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Diversity of *Bacteroidetes* in high-altitude saline evaporitic basins in northern Chile

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[1] The phylum *Bacteroidetes* represents one of the most abundant bacterial groups of marine and freshwater bacterioplankton. We investigated the diversity of *Bacteroidetes* in water and sediment samples from three evaporitic basins located in the highlands of northern Chile. We used both 16S rRNA gene clone libraries created with targeted *Bacteroidetes*-specific primers and separation of specifically amplified gene fragments by denaturing gradient gel electrophoresis (DGGE). DGGE analysis revealed a reduced richness of these organisms in samples from Salar de Huasco (two to four DGGE bands) increasing in Salar de Ascotán (two to seven DGGE bands) and Laguna Tebenquiche at Salar de Atacama (four to eight DGGE bands). Cluster analysis (WPGMA) of DGGE bands showed that bands from Salar de Huasco and Salar de Ascotán grouped together and samples from Salar de Atacama formed separate clusters in water and sediment samples, reflecting different *Bacteroidetes* communities between sites. Most of the sequences analyzed belonged to the family *Flavobacteriaceae* and clustered with the genera *Psychroflexus*, *Gillisia*, *Maribacter*, *Muricauda*, *Flavobacterium*, and *Salegentibacter*. The most abundant phylotype was highly related to *Psychroflexus* spp. and was recovered from all three study sites. The similarity of the analyzed sequences with their closest relatives in GenBank was typically <97% and notably lower when compared with type strains, demonstrating the unique character of these sequences. Culture efforts will be necessary to get a better description of the diversity of this group in saline evaporitic basins of northern Chile.

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1. Introduction

[2] Lakes located at high altitude (<3000 m) are characterized by their remoteness, geographical isolation, high UV radiation and low dissolved oxygen concentration [*Vila and Mühlhauser*, 1987]. High-altitude lakes are located in upland areas of the Andes, North and Central America, East Africa, Asia, and Europe. Most tropical high-altitude lakes are located in the Andes Range and exhibit an ecological continuity supporting both tropical and subantarctic flora and fauna [*Vila and Mühlhauser*, 1987]. The water bodies located in the Altiplano, the high plateau of the Andes, are characterized by high altitude and elevated salinities and are thus distinguished from other aquatic ecosystems such as mountain lakes or soda lakes [*Dorador*, 2007]. Contrastingly, lakes located in the European Alps and Pyrenees are characterized by low ionic content and are typically oligotrophic [*Mosello et al.*, 2002].

[3] The *Bacteroidetes* (previously referred to as *Cytophaga-Flavobacteria-Bacteroidetes* (CFB)) have been frequently observed in freshwater [*Kirchman*, 2002], as well as in aquatic environments with high salinity [*Bowman et al.*, 2000; *Humayoun et al.*, 2003], marine waters [*Kirchman*, 2002], and high-altitude, cold environments [*Dorador*, 2007; *Wu et al.*, 2006; *Dong et al.*, 2006; *Jiang et al.*, 2006; *Liu et al.*, 2006; *Demergasso et al.*, 2004; *Glöckner et al.*, 2000]. *Bacteroidetes* (together with *Proteobacteria*) are the most frequent groups in water and sediment samples collected from high-altitude lakes (Chilean Altiplano and Atacama Desert [*Demergasso et al.*, 2008; *Dorador*, 2007; *Demergasso et al.*, 2004]; Tibetan Plateau [*Zhang et al.*, 2007; *Jiang et al.*, 2007; *Wu et al.*, 2006; *Jiang et al.*, 2006; *Liu et al.*, 2006; *Dong et al.*, 2006]) and consist of 3 classes and at least 12 families. In a study examining microbial diversity across a salinity gradient in 16

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high-altitude lakes from the Tibetan plateau, *Proteobacteria* was the most abundant group, but the highest diversity was found in the phyla *Bacteroidetes* with *Psychroflexus* spp. representing the most frequent *Bacteroidetes* genera reported [*Wu et al.*, 2006].

[4] It has been demonstrated that isolates of *Cytophaga* from high-altitude lakes in the Argentinean Altiplano were more resistant to UV-B radiation than bacteria isolated from areas at sea level, an ability that might aid survival in the Altiplano with its elevated solar radiation [*Fernández Zenoff et al.*, 2006].

[5] The abundance and distribution pattern of *Bacteroidetes* in marine environments, combined with studies of enzymatic activity, has revealed the important role of this group in the marine carbon cycle. They are involved in the degradation of high molecular weight compounds such as cellulose, agar, and chitin in both dissolved and particulate fractions of marine organic matter [*Abell and Bowman*, 2005; *Kirchman*, 2002; *Cottrell and Kirchman*, 2000]. Recently, genomic studies on *Gramella forsetii* (family Flavobacteriaceae) revealed the presence of many genes encoding hydrolytic enzymes, the preference for polymeric carbon sources, and the distinct capability for surface adhesion [*Bauer et al.*, 2006].

[6] The taxonomy of *Bacteroidetes* is confused, and the group has undergone a series of reclassifications and amended descriptions in recent years [*Bernardet and Nakagawa*, 2006; *Garrity and Holt*, 2001]. The conserved inserts of proteins GyrB and SecA are characteristics of the *Bacteroidetes* and can be used as molecular markers for this group [*Suzuki et al.*, 2001; *Gupta*, 2004].

[7] Using specific 16S rRNA gene primers to target *Bacteroidetes* representatives [*Blümel et al.*, 2007], we analyzed the diversity of this phylum in three high-altitude evaporitic basins of the Chilean Altiplano. We also included *Bacteroidetes* sequences of cultures and clone libraries available from previous studies of other high-altitude evaporitic basins in northern Chile into our considerations.

2. Materials and Methods

2.1. Site Description and Sampling

[8] *Bacteroidetes* diversity was examined at three high-altitude saline evaporitic basins situated at latitudes between 20° and 21°S in the Chilean Altiplano and Atacama Desert, at altitudes between 2300 and 3800 m. Precipitation regimes and evaporation rates determine water availability and water content of these basins. Salar de Huasco (Hua) and Salar de Ascotán (Asc) [*Chong Díaz*, 1984; *Risacher et al.*, 2003; *Demergasso et al.*, 2004; *Dorador et al.*, 2008a, 2008b] are both salt flats, locally referred to as salares. Salar de Atacama is located at lower altitude and is the largest salar in Chile. Laguna Tebenquiche is one of the largest water bodies in the Salar de Atacama [e.g., *Zúñiga et al.*, 1991; *Demergasso et al.*, 2008]. Physical and chemical characteristics of these systems are shown in Table 1.

[9] Samples were collected from different sites and at different times. Temperature was recorded with a digital Hanna HI thermometer, pH was recorded with a Hanna HI 8314 m, and conductivity was recorded with an YSI 33 m. Environmental DNA was extracted from water and sediment samples from each site. Water samples were filtered at the site onto 0.2-μm and 25-mm diameter filters (Supor 200, Pall). The filtered volume varied between 0.05 and 1 L depending on the amount of suspended solids in the samples. Filters and sediment samples were maintained at −20°C for a few days until subsequent DNA extraction in the laboratory using previously described protocols [*Demergasso et al.*, 2004; *Dorador et al.*, 2008b].

2.2. Enrichment Cultures

[10] Fresh samples from Laguna de Tebenquiche and Salar de Ascotán were inoculated in (1) marine media at 1.9% of salt (Difco); (2) halophilic microorganisms media (MH) [*Quesada et al.*, 1984] at 8% of salt; and (3) in culture media for *Salinibacter ruber* containing 195 g L^{-1} NaCl [*Antón et al.*, 2002]. Liquid enrichment cultures in marine and MH media grew at 28°C, and *Salinibacter* enrichments grew at 37°C for 14 days. Liquid enrichments were transferred to agar plates with marine media (MA), MH, and *Salinibacter* media respectively. After 1 week, colonies were isolated and transferred 3 times in agar plates. The isolates were identified according their 16S rRNA gene sequences. Cultures were subsequently maintained in 67% glycerol at −80°C.

2.3. Denaturing Gradient Gel Electrophoresis

[11] Denaturing gradient gel electrophoresis (DGGE) was used to analyze the bacterial composition between sites and samples as described elsewhere [*Demergasso et al.*, 2004; *Dorador et al.*, 2008b]. Briefly, PCR products were generated with primers 9-27F and Cyt1020 R, and these products were used as a template to amplify a 550-bp fragment using the primers 341F-907R [*Muyzer et al.*, 1995]. DGGE was performed according to *Muyzer et al.* [1993]. The band pattern distributions between the samples were expressed in a binary matrix. Cluster analyses (WPGMA), based on percent similarity between the samples, were conducted using the Multivariate Statistical Package (MSVP version 3.12d; Kovach Computing Services, United Kingdom). Pairwise comparisons between samples were conducted using paired *t* tests [*Rothrock and Garcia-Pichel*, 2005].

2.4. PCR Amplification, Cloning, and Sequencing

[12] Fragments of *Bacteroidetes* 16S rRNA gene were amplified in a nested PCR approach. First, fragments of 1500 bp were obtained with primers Eub9-27F and Eub1542R [*Stackebrandt and Liesack*, 1993]. The general eubacterial primer 9-27F was used in combination with Cyt1020 R [*Blümel et al.*, 2007] to generate a 1000 bp product using the first round PCR products as templates in a nested PCR. Each PCR reaction contained 10 × PCR buffer with 1.25 mM $MgCl_2$ (Invitrogen), 200 μM dNTP mixture (Invitrogen), 1 pmol of each primer, 2.5 U Taq polymerase (Invitrogen), 10–100 ng template DNA, and water to a final volume of 50 μl. PCR was performed according to *Blümel et al.* [2007] using a touchdown protocol. For cloning, PCR reactions in triplicate were carried out, and PCR conditions were adjusted according to the manufacturer's instructions.

[13] Clone libraries of 16S rRNA gene were generated from water and sediment samples of site 5 of Tebenquiche Lake (Teb5) at the Salar de Atacama and sites V6 and J1 of Salar de Ascotán (Table 2). Samples for cloning were selected according to the DGGE band pattern in order to

Table 1. Physical and Chemical Parameters of the Study Sites[a]

	Salar de Ascotán				Salar de Atacama				Salar de Huasco			
Sampling Site	V10-Spring	V6-Spring	LT-Pond	J1-Pond	Laguna Tebenquiche	Laguna Chaxa	Laguna Cejar	H0-Spring	H1-Pond	H4-Pond	H6-Pond
Location (UTM) East	577782	577180	572171	577921	577363	5044353	580677	513007	512594	512004	515076
Location (UTM) North	7610573	7622870	7610653	7609328	7441644	7424617	7449674	7759436	7758373	7758252	7752432
Date sampling	Jun-06	Jun-06	Jun-06	Jun-06	May-06	May-06	May-06	Apr-06	Apr-06	Apr-06	Apr-06
Hour	12:27	14:00	15:15	11:35	15:07	10:15	11:20	12:30	11:30	10:30	9:00
Temperature (°C)	18	16	3	0	23.4	9.6	18.5	17.2	17.9	12.3	4.9
pH	7.55	6	6.5	5	7.85	7.8	7.47	7.6	8.8	8.5	8.6
UV 320 nm (mW cm^{-2})	0.249	0.105	0.128	0.132	0.14	0.155	0.215	ND	ND	ND	ND
O_2 (ppm)	8.8	10.8	7.5	8.9	5.33	4	3.76	5.8	7.7	3.7	0
Lake type	Oligosaline	Freshwater	Polysaline	Oligosaline	Hypersaline	Hypersaline	Hypersaline	Mesosaline	Mesosaline	Hypersaline	Hypersaline
Conductivity (mS cm^{-1})	2.85	1.04	18.5	3.90	102.5	83.3	153.9	11.8	12.4	100.8	138
TDS (mg L^{-1})	1390	493	10200	1750	9500	55800	21700	470	385	42765	66990
HCO_3^- (mg L^{-1})	146	90	498	261	328	1196	388	ND	ND	ND	ND
CO_3^{2-} (mg L^{-1})	1.2	12	74	ND	129	< 1	67	ND	ND	ND	ND
Ca^{2+} (mg L^{-1})	249	152	550	594	490	322	338	18.59	35.2	200.73	396.55
Mg^{2+} (mg L^{-1})	88	40	758	216	2114	5100	2172	12.13	9.33	187.81	316.85
NO_3^- (mg L^{-1})	4.18	4.16	8.24	3.89	8.86	16.1	3.37	0.755	0.048	ND	ND
K^+ (mg L^{-1})	96	67	1220	217	3790	3530	2850	5.87	3.43	1617.74	2795
Na^+ (mg L^{-1})	954	600	8922	2216	30500	81600	1930	70.41	64.15	9928.64	15389
$S0_4^{-2}$ (mg L^{-1})	366	181	3650	683	13100	16500	7180	25.52	30.79	3355.35	4323.5
Cl^- (g L^{-1})	1.85	1.14	3.01	4.56	12.9	28	8.18	ND	ND	ND	ND
As (mg L^{-1})	1.6	0.9	28	3.4	1.2	9.62	1.38	ND	ND	ND	ND

[a]Salar de Ascotán, Salar de Atacama and Salar de Huasco.

best represent the total diversity of the microbial communities. Purified amplicons were cloned into pGEM-T Easy Vector (Promega) according to the manufacturer's instructions. Ninety-six clones per sample were picked, and inserts were amplified with M13F/R primers. PCR products were used for one-shot sequencing (Macrogen). Sequences were checked for chimeras using Chimera check from RDP II (http://www.cme.msu.edu/). Rarefaction curves [*Simberloff*, 1972] were calculated with the RARFAC program (http://www.icbm.de/pmbio/downlist.htm) and used to evaluate whether sufficient numbers of clones were screened to estimate total diversity in each clone library.

2.5. Phylogenetic Analysis

[14] The closest relatives of the 16S rRNA gene sequences were determined by BLAST search (http://www.ncbi.nlm.nih.gov/blast) and the classifier tool in RDP II (http://www.cme.msu.edu/). Sequences were aligned with the alignment tool of the ARB package (http://www.arb-home.de), and phylogenetic relationships were calculated using maximum likelihood analysis in the program PHYML [*Guindon et al.*, 2005] with GTR (generalized time reversible) substitution model and 100 bootstrap resamplings. Pairwise similarities between sequences were determined using p distance in MEGA 4 [*Tamura et al.*, 2007] and the Blast 2 sequences tool [*Tatusova and Madden*, 1999].

[15] Trees were edited using MEGA 4 [*Tamura et al.*, 2007]. Sequences with similarities $\geq$97% were considered to represent the same phylotype [*Hughes et al.*, 2001].

[16] The nucleotide sequences of *Bacteroidetes* 16S rRNA gene have been deposited in GenBank with accession numbers FJ213784-FJ213838.

3. Results

3.1. Enrichment Cultures

[17] Enrichments were transferred to agar plates, and after 2 days, single colonies were obtained from agar plates with MA and MH media. According to their 16S rRNA gene sequences, seven isolates were identified as member of *Bacteroidetes* (Table 3). The isolates Teb2Bcf, Teb2Bf, and Teb5O1EL were isolated from samples from Laguna Tebenquiche and the isolates BAAM, BAMH, MA-41, and MA-504 from Salar de Ascotán. Most of these grew well at 1.9% w/v NaCl, but isolate BAMH grew at 8% w/v NaCl and had 97% similarity with *Psychroflexus torquis*, which exhibited an optimum growth at 2.3% w/v NaCl [*Bowman et al.*, 1998] and 92% with *P. gondwanense,* which exhibited an optimum growth at 5.8% w/v NaCl and a salinity range between 0 and 17.4% w/v NaCl [*Dobson et al.*, 1993; *Bowman et al.*, 1998]. All other isolates exhibited sequence similarities between 91 and 99% with their closest relatives in culture (Table 3).

3.2. *Bacteroidetes* Composition in High-Altitude Saline Evaporitic Basins

[18] Cluster analysis (WPGMA) of DGGE bands showed that bands from Salar de Huasco and Salar de Ascotán grouped together. Samples from Salar de Atacama formed separate clusters in water and sediment samples, reflecting different *Bacteroidetes* communities between the geographically separated locations (Figure 1). DGGE analysis

Table 2. Total Clones, Phylotypes, Species Richness Estimator S_{Chao1}, Coverage, and Shannon-Weaver Diversity Index for Clone Libraries From the Study Sites

	DGGE			*Bacteroidetes*-16S rDNA Clone Library						
Site	Sample	Bands	Shannon-Weaver Diversity Index (H′)	Total Clones	Total Phylotypes	Clones (*Bacteroidetes*)	Phylotypes (*Bacteroidetes*)	Species Richness S_{Chao1}	Coverage (%)	Shannon-Weaver Diversity Index (H')
					Salar de Atacama					
Laguna Tebenquiche	Teb5-w	7	2,08	25	4	20	3	5,85	92	0,82
Laguna Cejar	Cejas-w	6	1,61	-	-	-	-	-	-	-
Laguna Chaxa	Chaxa-w	4	1,39	-	-	-	-	-	-	-
Laguna Tebenquiche	Teb5-s	7	1,95	41	13	28	4	19,56	79	1,56
Laguna Cejar	Cejas-s	8	2,08	-	-	-	-	-	-	-
					Salar de Ascotán					
V6-spring	V6-w	7	1,79	30	16	27	13	29,51	64	2,40
V10-spring	V10-w	4	1,39	-	-	-	-	-	-	-
J1-spring	J1-w	3	1,10	57	10	57	10	49,74	84	0,78
LT-pond	LT-w	7	1,61	-	-	-	-	-	-	-
Cebollar-pond	Asc1b-w	2	1,61	33	15	11	8	29,35	67	2,03
V6-spring	V6-s	2	1,10	4	4	0	0	-	-	-
V10-spring	V10-s	3	1,61	-	-	-	-	-	-	-
LT-pond	LT-s	3	1,10	-	-	-	-	-	-	-
					Salar de Huasco					
H1-pond	H1-w	2	1,61	-	-	-	-	-	-	-
H0-spring	H0-s	2	ND	-	-	-	-	-	-	-
H4-pond	H4-s	3	1,10	-	-	-	-	-	-	-
H6-pond	H6-s	4	1,39	-	-	-	-	-	-	-

revealed a reduced richness (number of bands) of these organisms in samples from Salar de Huasco (two to four DGGE bands) increasing in Salar de Ascotán (two to seven DGGE bands) and Laguna Tebenquiche (four to eight DGGE bands) (Table 4). The *Bacteroidetes* communities at sample Teb5-w of Laguna Tebenquiche and Laguna Cejar were the most diverse (H′ = 2.08, seven DGGE bands) (Table 2). The less diverse samples were J1-w, V6-s, LT-s from Salar de Ascotán, and H4-s from Salar de Huasco (H′ = 1.10, three DGGE bands) (Table 2).

[19] We found significant differences between the sediment samples Teb5-s/LT-s (*t* test: $t = 2.19$, df = 18, $p < 0.042$), Teb5-s/H0-s (*t* test: $t = 2.88$, df = 18, $p < 0.0099$), Cejar-s/H0-s (*t* test: $t = 3.24$, df = 18, $p < 0.0045$), and V10-s/H0-s (*t* test: $t = 2.19$, df = 18, $p < 0.042$). All sequences of the excised DGGE bands were affiliated to the *Flavobacteriaceae*. Sequences related to *Psychroflexus* were recovered from the three study sites (Table 4).

3.3. *Bacteroidetes*-16S rRNA Gene Clone Libraries

[20] Six clone libraries were generated with *Bacteroidetes*-specific primers from water and sediment samples (Table 2). From a total of 190 clones obtained, 75% were classified into the phyla *Bacteroidetes*. Those clones were grouped into 38 phylotypes considering a similarity cutoff of 97%. Rarefaction analysis revealed saturation at between 4 and 16 phylotypes in all libraries not considering specificity to *Bacteroidetes* (data not shown). In addition, coverage indicated that more than 64% of total diversity was detected in the clone libraries. The richness estimator S_{Chao1} was higher than the number of observed phylotypes in all libraries but almost identical for one sample (Teb5-w). The *Bacteroidetes* community at sample V6-w of Salar de Ascotán was the most diverse (H′ = 2.26) and had the highest number of phylotypes (Table 2). The least diverse sample was J1-w from Salar de Ascotán (H′ = 0.78).

Table 3. Sequence Similarity of 16S rRNA Gene From Isolates of Salar de Ascotán and Laguna Tebenquiche at Salar de Atacama with GenBank Entries[a]

	Culture	Salinity	First Hit in BLAST		Closest Cultured Type Strain	
Isolates	Media	(NaCl %, w/v)	Closest Relative	Similarity (%)	Type Strain	Similarity (%)
Laguna Tebenquiche						
Teb2Bcf	MA	1,9	Clone A1795 (EU283523)	97	*Psychroserpens mesophilus* (DQ001321)	92
Teb2Bf	MA	1,9	*Muricauda aquimarina* strain SW-72 (AY445076)	99	*Muricauda aquimarina* strain SW-72 (AY445076)	99
Teb5O1EL	MA	1,9	*Tenacibaculum* sp. CL-TF13 (AY962294)	84	*Tenacibaculum discolor* (AM411030)	84
Salar de Ascotán						
BAAM	MA	1,9	Isolate EG26 (AM691089)	96	*Psychroflexus torquis* (AY167318)	96
BAMH	MH	8	Isolate EG26 (AM691089)	97	*Psychroflexus torquis* (AY167320)	97
AM-41	MA	1,9	*Cytophaga* sp. strain BSD S1 22 (AY259509)	91	*Leeuwenhoekiella blandensis* (DQ294291)	91
AM-504	MA	1,9	*Salegentibacter salegens* (M92279)	97	*Salegentibacter salegens* (M92279)	97

[a]BLAST Search.

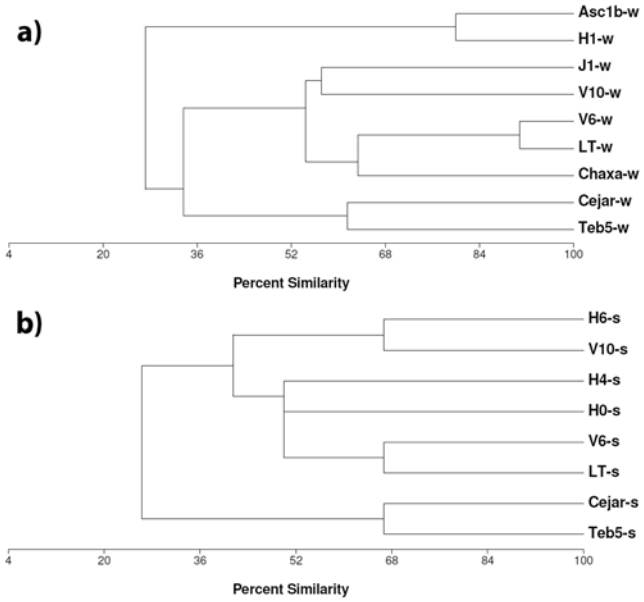

Figure 1. WPGMA clustering of DGGE band patterns of *Bacteroidetes* 16S rRNA gene from (a) water and (b) sediment samples of the studied sites. Abbreviations and samples are described in Table 2.

3.4. Phylogenetic Diversity

[21] Using BLAST search to find similarities of the phylotypes with sequences in GenBank, we found that more than 70% of the sequences exhibited a similarity lower than 97% with their closest relative (environmental or cultured strains), which could indicate novelty in the sequences [*Stackebrandt and Göbel*, 1994] (Table 4). The analysis of *Bacteroidetes* sequences indicated a diverse community matching at least two families. Thirty-eight phylotypes and seven isolates from this study were grouped into seven clusters including published sequences of *Bacteroidetes* retrieved from other high-altitude lakes [*Dorador*, 2007; *Demergasso et al.*, 2008] (Figure 2). Most of the clusters described in the phylogenetic tree did not match cultured representatives. *Flavobacteriaceae* was the most represented family including sequences and isolates belonging to the genus *Flavobacterium* spp. and the marine genera *Tenacibaculum* spp., *Psychroflexus* spp., *Salegentibacter* spp., *Gillisia* spp., *Muricauda*, and *Marinobacter* (Figure 2). *Sphingobacteriaceae* was represented by sequences highly similar with *Salinibacter* (Tables 3 and 4 and Figure 2).

[22] The cluster related to *Salinibacter* was formed by four sequences of site 12 of Laguna Tebenquiche [*Demergasso et al.*, 2008] similar to *Salinibacter ruber*, an extremely halophilic representative of the phylum *Bacteroidetes* that grows optimally at concentrations between 20 and 30% salts [*Antón et al.*, 2002]. Cluster 1 was formed by uncultured sequences from environmental samples, including the most abundant clone of the sediment sample of Laguna Tebenquiche (phylotype Teb5-s-9) which exhibited 93% sequence similarity with the most closely related cultured representative, the Cryomorphaceae *Brumimicrobium glaciale* isolated from polar habitats [*Bowman et al.*, 2003]. Cluster 2 was associated with members of *Sphingobacteriaceae* including an isolate from arsenic enrichment cultures from Salar de Ascotán (GenBank accession AM286268: J. Motok et al., unpublished data, 2006) and a sequence from water samples from Lago Chungará. The genus *Brumimicrobium* was represented by the phylotype Teb-s-41 from Laguna Tebenquiche and was distantly related with *Brumimicrobium mesophilum* (83–85% sequence similarity). Cluster 3 was formed by the phylotype V6-w-24 from Salar de Ascotán which exhibited 93% similarity with a clone retrieved from vermicompost with antifungal activity (GenBank accession EU593768: M. Yasir and Y. R. Chung, unpublished data, 2008) and 89% with *Owenweeksia hongkongensis*, member of *Flavobacteriales* [*Lau et al.*, 2005]. Cluster 4 was formed by three phylotypes from Salar de Ascotán and clustered with a clone retrieved from the delta of the Lena River in Siberia, Russia [*Liebner et al.*, 2008]; the phylotype V6-w-6 had 88% sequence similarity with *Prolixibacter bellariavorans*, an unclassified *Bacteroidetes* [*Holmes et al.*, 2007]. Cluster 5 was formed only by uncultured sequences distantly related with members of the family *Rikenellaceae* (83–93% similarity). The cluster included six phylotypes from Salar de Ascotán and one phylotype from Bofedal de Parinacota, a wetland located at 4000 m in the northernmost part of the Chilean Altiplano [*Dorador*, 2007]. The phylotype Asc1b-w-51 had 98% similarity with *Maribacter*, a genus isolated from marine water samples [*Nedashkovskaya et al.*, 2004], and the isolate Teb2Bf from Laguna Tebenquiche was highly similar (99% similarity) with *Muricauda aquimarina* isolated from a salt lake in Korea [*Yoon et al.*, 2005]. The genus *Leeuwenhoekiella* spp. was represented by the isolate MA-41 from Salar de Ascotán which showed a 91% sequence similarity with the genus *Leeuwenhoekiella* and 88% with *Zobellia.* Sequences related with *Salegentibacter* spp. included one clone sequences (J1-w-81) and the isolate MA-504 from Salar de Ascotán. MA-504 had 97% sequence similarity with *Salegentibacter salegens* isolated from a hypersaline antarctic lake [*Dobson et al.*, 1993]. The genus *Gillisia* spp. was represented by the most frequently found clone of the library J1-w (phylotype J1-w-61) which exhibited 97% similarity with *Gillisia limnaea* isolated from a microbial mat in Lake Fryxell in Antarctica [*Van Trappen et al.*, 2004]. Sequences related with *Psychroflexus* included two isolates, BAMH and BAAM (Table 3), and the phylotype J1-w-46 from Salar de Ascotán (Table 4) (Figure 2). The isolates exhibited 96–97% sequence similarity with the strain *P. torquis* ANT9232b, cultured from antarctic pack ice [*Brinkmeyer et al.*, 2003] and 95–96% with the type strain of *P. torquis* isolated from antarctic sea ice [*Bowman et al.*, 1998]. Four phylotypes from this study (two sites of Salar de Ascotán), the phylotype AT18OctC5 [*Demergasso et al.*, 2008] and two phylotypes from Salar de Huasco [*Dorador*, 2007], formed cluster 6, distantly related to *Psychroflexus* (90–91% sequence similarity with *P. torquis*). Inside this cluster, the phylotype J1-w-38 clustered with clone GSX1 retrieved from an oilfield in the area of Qinghai-Tibet in China (GenBank accession EF190068: B. Zheng et al., unpublished data, 2007). Cluster 7 included the phylotype Teb5-w-94, the most frequently identified clone from the water sample of Laguna Tebenquiche, having 98% sequence similarity with the clone LA1-B21N

Table 4. Phylogenetic Affiliation of *Bacteroidetes* Phylotypes[a]

		First Hit in BLAST			
Phylotype	Accession Number	Closest Relative	Sequence Coverage (%)	Similarity (%)	Habitat
		Laguna Tebenquiche			
clone Teb5-w-94 (18)	FJ213784	Clone LA1-B21N (AF513959)	100	98	Hypersaline Lake Laysan, Hawaii
clone Teb5-w-70	FJ213785	Clone Hua-w/3-2 (EF632863)	100	95	Salar de Huasco, Chilean Altiplano
clone Teb5-w-74	FJ213786	Clone LA1-B21N (AF513959)	100	94	Hypersaline Lake Laysan, Hawaii
DGGE Band Teb5-w-1 (2)	FJ213821	*Psychroflexus tropicus* (AF513434)	98	97	
clone Teb5-s-9 (25)	FJ213787	Clone MAT-CR-P1-G12 (EU246030)	100	91	Hypersaline microbial mat, Puerto Rico
clone Teb5-s-19	FJ213788	Clone E4aE04 (DQ103637)	98	97	Hypersaline endoevaporitic microbial, Eilat, Israel
clone Teb5-s-41	FJ213789	Clone NorSea50 (AM279197)	100	96	Marine water, Helgoland, Germany
clone Teb5-s-59	FJ213790	Clone VHS-B5-15 (DQ395033)	75	94	Harbor sediment, China
DGGE Band Teb5-s-5	FJ213822	Isolate GMD102 (DQ660394)	97	76	Marine sponge
DGGE Band Teb5-s-8	FJ213823	Clone WM13 (DQ415754)	97	90	Frasassi sulfidic cave stream biofilm
		Laguna Cejar			
DGGE Band Cejar-w-3	FJ213824	*Psychroflexus tropicus* (AF513434)	96	98	
DGGE Band Cejar-s-9	FJ213825	Clone WM13 (DQ415754)	97	91	Frasassi sulfidic cave stream biofilm
DGGE Band Cejar-s-10	FJ213826	Clone BD3-16 (AB015556)	75	76	Deep sea sediments
		Laguna Chaxa			
DGGE Band Chaxa-w-5	FJ213827	Clone E6aH07 (DQ103641)	97	74	Hypersaline endoevaporitic microbial, Eilat, Israel
DGGE Band Chaxa-w-6	FJ213828	Clone GN01-0.012 (DQ154838)	98	80	Hypersaline microbial mat
		Salar de Ascotán			
DGGE Band LT-w-11	FJ213829	Clone MAT-CR-H5-E08 (EU245295)	97	93	Hypersaline microbial mat, Puerto Rico
DGGE Band LT-w-12	FJ213830	*Psychroflexus* sp. strain YIM C238 (EU135715)	97	98	Haloalkaline soil, Qinghai
clone V6-w-73 (7)	FJ213791	*Flavobacterium* sp. WB3.1-53 (AM934654)	81	99	Hardwater creek, Westharz mountains, Germany
clone V6-w-90	FJ213792	*Flavobacterium gelidilacus* (AJ440996)	98	97	Microbial mats, Ace Lake, Antarctica
clone V6-w-1	FJ213793	*Flavobacterium* sp. WB2.1-83 (AM934639)	99	97	Hardwater creek, Westharz mountains, Germany
clone V6-w-3	FJ213794	Clone 24b08 (EF515416)	100	96	Upflow microbial fuel cell anode
clone V6-w-6	FJ213795	Clone LD_Ba_TopB16 (EU644258)	99	97	Lena Delta, Samoylov Island, Russia
clone V6-w-12	FJ213796	Isolate B76 (AY374109)	100	94	Rainbow trout
clone V6-w-16	FJ213797	Clone SS-18 (AY945898)	100	96	Sludge-seeded bioreactor
clone V6-w-17 (7)	FJ213798	*Flavobacterium* sp. WB3.1-53 (AM934654)	99	98	Hardwater creek, Westharz mountains, Germany
clone V6-w-18	FJ213799	*Gelidibacter* sp. strain IMCC1914	98	94	Coastal seawater
clone V6-w-22 (2)	FJ213800	Clone EFS-75 (EF190151)	100	95	Snow, Mount Everest
clone V6-w-24 (2)	FJ213801	Clone 3C003144 (EU801773)	100	91	Chesapeake Bay, United States
clone V6-w-56	FJ213802	Isolate ARK10164 (AF468425)	89	96	Arctic Sea ice-melt pond
clone V6-w-59	FJ213803	*Flavobacterium aquatile* (AM230485)	89	96	
DGGE Band V6-w-13	FJ213831	*Psychroflexus tropicus* (AF513434)	97	98	
DGGE Band V6-w-14 (2)	FJ213832	Clone MA72_2004T1d_E06 (EF378470)	97	98	Agricultural soil
DGGE Band V6-s-17	FJ213833	Clone DLE029 (EF127607)	97	98	Ice, Antarctica
clone J1-w-61 (48)	FJ213804	Clone SC78 (EU735617)	100	99	Jidong oilfield, China
clone J1-w-81	FJ213805	Clone SC145 (EU735631)	86	95	Jidong oilfield, China
clone J1-w-84	FJ213806	Clone Sc8 (EU375196)	82	97	Hydrocarbon polluted sand
clone J1-w-14	FJ213807	Isolate EG26 (AM691089)	85	98	
clone J1-w-18	FJ213808	Clone SC78 (EU735617)	73	99	Jidong oilfield, China
clone J1-w-21	FJ213809	Clone SC145 (EU735631)	100	95	Jidong oilfield, China
clone J1-w-25	FJ213810	Isolate Ko310 (AF550584)	98	96	
clone J1-w-35	FJ213811	Clone Asc-w-36 (EF632714)	100	98	Salar de Ascotán, Chilean Altiplano
clone J1-w-38	FJ213812	Clone GSX1 (EF190068)	100	96	Qinghai oilfield, China
clone J1-w-46	FJ213813	Isolate EG26 (AM691089)	99	96	
DGGE Band J1-w-16	FJ213834	*Flavobacterium* sp. WB3.4-76 (AM934664)	97	98	Hardwater creek, Westharz mountains, Germany
clone Asc1b-25 (2)	FJ213814	Clone ER-E4-17 (AY584739)	84	91	Chesapeake Bay, United States
clone Asc1b-28	FJ213815	Clone Asc-w-36 (EF632714)	100	91	Salar de Ascotán, Chilean Altiplano
clone Asc1b-32	FJ213816	Clone B16 (EU234211)	99	94	Effluent of wastewater treatment plant
clone Asc1b-51	FJ213817	*Maribacter* sp. strain MOLA 57 (AM990832)	86	98	Mediterranean Sea, France
clone Asc1b-53	FJ213818	Clone GSX1 (EF190068)	84	95	Qinghai oilfield, China
clone Asc1b-74 (2)	FJ213819	Clone Asc-s-26 (EF632650)	100	98	Salar de Ascotán, Chilean Altiplano
clone Asc1b-86 (2)	FJ213820	Clone GSX1 (EF190068)	86	96	Qinghai oilfield, China
		Salar de Huasco			
DGGE Band H1-w-19	FJ213835	Clone ES3-48 (DQ463267)	96	97	Songhuajiang River sediment
DGGE Band H4-s-20	FJ213836	Clone IRD18H08 (AY947982)	96	94	Massachusetts, Ipswich river
DGGE Band H6-s-21	FJ213837	Clone SB-5 (AF029041)	98	85	Benzene mineralizing consortium
DGGE Band H6-s-22	FJ213838	Clone ANTLV7_G11 (DQ521543)	98	87	Lake Vida, Antarctica

[a]Percent similarity with closest relatives in GenBank are shown.

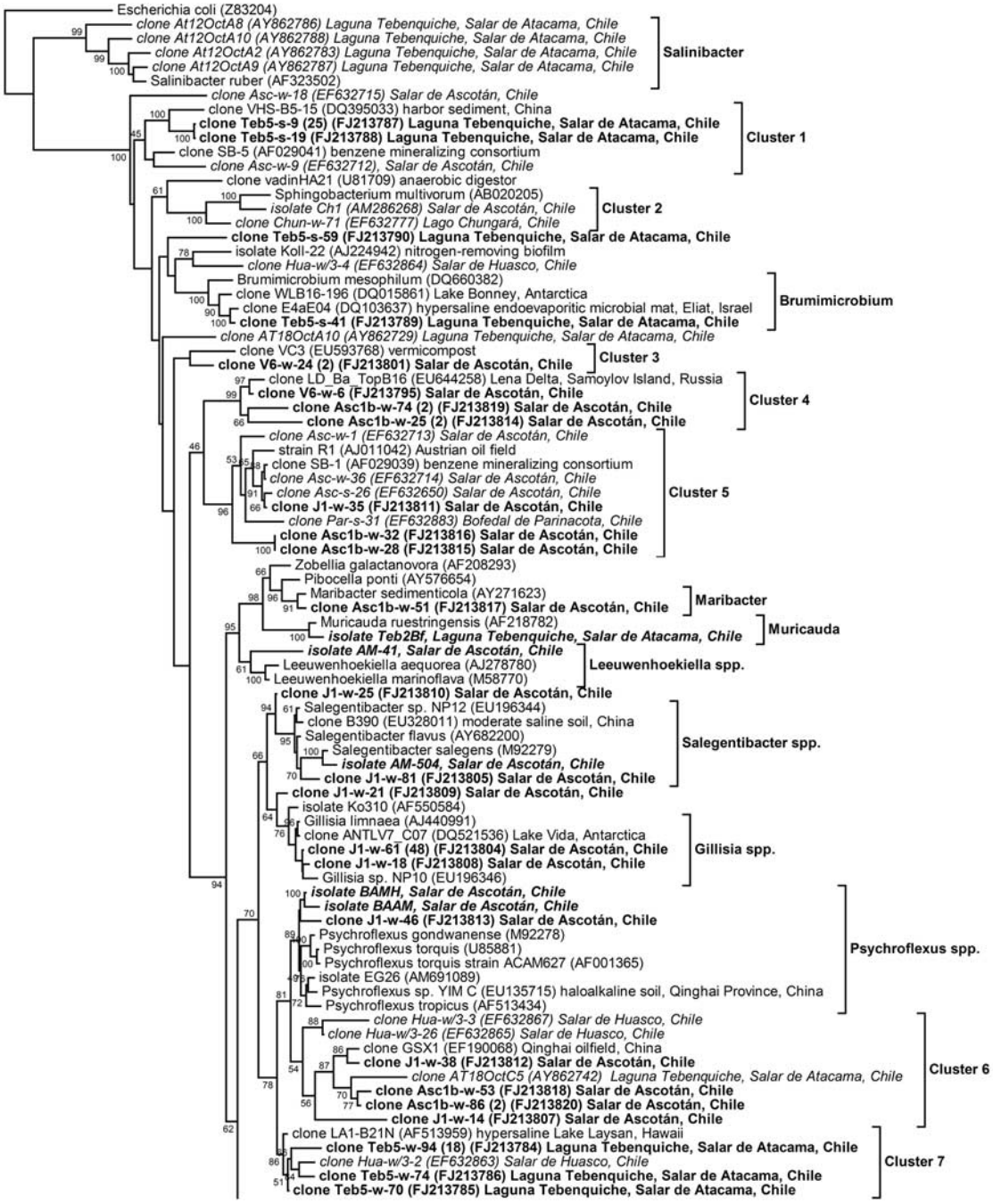

Figure 2. Phylogenetic tree based on partial 16S rRNA gene sequences (~800 bp) calculated by maximum likelihood analysis. The scale bar represents 10% nucleotide sequence difference. Bootstrap values greater than 45% are shown. Clone sequences from this study are in bold and coded as indicated in Table 2. Isolates obtained in this study are in bold and italics. Other *Bacteroidetes* sequences from evaporitic Andean basins are indicated in italics. The number of clones in each phylotype is shown in brackets except for phylotypes with only one clone. *Escherichia coli* (Z83204) was used as an outgroup.

retrieved from the hypersaline lake Laysan in Hawaii [*Donachie et al.*, 2004] and 92% with *Gillisia illustrilutea* isolated from marine antarctic habitats [*Bowman and Nichols*, 2005]. The phylotype V6-w-18 from Salar de Ascotán was associated with marine genera like *Psychroserpens*, *Gelidibacter* and *Bizionia* (94–96% similarity). The isolate Teb2Bcf from Laguna Tebenquiche had low similarity (92%) with *Psychroserpens mesophilus* [*Kwon et al.*, 2006]. Sequences related with *Tenacibaculum* spp., a genus described from marine environments [*Suzuki et al.*, 2001; *Bowman*, 2006], included the isolate Teb5O1EL from Laguna Tebenquiche (Table 3) and the phylotype Chun-w-24 retrieved from Lago Chungará [*Dorador*, 2007]. A bootstrap-supported clade contains several

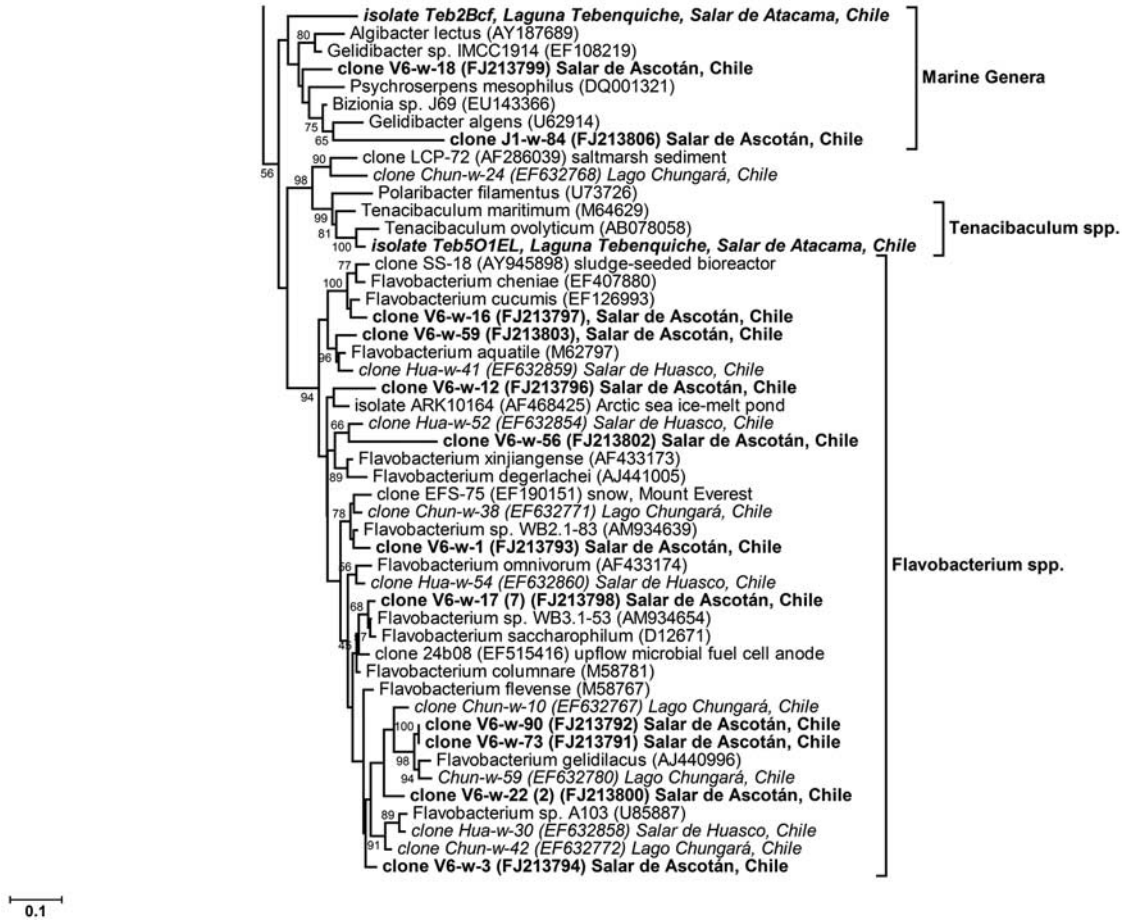

Figure 2. (continued)

sequences related to *Flavobacterium* spp. (Figure 2). Phylotypes V6-w-90 and V6-w-16 were highly similar with the type strains of *F. gelidilacus* and *F. cucumis* (97 and 96% sequence similarity respectively) (Table 4). Phylotypes retrieved from Salar de Ascotán (this study), Lago Chungará, and Salar de Huasco [*Dorador*, 2007] were highly related to the cultured species of *F. aquatile*, *F. flevense*, and *F. saccharophilum* (Figure 2).

4. Discussion

4.1. High Diversity of *Bacteroidetes* in High-Altitude Lakes of the Chilean Altiplano

[23] *Bacteroidetes* have been described as the most abundant group in many aquatic environments, including several high-altitude lakes of northern Chile. Previous results using PCR-based studies with eubacterial primers showed a *Bacteroidetes* community dominated by sequences similar to *Psychroflexus* [*Demergasso et al.*, 2004, 2008; *Dorador*, 2007] and *Flavobacterium* [*Dorador*, 2007], both genera belonging to the *Flavobacteriaceae* in environments with low salinity range. Similar results have also been found in high-altitude lakes from the Tibetan Plateau [*Wu et al.*, 2006]. Sequences similar to *Salinibacter* (*Sphingobacteriales*) dominated the only environment studied with higher salinity (30%) [*Demergasso et al.*, 2008]. Many of these previously described sequences did not match with described genera.

[24] In this study, using the antisense primer Cyt-1020R in a nested PCR approach [*Blümel et al.*, 2007], we found a much higher diversity of *Bacteroidetes* compared to previous reports of altiplanic basins. At least six described genera from the *Flavobacteriaceae*, and a number of additional clusters with unknown affiliation could be identified (Figure 2).

[25] Several sequences had close similarity with genera isolated from marine habitats (e.g., *Tenacibaculum*, *Psychroflexus*, *Salegentibacter*, *Gillisia*, *Maribacter*, *Muricauda*) and described as the marine clade of *Flavobacteriaceae* [*Bowman*, 2006]. These genera of *Bacteroidetes* (except *Psychroflexus*) were not recovered in previous studies using eubacterial 16S rRNA gene primers [e.g., *Demergasso et al.*, 2004, 2008; *Dorador*, 2007], highlighting that eubacterial primers did not match with many cultured and noncultured *Bacteroidetes*, resulting in marked underestimates of the diversity of this group in environmental samples [*Blümel et al.*, 2007] or simply the high spatial heterogeneity and sampling effort results in the underestimation of the genera cited before.

[26] Otherwise, the sequences related to *Salinibacter* (Figure 2) were recovered using eubacterial primers at the most saline site of Laguna Tebenquiche (site 12: 29.6%

NaCl) [*Demergasso et al.*, 2008] and were not found in other sites or lakes investigated. Apparently, the distribution of *Salinibacter* in high-altitude lakes is restricted to sites with high salt concentration [*Demergasso et al.*, 2004], considering that *Salinibacter* grows optimally at 150–300 g L^{-1} [*Antón et al.*, 2002], and the maximal salinities reported here reached 55.8 g L^{-1} (Table 2). The specificity of the primer used could also explain the lack of this group in our clone libraries. Clusters 9–14 grouped the marine groups described above and, interestingly, no sequences from Lago Chungará, the sole freshwater lake analyzed here, grouped into these clusters. Therefore, salinity may represent a crucial influence of the diversity and distribution of *Bacteroidetes* in high-altitude lakes.

4.2. Differentiation of *Bacteroidetes* Communities in Different Lakes

[27] *Bacteroidetes* communities appeared to be unique in each lake. Samples from Salar de Huasco and Salar de Ascotán tended to group together in terms of their composition (Figure 1), differing significantly from samples from Laguna Tebenquiche. The salares of Huasco and Ascotán are located in the same ecological range (e.g., climate, high altitude, high solar radiation) and exhibited lower evaporation rates and higher water inputs than salares located at lower altitude in the Atacama Desert [*Risacher et al.*, 2003], resulting in different conditions and selective pressures for microorganisms.

[28] Sample sites in each of the lakes differed mainly with regard to salt content, ranging between 0.39 and 55.8 g L^{-1} (Table 2). This variation was reflected in the *Bacteroidetes* communities recovered, a pattern also reported from other bacterial or archaeal groups in altiplanic lakes [*Demergasso et al.*, 2004; *Dorador*, 2007] and largely associated with local variations in salt concentration [*Demergasso et al.*, 2004, 2008]. For example, the greatest diversity was found in site V6 of Salar de Ascotán (0.5 g L^{-1}) which exhibited one of the lowest salinities of all the sites investigated. Most of the sequences retrieved clustered within *Flavobacterium*, a genus previously isolated in freshwater and seawater but never from hypersaline waters [*Bernardet and Bowman*, 2006] (Table 4 and Figure 2). Consequently, sequences from site V6 did not match with *Psychroflexus*, which tolerate salinities up to 10% NaCl (*P. tropicus*) [*Donachie et al.*, 2004]. Site J1 from the Salar de Ascotán (1.8 g L^{-1}) exhibited the lowest diversity and was dominated by members of *Gillisia*, isolated from microbial mats from Lake Fryxell in Antarctica [*Van Trappen et al.*, 2004].

[29] The environmental conditions of the lakes examined here include cold average temperatures and a broad range of salinities and make the high-altitude lakes of the north of Chile a unique habitat for the development of *Bacteroidetes*.

4.3. Possible Role of *Bacteroidetes* in High-Altitude Lakes

[30] Members of *Bacteroidetes* have been described of special interest not only because of their role in the mineralization of organic matter in the ocean, but also because of their association with some algae that may result in enhanced algal growth [*Grossart*, 1999]. Genera from the marine cluster of *Flavobacteriaceae* (also found in altiplanic lakes) are described as psychrophilic and saline microorganisms whose habitats are present in the Altiplano. The average optimal salinities for this group ranged between 2.5 (*Gillisia*) and 20% of total salt (*Psychroflexus tropicus*) [*Bowman*, 2006]. Apparently, their role in nonmarine environments is similar to their role in the ocean [*Bowman*, 2006]. Therefore, this group could be the responsible for the degradation of high molecular weight compounds in altiplanic lakes, including hypersaline sites revealed by the presence of *Salinibacter* in Laguna Tebenquiche [*Demergasso et al.*, 2008].

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