Molecular taxonomy and community dynamics of Actinobacteria in marine sediments off central Chile

Taxonomía molecular y dinámica comunitaria de Actinobacteria en sedimentos marinos de Chile central

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Resumen.— Se usó pirosecuenciación de la región V6 del gen 16S del ARNr para caracterizar la diversidad y la dinámica espacio-temporal de unidades taxonómicas operacionales (OTUs) del filo Actinobacteria, los que fueron aislados desde sedimentos provenientes del Sulfureto de Humboldt frente a Chile central. Este substrato es rico en compuestos azufrados y material orgánico lo que mantiene una vasta comunidad microbiana que experimenta cambios estacionales en respuesta a regímenes oceanográficos contrastantes. Se identificaron 498 OTUs distribuidas en 7 órdenes, 47 familias, 122 géneros, (5 de los cuales son ampliamente reconocidos por sus aplicaciones biotecnológicas), y 56 especies. El análisis temporal reveló que algunos OTUs presentan diferencias significativas en abundancia, índices de diversidad y riqueza, las que generaron una agrupación de las muestras asociada a la fecha de muestreo (estación del año) y no a la profundidad del sitio de muestreo. Debido a que las Actinobacteria son mayormente aeróbicas, las altas concentraciones de oxígeno disuelto que ocurren en la zona en el otoño-invierno austral, representan condiciones ambientales beneficiosas para este filo, no así las de primavera-verano austral cuando prevalece la hipoxia. El presente trabajo se benefició de la aplicación de métodos cultivo-independientes (métodos moleculares) para evaluar la diversidad taxonómica y examinar la dinámica de uno de los grupos de bacterias presentes en el Sulfureto de Humboldt reportado como una fuente potencial e inexplorada de metabolitos secundarios.

Palabras clave: Actinobacteria, Sulfureto de Humboldt, ecología bacteriana

Abstract.— We used amplicon sequencing of the 16S rRNA gene to characterize the diversity and assess temporal and spatial patterns of Actinobacteria operational taxonomic units (OTUs) extracted from sediments of the Humboldt Sulfturetum located off the coast of central Chile. The sediment of this zone is rich in sulfur compounds and organic material and supports a vast microbial community that experiences seasonal changes in response to contrasting oceanographic regimes. We distinguished 498 OTUs distributed among 7 orders, 47 families, and 122 genera (5 of these have been widely recognized for their biotechnological applications), and 56 species. The temporal analyses indicated that some OTUs underwent significant temporal changes in abundance, richness, and diversity that allowed samples to be grouped by sampling dates (seasons) but not by sampling depth or location. Since Actinobacteria are mostly aerobic, higher concentrations of dissolved oxygen near the bottom during the austral autumn-winter seasons result in a more benign environment for this phylum than the upwelling-favorable spring-summer seasons when waters over the shelf are oxygen-deficient. To evaluate the taxonomic diversity and inquire into the community dynamic of Actinobacteria present in the Humboldt Sulfturetum and reported as a potentially untapped source for secondary metabolites this work benefited from culture-independent (molecular) techniques.

Key words: Actinobacteria, Humboldt Sulfturetum, bacterial ecology

INTRODUCTION

The benthic habitat of the Humboldt Current System in the Southeast Pacific off Chile is characterized by an oxygen minimum zone (Gallardo 1963) and seasonally variable dissolved oxygen conditions (Ahumada & Chuecas 1979, Gallardo et al. 1995, Paulmier et al. 2006, Sobarzo et al. 2007, Fuenzalida et al. 2009). This habitat supports a vast and diverse community of giant bacteria (Gallardo 1963, Gallardo 1975, 1977a, b; Fossing et al. 1995, Gallardo & Espinoza 2007a, b); smaller prokaryotes (Tremberger et al. 2010); and microbial euksaryotes (Høgslund et al. 2008), and is now known as the Humboldt Sulfturetum (HS: Gallardo et al. 2013a, b). Despite efforts, knowledge about the taxonomy, diversity, dynamics, and biotechnological value of this complex microbial community (Li et al. 2013), is still in its infancy.
Among major bacterial lineages the phylum Actinobacteria represents one of the richest taxa as revealed by culture and molecular approaches (Rappe & Giovannoni 2003); it comprises 5 sub-classes, 9 orders, 55 families, and 240 genera, from which ca. 3,000 species are currently known (Goodfellow & Fiedler 2010). They include aerobic and facultative anaerobic gram-positive bacteria with a high DNA G+C content, ranging from 51% in some Corynobaenia to more than 70% in Streptomyces and Frankia (Hogg 2005). Within the Actinobacteria, Actinomycetes are a recurrent component of marine systems comprising taxa with highly variable physiological and metabolic properties, which form stable and persistent communities (Jensen et al. 2005). They are morphologically diverse (e.g., rod or coccioid, fragmented hyphae or differentiated branched mycelia; Adegboye & Babalola 2012) and possess an unparalleled ability to produce secondary metabolites (Cho et al. 2006, Manivasagan et al. 2013). These compounds often serve as leads for the development of new pharmaceutical drugs with clinical applications, e.g., bonactin, antibacterial and antifungal; aureoverticillactam, anticancer (Bérdy 2005, Fiedler et al. 2005, Lam 2006).

Next-generation amplicon sequencing of 16S rRNA genes provides a powerful, culture-independent tool to assess temporal and spatial changes among bacterial communities (Fandino et al. 2001, Galand et al. 2009, Caporaso et al. 2011, Ulloa et al. 2012, Sule et al. 2013) as well as to explore and estimate bacterial richness and diversity (Jensen et al. 2005, Deutschbauer et al. 2006, Sogin et al. 2006, Ward & Bora 2006, Amaral-Zettler et al. 2010, Goodfellow & Fiedler 2010, Zinger et al. 2011, Bik et al. 2012). Amplicon pyrosequencing of the V6 region of the 16S rRNA gene (V6 pyrotags, hereafter) was used to assess the taxonomic composition and community structure of Actinobacteria found in the HS off central Chile. The first goal was to classify the local taxonomic diversity of this group using global alignment for sequence taxonomy (GAST) and identify relevant taxa. The second goal was to test for spatial and temporal patterns among sampling sites (stations) in the relative abundance composition of operational taxonomic units (OTUs). The designation of OTUs based on molecular criteria is the method of choice among bacterial ecologists to assess diversity (Pedrós-Alió 2012). Since off central Chile the diversity within macrobenthic communities showed a negative relationship with oxygen concentration and depth (Gallardo et al. 1995), it was hypothesized that the Actinobacteria community could also show such related patterns. In addition, and because Actinobacteria are primarily aerobic (Hogg 2005), it was hypothesized that any temporal changes in the Actinobacteria community could be linked to the two different seasonal oceanographic regimes in the study area with low-oxygen over the shelf due to increased upwelling during the spring-summer and high oxygen due to diminished upwelling and the presence of oxygenated surface waters over the shelf during autumn-winter (Ahumada & Chuecas 1979, Gallardo et al. 1995, Paulmier et al. 2006, Sobarzo et al. 2007, Fuenzalida et al. 2009).

**MATERIALS AND METHODS**

**STUDY ZONE AND SAMPLING**

This study involves the Bay of Concepcion (BoC) and the adjacent continental shelf off central Chile (Fig. 1). Four samplings stations were visited in the study area (Table 1), three located inside the BoC (station 1, 15 m; station 4, 27 m and station 7, 35 m depth), and one in the adjacent continental shelf (station 18, 88 m depth). At each location triplicate samples were obtained at four periods: December (end of austral spring) 2007, April (end of austral summer) 2008, September (end of austral winter) 2008, and January (austral summer) 2009.

The sediment was collected using an Oktopus mini-multicorer equipped with 6 plexiglass tubes (9 cm diameter, 40 cm long) and a mono-corer with a single 1 m long, 5 cm in diameter plexiglass tube. Each sediment sample was immediately subsampled onboard into smaller acrylic tubes and transported refrigerated to the laboratory where the first 5 cm of each replicate were mixed and, from the resulting mix, a final aliquot of 0.5 g was used for DNA extraction. In total, 16 (4 stations x 4 seasonal) samples were extracted.

**DNA EXTRACTION AND PYROSEQUENCING OF 16S rRNA BACTERIAL GENES**

The 0.5 g aliquot samples obtained as above were washed 3 times with phosphate buffered saline and extracted using the PowerSoil DNA isolation kit (MoBio Laboratories, Inc). DNA quality and concentration were evaluated by absorbance readings taken at A260 and A280 in an Infinite F200pro (Tecan Group Ltd., Switzerland). Three DNA extractions were performed from each sampling site which were subsequently pooled and lyophilized using a Speed Vac System. Massive and parallel tag sequencing of the hypervariable V6 region of the 16S rRNA bacterial gene (Sogin et al. 2006, Huber et al. 2007, Huse et al. 2007) among pooled isolates was done in a 454 GS-FLX Roche housed at the Marine Biology Laboratory, Woods Hole, Massachusetts, USA (Fakruddin & Chowdhury 2012).
Table 1. Sampling sites, dates, depth, and V6 pyrotag numbers in benthic samples collected in the Bay of Concepcion ('BoC stations' 1, 4 and 7) and open ocean ('off BoC' station 18), central Chile (sampling station numbers correspond to those of the 1994 *Thioploca*-Chile Expedition (see Gallardo et al. 2013a)) / Sitios de muestreo, fechas, profundidad y número de pyrotags V6 en las muestras bentónicas recolectadas en la Bahía de Concepción (estaciones BoC 1, 4 y 7) y en mar abierto (estación 18 off BoC), Chile central (los números de las estaciones de muestreo corresponden a las de la Expedición *Thioploca*-Chile 1994 (ver Gallardo et al. 2013a))

<table>
<thead>
<tr>
<th>Station Nr.</th>
<th>Date</th>
<th>Lat. °S</th>
<th>Long. °W</th>
<th>Depth (m)</th>
<th>Total V6 pyrotags</th>
<th>Actinob. V6 pyrotags</th>
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<tr>
<td>1</td>
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<td>36.69</td>
<td>73.07</td>
<td>12</td>
<td>11166</td>
<td>172</td>
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<td>12</td>
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<td>307</td>
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<tr>
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<td>73.07</td>
<td>12</td>
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<td>73.07</td>
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<td>9713</td>
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<tr>
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<td>73.04</td>
<td>25</td>
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<td>73.04</td>
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<td>7</td>
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<td>35</td>
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<td>73.12</td>
<td>88</td>
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<td>1426</td>
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<td>73.12</td>
<td>88</td>
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<td>73.12</td>
<td>88</td>
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</table>
DNA TAXONOMIC ANALYSES

After low quality sequences were trimmed (Huse et al. 2007), each V6 pyrotag was taxonomically assigned using a (GAST) pipeline (Sogin et al. 2006, Huse et al. 2008). All selected V6 sequences from all samples that were assigned to the phylum Actinobacteria were then distributed into the genus level.

COMMUNITY STRUCTURE: OTU DIVERSITY AND RICHNESS

V6 pyrotags were clustered into OTUs following Huse et al.’s (2008) method, which reduces OTUs overestimation (Huse et al. 2010) and is analogous to the PyroNoise method (Quince et al. 2011). High-quality V6 pyrotags (N= 269,752) were organized in 19,750 OTUs using a 97% sequence similarity criterion (Huse et al. 2010). This method was adopted because bacterial sequences with similarities greater than 97% are typically assigned to the same species (Rosselló-Mora & Amann 2001). The number of selected V6 pyrotags assigned by GAST to the phylum Actinobacteria amounted to 8,218.

Diversity for each of the 16 samples was estimated using the reciprocal Simpson’s index (D), according to: 
\[ D = 1/N - S(N_i (N_i - 1))/(N(N-1)), \]
where \( N_i \) is the abundance of the \( i \)th OTU in each sample and \( N \) is the total number of OTUs. Inverse Simpson is sensitive to the level of OTUs dominance (Hansel et al. 2008) and effectively distinguishes between dominant and uniform diversity patterns (Zhou et al. 2002). Additionally, OTUs expected richness for each sample using CatchAll, which implements a parametric estimation method, was calculated (Bunge 2011). This analysis uses frequency count data to compute ‘real’ richness to account for potentially overlooked or unseen species richness.

Testing for spatial and temporal patterns proceeded in 2 steps: (A) the degree of correlation between OTUs relative abundances (semi-metric Bray-Curtis distance) and OTUs presence/absence matrices (metric Jaccard distance) using a Mantel test (Mantel & Valand 1970) in R (R Core Team 2014) was measured. This analysis should assess whether changes in OTUs relative abundances were correlated with OTUs richness, which may be influenced by many V6 pyrotags of low relative abundance. Spearman’s correlation coefficient (\( \rho \)) between observed and randomized data matrices after 99,999 permutations was calculated using the vegan package (Oksanen et al. 2012) in R; (B) OTUs data sets were analyzed for spatial (Stations) and temporal patterns (dates of sampling-season) using a permutation multivariate analysis of variance, PERMANOVA (Anderson 2001, McArdle & Anderson 2001). PERMANOVA on OTUs data sets in vegan using 99,999 permutations were performed. Two-dimensional ordination plots were based on nonmetric multidimensional scaling (NMDS) (Kruskal 1964) using vegan’s metaMDS procedure. GAST and OTUs data sets are available in the project ID ICM_VAG_Bv61.

RESULTS

MOLECULAR TAXONOMY

GAST taxonomy classified 8,218 V6 pyrotags in the Actinobacteria phylum. The proportions of V6 pyrotags assigned by GAST at the different taxonomic levels are presented in Fig. 2. Within the phylum, 498 OTUs were distributed among 7 orders, 47 families, 122 genera, and 56 species. Actinomycetales was the dominant order (3,309 V6 pyrotags), whereas Acidimicrobicaeae was the dominant family with more than 600 V6 pyrotags. At the genus level, *Mycobacterium*

\[ <http://vamps.mbl.edu> \]
accounted for the majority of V6 pyrotags with more than 350 reads, followed by *Conexibacter* and *Streptomyces*. Many genera found in this community were classified as important producers of secondary metabolites with known biotechnological applications (Table S1).

Several V6 pyrotags had no matches to any genus (4,662) or species (8,523), and remained unclassified. All genera were plotted against their relative abundance after sample pooling (Fig. 3).

![Figure 3. Relative abundance of HS Actinobacteria genera as identified through GAST](image-url)
**Community structure: OTUs diversity and richness**

Actinobacteria showed changes in richness and diversity among samples (Fig. 4). In general, for all sampling sites the highest abundance of OTUs and species richness were observed in samples collected in September 2008 (transition between austral winter and austral spring). Conversely, the lowest number of pyrotags (a proxy for relative abundance) was seen in January 2009 in all stations (austral summer) (Table 1). Expected species richness from CatchAll varied between 744 ± 99 (Sta. 4 in September (end of austral winter) 2008) and 80 ± 15 (Sta. 1 in January (austral summer) 2009) and this trend was consistent with relative abundance and richness values. Mean expected richness was 300 OTUs, while the mean observed richness was 137 OTUs, indicating that approximately 45% of all OTUs from the phylum Actinobacteria potentially present at the study zone were sampled. Also, diversity ($D$) was correlated with relative abundance and richness, showing the highest diversity values in September (end of austral winter) 2008.

A Mantel test showed a strong and significant correlation ($r= 0.9037, P < 0.005$) between OTUs relative abundance (Bray-Curtis) and presence/absence (Jaccard) matrices. PERMANOVA analyses on the Bray-Curtis matrix suggested significant differences among dates (season) of sampling (F= 3.9426, $P < 0.05$), but not among stations (depth) (F= 1.2336, $P > 0.05$; Table 2). These results were consistent with groups obtained through NMDS (Fig. 5). By date, samples clustered into 3 groups: (i) January (austral summer) 2009, (ii) December (end of austral spring) 2007-April (end of summer) 2008, and (iii) September (end of austral winter) 2008. In addition, among sampling sites 2 groups were found: a ‘BoC group’ (Sta. 1, 4 and 7) and an ‘off BoC group’ (Sta. 18).

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**Table 2. Results of PERMANOVA based on Bray-Curtis distances among samples using two grouping factors: sampling station and date of sampling / Resultados del análisis PERMANOVA basado en distancias de Bray-Curtis entre las muestras utilizando dos factores: estación de muestreo y fecha de muestreo**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Df</th>
<th>Sum of Sqs</th>
<th>Mean Sqs</th>
<th>F Model</th>
<th>$R^2$</th>
<th>$P (&gt;F)$</th>
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</thead>
<tbody>
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<td>Station</td>
<td>3</td>
<td>0.634</td>
<td>0.21135</td>
<td>1.2336</td>
<td>0.15088</td>
<td>0.2112</td>
</tr>
<tr>
<td>Date</td>
<td>3</td>
<td>2.0264</td>
<td>0.67546</td>
<td>3.9426</td>
<td>0.4822</td>
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<tr>
<td>Residuals</td>
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<td>1.5419</td>
<td>0.17132</td>
<td>0.36692</td>
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<td></td>
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<td>Total</td>
<td>15</td>
<td>4.2023</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Discussion**

In this study, we used amplicon sequencing of the hypervariable V6 region of the 16S rRNA to assess taxonomic diversity among Actinobacteria and elucidate temporal and spatial patterns in community structure. Actinobacteria is considered one of the four most abundant phyla in marine sediments (Zinger et al. 2011). In the studied sulfuretum Actinobacteria had a low (7th) ranking with only 2.9% of all pyrotags (data not shown). This overall low abundance differs from estimates by Duncan et al. (2014) for Actinobacteria isolated from various marine sediments, a feature that can be explained by the normal temporal interplay of oxygen-rich and oxygen-poor benthic conditions prevailing in the HS. Below, molecular taxonomy and community structure related findings of this abundant taxon are separately discussed.

**Molecular Taxonomy and Biotechnological Potential of the Actinobacteria from the HS**

Culture-independent methods such as amplicon sequencing of DNA isolates have improved the understanding of ecological and diversity patterns in bacterial communities by using phylogenetic-based approaches (Hugenholtz et al. 1998). Because culture methods recover only between 1 and 10% of the total diversity recovered using DNA culture-independent methods (Bérdy 2005, Vartoukian et al. 2010), the latter present clear advantages to characterize unexplored environments (Das et al. 2006, Sogin et al. 2006). Vaz-Moreira et al. (2011) showed that culture-independent methods were more cost-effective than traditional culture methods. Furthermore, Duncan et al. (2014) suggest that culture-independent methods are the most efficient in recovering the taxonomic diversity of microbial groups. Yet, describing new bacterial species and their phenotype will often require culturing them. New techniques were developed for culturing the ‘as yet uncultivated’ bacteria during the last decade (Vartoukian et al. 2010). These include: (i) use of simulated environments, (ii) co-culturing using ‘helper strains’, and (iii) single-cell isolation techniques that might help grow environmental bacteria never cultured before (Ishii et al. 2010, Vartoukian et al. 2010, Stewart 2012). In general, all these approaches promise to close the gap between culturable and unculturable bacteria (Aoi et al. 2009, Liu et al. 2009, Ishii et al. 2010, Nichols et al. 2010, Park et al. 2011).

In this study Actinomycetales was the dominant order. Over 10,000 bioactive compounds have been isolated from species of this order (Bérdy 2005). In this study 122 different genera were found, including *Streptomyces*, a genus known by its unmatched potential to produce secondary metabolites with...
COMMUNITY STRUCTURE OF THE HS ACTINOBACTERIA

Among the most striking ecological results of this study were the temporal changes in relative abundance and richness of Actinobacteria OTUs, which are linked to seasonal changes of upwelling conditions in the sea off central Chile. It was early found, (Ahumada & Chuecas 1979), and later confirmed, that normally (‘no El Niño regime’) in the study area there is an alternation between two different regimes that affect the benthos: low-oxygen in spring-summer and high oxygen in autumn-winter (Gallardo et al. 1995, Paulmier et al. 2006, Sobarzo et al. 2007, Fuenzalida et al. 2009). Given that Actinobacteria are either aerobic or facultative anaerobes (Hogg 2005), the relative abundance and OTUs’s richness should increase during the austral autumn-winter seasons, and decrease, during the austral spring-summer seasons, as it was found in this study. In freshwater, dissolved oxygen is also strongly correlated with seasonal changes and vertical stratification of bacterial communities (Martínez-Alonso et al. 2008, Rotaru et al. 2012, Garcia et al. 2013). Pelagic marine and fresh-water Actinomycetales communities showed seasonal differences with depth and seasons presumably associated with changes in nutrient availability (Yoshida et al. 2008). Gallardo et al. (1995) surveyed the HS macrobenthic biota, including the megabacteria Candidatus Marithioploca spp. (ex-Thioploca, see Teske & Salman 2014). This study found that the former’s response to the seasonal and depth variations in dissolved oxygen are in tune with those shown in this study by Actinobacteria but not with respect to the behavior of the megabacteria Candidatus Marithioploca spp. which require the spring-summer reduced oxygen bottom conditions (Gallardo et al. 2013a).

Although evidence for spatially heterogeneous Actinobacteria OTUs was not supported by PERMANOVA analyses, 2 clusters were recognized: one represented by shallower ‘BoC Stations’ 1, 4, and 7, and another by the deeper open ocean ‘off BoC Station’ 18. It is hypothesized that this is an emerging bathymetric pattern that deserves further attention.

Both temporal and spatial patterns can be related to the movements, presence, and changes of water masses (Agoué et al. 2011, Salazar et al. 2015). Alves et al. (2015) found that parameters such as temperature, dissolved organic carbon, and depth appear to strongly influence the abundance and diversity of marine bacterial communities, and that the community structure might be related to features of the water masses present. They further suggest that the microbial component can help characterize each water mass. Brown et al. (2014) showed that bacterial communities in marine environments are highly structured and that biogeographic patterns reflect affinities for different water masses among bacteria. Water masses carrying different concentrations of oxygen, thus allowing for either organic-poor or organic-rich (reduced) benthic environments could structure benthic bacteria assemblages, and thus explain their eventual spatial and temporal variability as it has been confirmed by the present study.

CONCLUSIONS

As suggested by the important percentage of un-annotated tags, the lack of database information on uncultured bacteria needs to be addressed in order to provide more comprehensive assessments of bacterial diversity and ecology. Growth of genetic and taxonomic databases should enable more informed conclusions based on molecular, culture-independent data. In this study, using culture-independent methods the diversity and dynamics of the Actinobacteria community collected from marine sediments in the HS were characterized. Results indicated that the highly dynamic seasonal environment of the Humboldt Current system can explain temporal and spatial patterns in the Actinobacteria community structure. Molecular taxonomy based on V6 pyrotags promises to illuminate community diversity among other bacterial phyla found in sediments at the BoC and adjacent continental shelf. A large proportion of the OTUs had no matches in any database and remained anonymous. Yet, we found 5 genera that have been widely recognized for their biotechnological applications. Therefore, the HS appears as an untapped source of secondary metabolites.

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**LITERATURE CITED**


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Editor: Claudia Bustos D.
<table>
<thead>
<tr>
<th>Genus</th>
<th>N of V6 pyrotag</th>
<th>N of OTUs</th>
<th>Metabolites</th>
<th>Uses</th>
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<tr>
<td>Streptomyces</td>
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<td>Aureoverticillactam, Frigocyclinone, Lajollamycin, Bonactin, Caprolactones, Chinomycins, 3,6-disubstituted indoles, Glaciapyroles, Gutingimycin, Himalomycins, Komodoquinone A, Trioxacarmins, Albidopyrone, Abyssomicins B, C, atrop-C, D, G and H Benzoazine NTK 935, Caboxamycin</td>
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<td>10</td>
<td>Diazepinomicin (ECO-4601)</td>
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<tr>
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<tr>
<td>Dermacoccus</td>
<td>2</td>
<td>1</td>
<td>Dermacozines</td>
<td>Antitumour; antiprotozoal and free radical scavenging activities</td>
</tr>
<tr>
<td>Tsukamurella</td>
<td>1</td>
<td>1</td>
<td>Lipocarbazoles A1-A4</td>
<td>Strong free radical scavenging activity</td>
</tr>
</tbody>
</table>