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NOTE

Phylotype diversity of deep-sea hydrothermal vent prokaryotes trapped by 0.2- and 0.1-μm-pore-size filters

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Abstract Eleven 16S rRNA gene clone libraries including 34 archaeal and 72 bacterial phylotypes were constructed from total 708 clones of hydrothermal vent prokaryotes trapped by 0.2- and 0.1-μm-pore-size filters. Crenarchaeota and Proteobacteria phylotypes dominated the archaeal and bacterial populations, respectively. Novel unaffiliated phylotypes occurred only in the 0.1-μm-trapped populations.

Keywords 16S rRNA gene · Sterivex filters · Clone libraries · Archaea · Bacteria

Isolates and environmental 16S rRNA gene sequences of microorganisms that pass filters with a pore size of 0.2 μm (0.2-μm filters) have been characterized (Haller et al. 1999; Vybiral et al. 1999; Elsaied et al. 2001; Velimirov 2001; Hahn 2003, 2004; Hahn et al. 2003, 2004; Boenigk et al. 2004). The 0.2-μm-passable (0.1-μm-trapped) phylotypes of deep terrestrial aquifers include novel bacterial lineages (Miyoshi et al. 2005). Hydrothermal vents also provide accesses to marine subsurface inhabitants. The submarine volcano Suiyo Seamount was drilled (Urabe et al. 2000), and the 0.2-μm-trapped populations show dominance of Proteobacteria in hydrothermal plumes (Sunamura et al. 2004), occurrence Aquificae and Nanoarchaeota in vent fluids (Nakagawa et al. 2004) and in situ growth of Alpha-/Epsilonproteobacteria and Euryarchaeota in boreholes (Higashi et al. 2004). Here we add 16S rRNA gene sequences from the 0.2-μm-passable (0.1-μm-trapped) populations collected from hydrothermal vents in Suiyo Seamount and Mariana Trough (MT). Phylotype diversities of the clone libraries are compared.

Hydrothermal fluid samples for DNA extraction were collected from MT and Suiyo Seamount (Table 1) into gamma-ray-sterilized polythene bags through impeller pumps. Possibility of entrainment of ambient seawater was not ruled out. Immediately after retrieval, 0.2-μm- and 0.1-μm-Sterivex filters (Millipore Corp., Bedford, MA, USA) were consecutively used to trap microorganisms (Miyoshi et al. 2005) and frozen onboard at –20°C. The Teflon and Tygon tubes and polypropylene bottles were washed with HCl and autoclaved before use.

Bulk DNA was extracted directly in the Sterivex housings (Miyoshi et al. 2005), and the DNA purity and quantity of sample DNA was determined by A_{260}/A_{280} as shown in Table 1. DNA preparations were used for the PCR amplification of near-full length 16S rRNA gene sequences, using the Archaea-specific Arch21F, Bacteria-specific 27F, and universal 1492R primers (DeLong 1992) on a TaKaRa PCR Thermal Cycler with Ex*Taq* (TaKaRa Bio Inc., Otsu, Japan) as follows: 1 cycle at 96°C for 3 min, and 33 cycles at 95°C for 30 s, at 53°C for 30 s (25 s for bacteria), and at 72°C for 50 s; this was followed

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Table 1 Samples of 0.2-μm-trapped (E) and 0.2 μm-passed-but-0.1-μm-trapped (N) microorganisms collected from hydrothermal vents in the Mariana Trough (MT) and Suiyo Seamount (SSM) by the manned submersible *Shinkai 6500* (6K) and the unmanned vehicle *Hyper Dolphin* (HPD)

Sample code	Dive no.	Latitude/longitude	Water depth (m)	Fluid temperature (°C)	Filtered volume (l)	Filter pore (μm)	Bulk DNA extracted (μg)
MT793-E	6K-793	12°57.172′N	2,850	~106	4.0	0.2	11.9
MT793-N		143°37.167′E			8.5	0.1	0.64
MT797-E	6K-797	12°55.129′N	2,767	~50	4.4	0.2	5.9
MT797-N		143°38.981′E			6.2	0.1	ND[a]
SSM263-E	HPD-263	28°34.290′N	1,390	4.1–62.0	4.4	0.2	ND[a]
SSM263-N		140°38.634′E				0.1	0.15
SSM264-E	HPD-264	28°34.290′N	1,385	4.1–104.8	7.0	0.2	4.39
SSM264-N		140°38.588′E				0.1	0.27

[a] Not determined due to the brownish color of the DNA suspension

by 72°C for 3 min. It should be noted that the archaeal and bacterial PCR clone libraries from the 0.2-μm-trapped MT793-E and MT797-E fractions were excluded from detailed phylogenetic analyses, because the sample fluids were collected and treated separately from other samples. In addition, shorter archaeal 16S rRNA gene sequence (ca. 800 bp) were amplified from those samples, due to the use of a different primer set Arch109F and Arch915R (Großkopf et al. 1998; Stahl and Amann 1991).

The PCR products were purified by agarose gel electrophoresis and cloned into pCR2.1-TOPO in *Escherichia coli* TOP10 transformants (Invitrogen Corp., San Diego, CA, USA). All the inserts were directly PCR-amplified with the M13f and M13r primers, and purified using QIAquick (Qiagen, Hilden, Germany) for sequencing on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDyeTerminator V3.1 Cycle Sequencing Kit (Applied Biosystems) with the primers T7, ARC344F, Arch958R, 357F, 1100R and T7R (DeLong 1992; Raskin et al. 1994).

The determined sequences were aligned by ClustalW, and the sequences having >97% similarity were grouped into a phylotype after chimera-check and registered in GenBank/EMBL/DDBJ (Tables 2, 3). Nucleotide mismatches within a phylotypes consisting of multiple clones were represented by the preferred nucleotides in the complete sequences. The phylotype sequences were homology-searched by FASTA, aligned to construct phylogenetic trees by TreeView based on the algorithms of neighbor-joining, minimum evolution and maximum parsimony. Clone library similarities were determined by *P*-values from LIBSHUFF (Singleton et al. 2001) and PHYLIP DNADIST (Felsenstein 1989). Two libraries having similar (or different) clone compositions would yield *P*-values <0.05 (or >0.05) reciprocally. A one-way *P*-value >0.05 (for example, $X \rightarrow Y$) suggests that the library Y is a *subset* of the library X (in other words, X is the *superset* of Y), i.e., $Y \subset X$. Contrary, a *P*-value >0.05 for $Y \rightarrow X$ suggests that X is a *subset* of Y (Y is the *superset* of X), i.e., $X \subset Y$. Otherwise, a *P*-value <0.05 would indicate that the X and Y are not in a binary subset-superset relation and regarded as dissimilar sets.

Initially total 814 clones were sequenced to yield 106 chimeras and 708 non-chimeras, the latter yielding 34 archaeal and 72 bacterial phylotypes (Tables 2, 3) from eight sample fluids listed in Table 1; the sample MT797-N yielded no archaeal sequences. Suspected contaminants were bacterial phylotypes (Sc-EB04 and Sc-NB05; Table 3) related to *Enterobacter* sp. and *Propionibacterium acnes*.

Total 34 archaeal phylotypes included 24 Crenarchaeota, 9 Euryarchaeota and 1 unaffiliated units (Table 2). The unaffiliated was the 0.1-μm-trapped Ma-NA02, forming a novel deep branch loosely related to Euryarchaeota (Fig. 1). Three Crenarchaeota phylotypes, 0.1-μm-trapped Sd-NA and 0.2-μm-trapped Sd-EA01/-EA05, were unaffiliated with any known sequences at <90% similarities, forming unique branches (Fig. 1). The other 18 Crenarchaeota phylotypes were affiliated with the planktonic Marine Group I, 16 of which were closely related to the deep-sea cluster (López-García et al. 2004); and, three phylotypes were affiliated with *Crenarcheales*. All Euryarchaeaota phylotypes were related at >95% similarities to known sequences such as: *Pyrococcus horikoshii* that was also present in the 0.2-μm-trapped fraction of the same sample fluid; the Okinawa Trough hydrothermal vent clones pMC2A228 and pIVWA104 (Takai and Horikoshi 1999); and, planktonic marine group II (DeLong et al. 1994). No phylotypes were affiliated with Korarchaeota and Nanoarchaeota.

Table 2 Archaeal clone number distributions and phylogenetic affiliations of phylotypes among the prokaryotes trapped by 0.1- and 0.2-μm-pore-size filters from hydrothermal vent samples collected from the Mariana Trough and the Suiyo Seamount

Phylotype	Marina Trough			Suiyo Seamount				Accession number	Closest sequence/organism (similarity >95%)	Sim[f] (%)
	0.1 6K 793	0.2 6K 793	0.2 6K 797	0.1 HPD 263	0.1 HPD 264	0.2 HPD 263	0.2 HPD 264			
Crenarchaeota										
Marine group I										
MaSc-NEA01	42			4		1		AB193956	Clone DA[c]-EC39 (AY316120)	97.8
MaScd-NEA	3			8	7	6	2	AB193974	Clone ST-12K2A (AJ347777)	99.0
MaSc-NEA02	1			5		1		AB193968		
MaSc-NEA03	1			6		1		AB193971	Clone DA-EC39 (AY316120)	95.1
Ma-NA01	1							AB193960	Clone ST-12K2A (AJ347777)	95.1
Scd-NEA01				13	13	15	22	AB193972	Clone DA-EC39 (AY316120)	97.6
Scd-NEA02				14	8	3	5	AB193965	Clone pIVWA1 (AB019727)	99.1
Scd-NA				4	1			AB193967	Clone pIVWA108 (AB019726)	99.4
Sc-EA01						1		AB193980	Clone DA-EC39 (AY316120)	96.8
Scd-EA						2	2	AB193984	Clone DA-EC39 (AY316120)	97.1
Sc-EA02						2		AB193986	Clone 74A4 (AF393466)	98.3
Sc-EA03						1		AB193988	Clone pIVWA2 (AB019730)	97.7
Sd-EA03							1	AB193999	Clone DA-EC39 (AY316120)	95.5
Sd-EA04							2	AB194000	Clone DA-EC39 (AY316120)	96.3
Sd-EA05							1	AB194001	Clone 4B7 (U40238)	98.3
Sd-EA06							2	AB194002	Clone DA-EC39 (AY316120)	97.2
Sd-EA07							2	AB194004	Clone DA-EC39 (AY316120)	99.6
Sd-EA08							1	AB194005	Clone DA-EC39 (AY316120)	96.3
Group I										
Sd-NA					1			AB193977		
Sc-EA05						1		AB193991		
Sd-EA01							1	AB193994		
Cren[a]										
Mafp-EA01		1	29					AB278136		
Maps-EA04			6					AB278137		
Maps-EA06			5					AB278138		
Euryarchaeota										
Thermo[b]										
Ma-NA03	1	11						AB193962	*P. horikoshii*[d] (AP000001)	96.1
Mafs-EA01		30						AB278139	Clone Tc-S-85 (AB095157)	96.5
Maps-EA05			3					AB278140	*T. stetteri*[e] (AY099186)	96.6

Table 2 continued

Phylotype	Marina Trough			Suiyo Seamount				Accession number	Closest sequence/organism (similarity >95%)	Sim[f] (%)
	0.1 6K 793	0.2 6K 793	0.2 6K 797	0.1 HPD 263	0.1 HPD 264	0.2 HPD 263	0.2 HPD 264			
Marine group II										
Scd-NEA03				4	1	5	2	AB193995	Clone pIVWA104 (AB019757)	96.3
Sd-NEA					1		2	AB193978	Clone DA-JyKC7 (AY534910)	97.3
Sd-EA02							3	AB193998	Clone pIVWA104 (AB019757)	98.6
Sc-EA04						1		AB193990	Clone pMC2A228 (AB019735)	97.7
Maps-EA02			5					AB278141		
Maps-EA03			5					AB278142		
Unaffiliated										
Ma-NA02	1							AB193961		
Total	50	42	53	58	32	40	48			

Note that sequences from 6K-793 and 6K-797 clone libraries derived from separate samples and thus are not included further phylogenetic analyses

[a] Crenarchaeales

[b] Thermococales

[c] Deepant

[d] *Pyrococcus horikoshii* OT3

[e] *Thermococcus stetteri* DSM 5262

[f] Similarity

Table 3 Bacterial clone number distributions and phylogenetic affiliations of phylotypes among the prokaryotes trapped by 0.1- and 0.2-μm-pore-size filters in hydrothermal vent samples from Mariana Trough and Suiyo Seamount

Phylotype	Mariana Trough				Suiyo Seamount				Accession number	Closest sequence/organism (similarity >95%)	Sim[d] (%)
	0.1 6K793	0.1 6K797	0.2 6K793	0.2 6K797	0.1 HD263	0.1 HD264	0.2 HD263	0.2 HD264			
Proteobacteria											
Aquificae											
Alpha											
Mafs-EB01			6						AB278143	*Balnearium lithotrophicum* (AB105049)	96.8
Ma-NB01	1								AB193876	Clone ZA3911c (AF382131)	97.2
MabSc-NB	3	1			1				AB193878	*Sphingomonas* sp. SKJH-30 (AY749436)	98.4
Ma-NB02	1								AB193879	Clone ZD0410 (AJ400351)	97.2
MabScd-NB	4	3			2	3			AB193929	Clone ZA3320c (AF382134)	98.8
Ma-NB04	2								AB193883	Clone ZA3911c (AF382131)	95.4
Mab-NB	1	6							AB193884	Clone AEGEAN_248 (AF406553)	97.5
Ma-NB07	1								AB193887	Clone SAR464 (U75254)	96
Ma-NB09	1								AB193889	Clone EBAC750-02H05 (AY458637)	95.6
Ma-NB10	1								AB193890	Clone SAR464 (U75254)	97.4
Ma-NB11	1								AB193891	*Sphingomonas yabuuchiae* (AB071955)	98.8
Ma-NB12	1								AB193892	Clone ZD0410 (AJ400351)	98.7
Mb-NB01		4							AB193894	Clone ZD0409 (AJ400350)	95.1
Mb-NB02		3							AB193895	Clone EBAC750-02H05 (AY458637)	97.8
Mb-NB03		7							AB193896	Clone ZA3911c (AF382131)	98.5
MbSd-NB		2				1			AB193901		
Mb-NB07		2							AB193902	Clone SAR211 (U75256)	96.1
Mb-NB08		1							AB193903	Clone EBAC750-02H05 (AY458637)	96.7
Mb-NB10		1							AB193906	Clone EBAC750-02H05 (AY458637)	97.9
Mb-NB11		3							AB193908	Clone SAR464 (U75254)	97.1
Sc-NB02					2				AB193912	Clone EBAC750-11E01 (AY458633)	98.5
Scd-NB02					3	1			AB193926	Clone MB12A07 (AY033312)	99.2
Sc-NB08					1				AB193924		
Sd-NB04						1			AB193936	*Sphingomonas* sp. JSS-7 (AF131295)	99.7
Sc-EB05							1		AB193945		
Beta											
Sd-NB03						1			AB193935		
Sc-EB01							1		AB193940	*Variovorax paradoxus* Iso1 (AY127900)	99.4
Sc-EB02							2		AB193942	Clone Sto2-2 (AY138235)	99.6
Sc-EB03							2		AB193943	Clone BW5 (AF394170)	97.2
Sc-EB06							3		AB193946	Clone SUP24-24 (AB112464)	99.7
Gamma											
MaSc-NB	12				2				AB193877	Clone F8 (AY077611)	99.1

Table 3 continued

Phylotype	Mariana Trough				Suiyo Seamount				Accession number	Closest sequence/organism (similarity >95%)	Sim[d] (%)
	0.1 6K793	0.1 6K797	0.2 6K793	0.2 6K797	0.1 HD263	0.1 HD264	0.2 HD263	0.2 HD264			
Ma-NB05	1								AB193885	*Acinetobacter* sp. TUT1001 (AB098569)	99.5
Ma-NB06	1								AB193886	*Stenotrophomonas maltophilia* (AJ293468)	99.3
Scd-NEB01					32	16	36	33	AB193910	Clone SUP05-21 (AB112459)	99.7
Scd-NB01					2	1			AB193931	Clone EBAC080-L31E09 (AY458646)	98.9
Sd-NB01						4			AB193927	*Moraxella osloensis* (X95304)	99.5
Sd-NB06						1			AB193938		
Sc-EB04							1		AB193944	*Enterobacter* sp. B901-2 (AB114268)	98.7
Sc-EB07							1		AB193947	*Pseudomonas veronii* INA06 (AB056120)	97
Sc-EB08							1		AB193948	Clone SUP05-21 (AB112459)	97.2
Sd-EB01								3	AB193952	*Pseudomonas* sp. WBC-3 (AY040872)	99.7
Sd-EB02								1	AB193953	Clone SUP05-21 (AB112459)	97.7
Mafs-EB04			9						AB278144		
Mafs-EB05			4	3					AB278145	*Alviniconcha hessleri* gill endosymbiont (AB214932)	98.9
Maps-EB02				8					AB278146	*Bathymodiolus* sp. gill endosymbiont (AJ745718)	98.7
Delta											
Mb-NB12		1							AB193909	Clone EBAC750-01B07 (AY458630)	98.6
Sc-NB01					3				AB193911	Clone EBAC750-01B07 (AY458630)	99.5
Masf-EB06			5						AB278147		
Epsilon											
Mb-NB05		1							AB193899	Clone JTB146 (AB015257)	95.9
Scd-NEB02					2	4	5	2	AB193933	Clone SUP01-02 (AB112447)	99.9
Sc-EB09							1		AB193949		
Sd-EB03								1	AB193954	Clone SUP01-02 (AB112447)	97
Sd-EB04								1	AB193955		
Mafs-EB02			17	15					AB278148		
Mafs-EB03			8	6					AB278149		
Maps-EB01				10					AB278150		
Maps-EB04				7					AB278151		
Actinobacteria											
Ma-NB03	6								AB193880	Clone ZA3111c (AF382115)	97.6
MabSd-NB	4	3				8			AB193930	Clone ZA3111c (AF382115)	99.9
Sc-NB05					1				AB193919	*P. acnes*[b] (AB042288)	99.5
Sphingobacteria											
Maps-EB03				5					AB278152		
Firmicutes											
Ma-NB08	1								AB193888	*S. sanguinis*[c] (AF543290)	99.3
GNS[a]											
Sc-NB06					1				AB193920		

Table 3 continued

Phylotype	Mariana Trough				Suiyo Seamount				Accession number	Closest sequence/organism (similarity >95%)	Sim[d] (%)
	0.1 6K793	0.1 6K797	0.2 6K793	0.2 6K797	0.1 HD263	0.1 HD264	0.2 HD263	0.2 HD264			
Candidate division											
OD1											
Mb-NB06		1							AB193900		
Mb-NB09		1							AB193905		
Sc-NB03					1				AB193914		
Sc-NB07					1				AB193923		
OP11											
Mb-NB04		3							AB193897		
Sc-NB09					1				AB193925		
SB1											
Sc-NB04					1				AB193918		
Sd-NB02						1			AB193932		
Sd-NB05						2			AB193937		
Total	42	43	51	54	56	44	54	41			

Note that sequences from 6K-793 and 6K-797 clone libraries were derived from separate samples and thus are not included further phylogenetic analyses

[a] Green non-sulfur bacteria

[b] *Propionibacterium acnes* ATCC6919

[c] *Streptococcus sanguinis* ChDC OS38

[d] Similarity

Total 72 bacterial phylotypes included 24 Alphaproteobacteria, 15 Gammaproteobacteria, 9 Epsilonproteobacteria, 5 Betaproteobacteria, 3 Deltaproteobacteria, 3 Actinobacteria, 1 Aquificae, 1 Sphingobacteria, 1 Firmicutes, 1 green non-sulfur bacteria and 9 candidate divisions (Table 3). The phylotypes ascribed to the candidate divisions were only loosely related to OP11, OD1 and SB1 at 75–92% similarities, and represented novel candidate division branches. These phylotypes were found only in the 0.1-μm-trapped libraries, while the 0.2-μm-trapped libraries were dominated by Proteobacteria (Table 3).

The 0.1- and 0.2-μm-trapped libraries from the same sample fluids were largely dissimilar, with only three combinations yielded *P*-values >0.05 (bolded and underlined values in Tables 4, 5). The three combinations (SSM263-EA/NA, SSM264-EA/NA and SSM264-EB/NA) showed that the 0.2-μm-trapped libraries are subsets of the 0.1-μm-trapped counterparts, i.e., SSM263-EA $\subset$ NA, SSM264-EA $\subset$ NA and SSM264-EB $\subset$ NB. These subset–superset relations indicate that the 0.1-μm-trapped NB libraries contained higher diversities in 16S rRNA gene sequences. The other libraries were dissimilar in phylotype compositions with *P*-values <0.05. For bacterial libraries in general, 0.1-μm-trapped libraries showed higher phylotype diversities than 0.2-μm-trapped ones, as demonstrated by evolutionary distances and coverages (data not shown), as previously reported for deep aquifer populations (Miyoshi et al. 2005). Similar but less clear tendency was found in the archaeal libraries. The site-to-site comparison showed higher archaeal diversity in Suiyo Seamount but higher bacterial diversity in MT.

Seven representative phylotypes of the Alphaproteobacteria-dominated MT libraries (Table 3) were affiliated with the SAR11 clade (Fig. 1). SAR11 is the most abundant marine bacterium and is very small, i.e., <1 μm in size (Morris et al. 2002; Rappé et al. 2002). Since SAR11-related clones were found even in the 0.1-μm-trapped libraries, total abundance of SAR11 should be higher than the global estimate of 2.4×10^{28} cells trapped by 0.2-μm-filters (Morris et al. 2002).

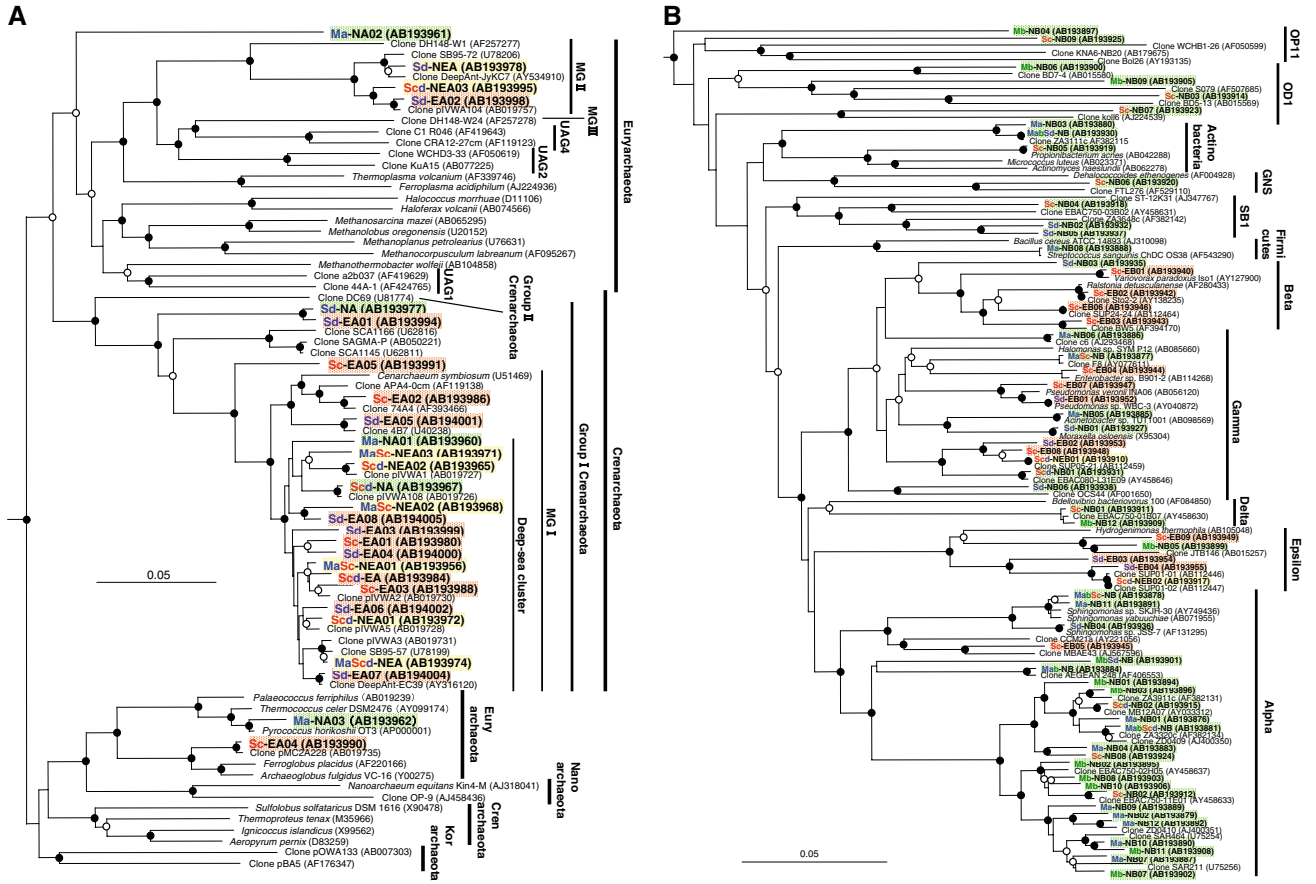

Fig. 1 Neighbor-joining phylogenetic tree based on nearly-complete archaeal (a) and bacterial (b) 16S rRNA gene sequences in phylotypes of hydrothermal vent samples from the Mariana Trough and the Suiyo Seamount. Sample code designations listed in Table 1 that are followed by **a** to indicate the archaeal phylotypes. The 16S rRNA gene sequences of the Aquaficales Aquifex aeolicus (AJ309733) and Hydrogenobacter hydrogenophilus (Z30242) were used as outgroups but were pruned from the tree. *Green*, *red* and *yellow* backgrounds indicate the phylotypes trapped by the 0.1-pore-size filter, 0.2-μm-pore-size filter and both filters, respectively. Bootstrap values at branching nodes after 1,000 resampling are represented by *filled circles* (>75%) and *open circles* (75%> ○ >50%), and *no circles* (<50%). Sample location codes such as Ma and Sc are indicated in *different colors*

Table 4 LIBSHUFF *P*-value distribution in the archaeal 16S rRNA gene clone libraries derived from hydrothermal vents in the Mariana Trough and Suiyo Seamount

Library code	Clone number	Heterologous (*Y*)				
		MT793-NA	SSM263-NA	SSM264-NA	SSM263-EA	SSM264-EA
Homologous (X)						
MT793-NA	50	–	**0.074**	**0.510**	0.020	0.001
SSM263-NA	58	0.001	–	**0.077**	0.021	0.003
SSM264-NA	32	0.001	**0.436**	–	**0.176**	**0.209**
SSM263-EA	40	**0.660**	**0.772**	**0.435**	–	**0.886**
SSM264-EA	48	**0.092**	0.003	**0.239**	0.030	–

The *P*-values >0.05 are bolded, and further underlined if resulting from the same sample fluids

Table 5 LIBSHUFF *P*-value distribution in the bacterial 16S rRNA gene clone libraries derived from hydrothermal vents in the Mariana Trough and Suiyo Seamount

Library code	Clone number	Heterologous (*Y*)					
		MT793-NB	MT797-NB	SSM263-NB	SSM264-NB	SSM263-EB	SSM264-EB
Homologous (X)							
MT793-NB	42	–	0.001	0.001	0.001	0.001	0.001
MT797-NB	43	0.004	–	0.001	0.001	0.001	0.001
SSM263-NB	56	0.001	0.001	–	**0.139**	0.001	0.001
SSM264-NB	44	0.001	0.010	0.001	–	0.001	0.001
SSM263-EB	54	0.001	0.001	0.007	0.045	–	0.009
SSM264-EB	41	0.001	0.001	**0.280**	**0.251**	**0.444**	–

The *P*-values >0.05 are bolded, and further underlined if resulting from the same sample fluids

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