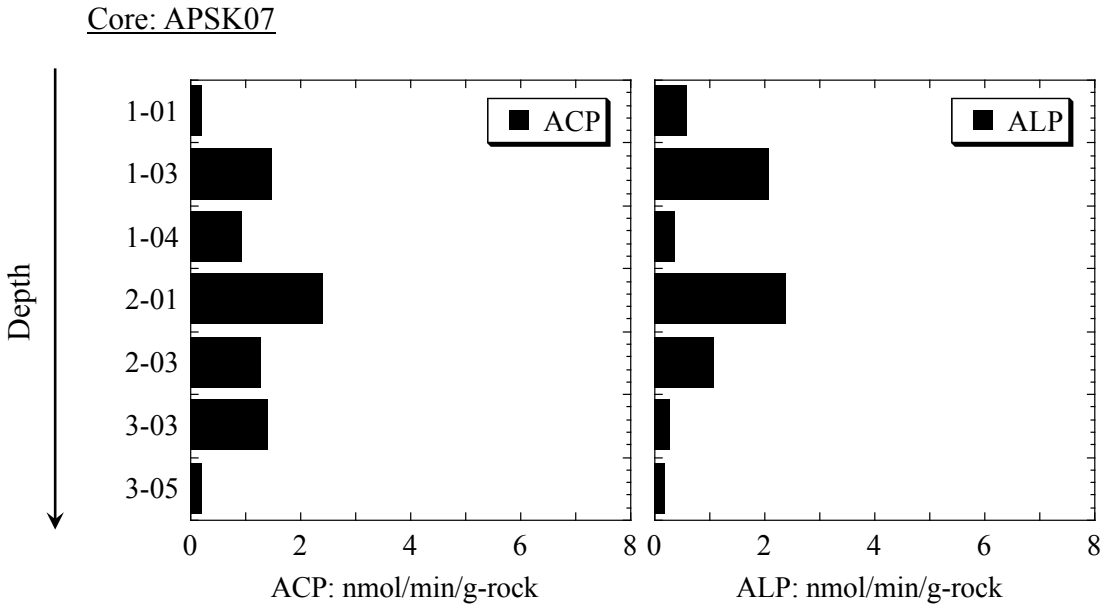
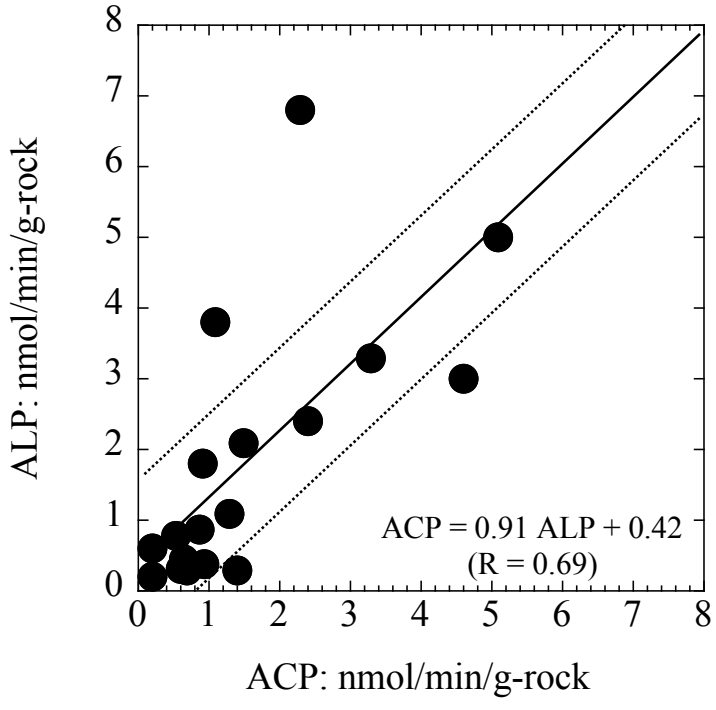


Figure 5



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Figure 6

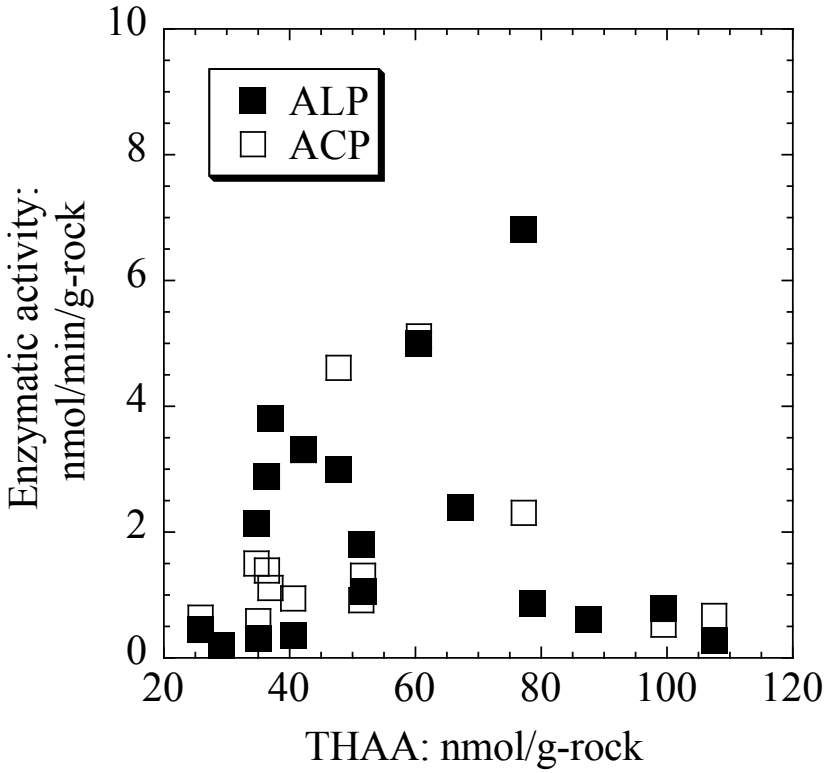


Table 1

	Enzymatic activity		Amino acids	
core: APSK05	ACP	ALP	THAA	D/L
1-01	0.53	0.77	99.5	0.21
1-02	0.86	0.86	78.6	0.25
2-02	0.68	0.29	107.4	0.08
2-03	2.30	6.80	77.3	0.23
3-01	4.60	3.00	47.7	0.07
3-03	3.30	3.30	42.2	0.17
4-02	5.10	5.00	60.5	0.07
5-02	1.10	3.80	37.1	0.16
5-04	0.64	0.45	26.0	0.04
5-06	0.59	0.32	35.0	0.09
5-09	0.92	1.80	51.5	0.06

	Enzymatic activity		Amino acids	
core: APSK07	ACP	ALP	THAA	D/L
1-01	0.19	0.61	87.6	0.32
1-03	1.50	2.10	34.8	0.23
1-04	0.94	0.37	40.6	0.10
2-01	2.40	2.40	67.1	0.50
2-03	1.30	1.10	51.7	0.25
3-03	1.40	0.29	36.5	0.17
3-05	0.19	0.19	29.1	0.13