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Composition of archaeal, bacterial, and eukaryal RuBisCO genotypes in three Western Pacific arc hydrothermal vent systems

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Abstract We studied the diversity of all forms of the RuBisCO large subunit-encoding gene *cbbL* in three RuBisCO uncharacterized hydrothermal vent communities. This diversity included the archaeal *cbbL* and the forms IC and ID, which have not previously been studied in the deep-sea environment, in addition to the forms IA, IB and II. Vent plume sites were Fryer and Pika in the Mariana arc and the Suiyo Seamount, Izu-Bonin, Japan. The *cbbL* forms were PCR amplified from plume bulk microbial DNA and then cloned and sequenced. Archaeal *cbbL* was detected in the Mariana samples only. Both forms IA and II were amplified from all samples, while the form IC was amplified only from the Pika and Suiyo samples. Only the Suiyo sample showed amplification of the form ID. The form IB was not recorded in any sample. Based on rarefaction analysis, nucleotide diversity and average pairwise difference, the archaeal *cbbL* was the most diverse form in Mariana samples, while the bacterial form IA was the most diverse form in the Suiyo sample. Also, the Pika sample harbored the highest diversity of *cbbL* phylogenetic lineages. Based on pairwise reciprocal library comparisons, the Fryer and Pika archaeal *cbbL* libraries showed the most significant difference, while Pika and Suiyo showed the highest similarity for forms IA and II libraries. This suggested that the Fryer supported the most divergent sequences. All archaeal *cbbL* sequences formed unique phylogenetic lineages within the branches of anaerobic thermophilic archaea of the genera *Pyrococcus*, *Archaeoglobus*, and *Methanococcus*. The other *cbbL* forms formed novel phylogenetic clusters distinct from any recorded previously in other deep-sea habitats. This is the first evidence for the diversity of archaeal *cbbL* in environmental samples.

Keywords Deep-sea hydrothermal vents · RuBisCO *cbbL* · Archaea · Bacteria · Eukarya · Statistical analyses · Phylogenetic analyses

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Introduction

The discovery of deep-sea hydrothermal vents 28 years ago opened a window to largely diverse and hitherto unknown autotrophic microbial communities. Most microbial communities that have the ability to fix inorganic carbon dioxide possess the key Calvin-Benson cycle enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39). Recent research has detected RuBisCO in some archaeal and bacterial species that have never been reported to posses the Calvin cycle (Eisen et al. 2002; Finn and Tabita 2003).

The main constituent of the RuBisCO enzyme is the large subunit encoded by the gene *cbbL* (Kusian and Bowien 1997). Diversity in *cbbL* has been used to classify RuBisCO into different forms, from forms I and II identified earlier to the more recently identified archaeal RuBisCO (referred as form III) and form IV (RuBisCO-like *cbbL* protein) (Ashida et al. 2003). The completion of several genome projects has offered indications that euryarchaea contain putative RuBisCOs, which are distinguished from the aforementioned form I and II enzymes. The RuBisCO homologues have been detected in the genomes of *Methanocaldococcus jannaschii* and *Archaeoglobus fulgidus* (Bult et al. 1996; Klenk et al.

1997). Both archaeal species are clearly different from typical organisms known to fix carbon dioxide through the Calvin cycle. In addition to these organisms, some thermophilic archaea of the genus *Pyrococcus*, renamed *Thermococcus* (Ezaki et al. 1999), contain form III enzyme (Kitano et al. 2001).

The existence of RuBisCO proteins in archaea is not limited to thermophiles. The complete genomes of *Methanosarcina acetivorans* (Galagan et al. 2002), *M. mazei* (Deppenmeier et al. 2002), and *M. barkeri*, mesophilic heterotrophic methanogens, have also been found to contain RuBisCO genes. Most archaeal RuBisCOs are closer to those of form II than form I (Watson et al. 1999). As an exception, RuBisCO activity (not genes) was detected in the extreme halophilic archaeon *Haloferax mediterranei*, and found to resemble the form I protein found in higher plants (Rajagopalan and Altekar 1994). The diversity and role of RuBisCO in archaea are still unclear. Recently, RuBisCO was discovered in the autotroph *Chlorobaculum tepidum*, which uses the reverse tricarboxylic acid (rTCA) cycle as an alternative CO_2 fixation pathway (Li et al. 2005). Although some species of deep-sea autotrophic Epsilonproteobacteria that use rTCA lack RuBisCO genes (Campbell and Cary 2004; Takai et al. 2005), the existence of RuBisCO in Epsilonproteobacteria is still undergoing concept. The occurrence of RuBisCO genes in these archaea and bacteria that lack the Calvin cycle may indicate that these microorganisms either harbor other autotrophic pathways or have an ancestral autotrophic character, which exists in the form of RuBisCO genes. These new findings of *cbbL* genes suggested the lateral gene transfer of *cbbL* among archaea, bacteria and eukarya (Delwiche and Palmer 1996) and opened the possibility of studying the composition of *cbbL* gene variants in a wide range of organisms.

RuBisCO form I has been classified into four types, IA–ID, based on the inferred amino acid homology of the *cbbL* genes (Watson and Tabita 1997). Forms IA and IC occur primarily in Proteobacteria. Forms IB and ID occur predominately in cyanobacteria and eukarya chloroplasts (Tabita 1999). Form II is widespread in some anoxygenic Alphaproteobacteria and species of thiobacilli, which also have the ability to possess form IA (English et al. 1992).

All previous studies on the diversity of *cbbL* from deep-sea environments focused only on the bacterial forms IA/IB and II. Form IA was recorded in free-living hydrothermal vent Proteobacteria (Elsaied and Naganuma 2001; Elsaied et al. 2002b; Campbell and Cary 2004) and in the symbionts of several deep-sea hydrothermal vent mollusks and some Pogonophora species (Stein et al. 1990; Kimura et al. 2003; Schwedock et al. 2004). Form IB has only been recovered from a vent plume at a Mid-Okinawa Trough hydrothermal vent. Form II has been recorded in free-living autotrophic microbial communities at only two hydrothermal vent sites (Elsaied and Naganuma 2001; Campbell and Cary 2004). On the other hand, form II was detected as the predominant form in cold seeps, symbionts of tubeworms and some deep-sea clams (Robinson et al. 1998; Elsaied et al. 2002a). Generally, neither form IC nor ID has been studied in deep-sea environments.

To conceptually map the diversity of *cbbL* gene in an environmental sample, investigation of all known *cbbL* forms is required. To our knowledge, the present study is the first aimed at investigating the composition of RuBisCO *cbbL* gene variants from archaea, bacteria and eukarya living in deep-sea hydrothermal vents. We used newly designed PCR primer sets to amplify archaeal *cbbL*, and the *cbbL* forms IC and ID. Here, we report the diversity of RuBisCO *cbbL* gene within and between the samples using statistical analyses. Also, we draw the first phylogenetic tree for archaeal *cbbL*, which recovered from environmental samples.

Materials and methods

Sample collection and preparation for DNA analysis

The vent plumes were collected from three western arc distinct hydrothermal vent sites. Fryer (12°57.7114′ N; 143°38.0839′ E) and Pika (12°55.1433′ N; 143°38.9283′ E) sites are located at the Mariana arc and represent old and newly discovered spreading areas, respectively (Utsumi et al. 2004). The hydrothermal vents at Fryer and Pika are characterized as white and black smokers, respectively (Utsumi et al. 2004). The third site is located at the Suiyo Seamount, a submarine black smoker volcano found in the Izo-Bonin arc (28°.5666667′ N; 140°.65′ E), Japan (Sunamura et al. 2004). The samples were collected through two Japanese Archaean Park project cruises, one with the manned submersible Shinkai 6500, dives 793 (Fryer site) and 797 (Pika site), and the second with Shinkai 2000, dive 1,233 to the Suiyo Seamount. At each site, approximately 1,000 ml of hot plume was collected a few centimetres distant to the eruptive vent and filtered on a single cylindrical 0.2 μm filter membrane unit (type Sterivex-GS, Millipore Corp., USA). Filters were washed with 10 ml sterile SET buffer (20% sucrose, 50 mM EDTA, 50 mM Tris–HCl, pH 7.6). The inlet and outlet of the filters were capped, and the filters were stored at −30°C until processed.

DNA extraction and PCR amplification of the *cbbL*

Bulk microbial DNA was extracted essentially within the Sterivex-GS filter housing according to Elsaied and Naganuma (2001). New PCR primer sets were designed to amplify the archaeal, IC and ID *cbbL*s (Table 1). The archaeal *cbbL* primers were designed from multiple alignments of *cbbL* sequences recorded through whole genome sequences of *M. jannaschii* (**U67564**), *M. acetivorans* (**AE011176**), *M. mazei* (**AE013355**), *A. fulgidus* (**AE000989**), *Thermococcus kodakaraensis* (**AB018555**), *Pyrococcus furiosus* (**AE010224**), *Pyrococcus horikoshii*

Table 1 Primers used for amplification of different forms of *cbbL*

Primer	Target *cbbL* form	Sequence	Tested species or references
Arch-375f	Archaea	5′-GCH GGR AAY ATY TTY RGM ATG AAR-3′	*Methanocaldococcus jannaschii*
Arch-891r		5′- KGC NGM ATG CAT RSM NCK GTG GSC RTG-3′	
IAB-595f	IA and IB	5′-GAY TTM ACT AAR GAT GAY GA-3′	Elsaied and Naganuma (2001)
IAB-1385r		5′- TCG AAC TTG ATT TCT TTC CA-3′	
IC-537f	IC	5′-ACS AAG CCC AAG CTG GGC CTG TCG GGC-3′	*Wautersia eutropha*
IC-1212r		5′-GAT GGT GCC GCC GCC GAA CTG-3′	
ID-537f	ID	5′-GTA AAA CCT AAA TTA GGT YTA TCT GGT-3′	*Olisthodiscus luteus*
ID-1212r		5′-AAT AGT ACC ACC ACC AAA TTG -3′	
II-537f	II	5′-ATC ATC AAR CCS AAR CTS GGC CTG CGT CCC-3′	Elsaied and Naganuma (2001)
II-1113r		5′-GGC GTT CAT GCC GCC GSW GAT GAT CGG SGT-3′	

(**BA000001**), and *Pyrococcus abyssi* (**AJ248286**). The primers Arch-375f and Arch-891r represented the sequence positions from 354 to 375 and from 865 to 891, respectively, of the *cbbL* open reading frame of *Synechococcus* PCC 6301 (Shinozaki et al. 1983). The PCR mixture composition was according to a previous study (Elsaied and Naganuma 2001). PCR was performed with an initial denaturation step of 3 min at 95°C. The reaction was continued with 30 cycles of 1 min at 95°C, 2 min at 46°C, and 3 min at 72°C, with a final extension of 10 min at 72°C.

The primer set IC-537f/IC-1212r (Table 1) was designed from multiple alignment of the *cbbL* form IC sequences incorporated to date in the DNA databases and related to the genera *Alcaligenes*, *Nitrosospira*, *Bradyrhizobium*, *Ralstonia*, *Xanthobacter*, *Rhodobacter* and several uncultured clones recovered from environmental samples as reported by Nanba et al. (2004). The ID-537f/ID-1212r primer set was constructed from multiple alignment of *cbbL* form ID sequences incorporated to date in the DNA databases and belonged to the genera *Olisthodiscus*, *Trematocarpus*, *Pterocladiella* (Rhodophyta) and uncultured stramenopile clones (Paul et al. 2000). Both primer sets IC-537f/IC-1212r and ID-537f/ID-1212r corresponded to positions 511 to 537 (forward primer) and 1191 to 1212 (reverse primer) of the *cbbL* gene of *Synechococcus* PCC 6301 (Table 1). The PCR mixture and conditions for amplification of the *cbbL* forms IC and ID were the same as those of archaeal *cbbL* except that the annealing temperatures for primers IC-537f/IC-1212r and ID-537f/ID-1212r were 54 and 46°C, respectively. The primers and PCR conditions used for amplification of form IA/IB and form II were as described by Elsaied and Naganuma (2001).

Construction of clone libraries and sequence analyses

The products of triplicate PCR reactions for each *cbbL* amplification were combined and cloned into *Escherichia coli* using a TOPO TA cloning kit according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). White transformed clones that were screened to contain the correct insert size were grown overnight in Luria–Bertani broth prior to plasmid extraction with a Qiagen plasmid purification kit (Qiagen, USA). Diversity of the inserts in the plasmids was analyzed by direct sequencing using vector primer T7 and an ABI model 377 sequencer (Applied Biosystems, Foster City, CA).

Clone sequences were introduced to FASTA to determine their similarity to known *cbbL* sequences. Sequences for which the *cbbL* most closely matched were submitted to Transeq (European Bioinformatics, http://www.ebi.ac.uk/emboss/transeq/) to obtain the inferred amino acid sequences. The correct reading frames were determined from the presence of diagnostic motifs, including forward and reverse primer sites. Inferred amino acid sequences were aligned using Clustal W software (DDBJ, http://www.ddbj.nig.ac.jp/search/clustalw-e.html). Sequences that had 100% amino acid identity within each library were grouped into an Operational RuBisCO Unit (ORU) according to Elsaied and Naganuma (2001). Also, nucleotide sequences of the ORUs were aligned according to the amino acid alignments.

The diversity of the ORUs was analyzed by several methods. (1) Rarefaction analyses (Simberloff 1978) were used to determine the total expected number of ORUs in the samples as described by Hurlbert (1971). Calculations were performed with the Analytic Rarefaction freeware program (http://www.uga.edu/_strata/software/Software.html). (2) Nucleotide diversity and average pairwise differences between ORUs for each library were estimated using the freeware program Arlequin (Schneider et al. 2000). The two most similar ORUs within a clone library differed in one amino acid. This pairwise difference can be extended if the nucleotides are used as a diversity tool. To extrapolate similarities or differences within clone libraries constructed for each RuBisCO form, we performed statistical analyses based on ORU pairwise nucleotide differences. These biodiversity analyses were possible because the clone libraries were created under almost identical conditions. For a given set of aligned ORU sequences within a library, nucleotide diversity is a measure of the probability that two randomly chosen homologous nucleotides will differ. Average pairwise difference is a measure of the number of nucleotide differences observed when each

ORU sequence is compared with all other corresponding ORU sequences (Schneider et al. 2000). (3) Analysis of molecular variance (AMOVA) as implemented with Arlequin was used to estimate the significance of differences in population pairwise fixation indices (F_{ST} values) among *cbbL* libraries. (4) The computer program LIBSHUFF (Singleton et al. 2001) was used to estimate homologous and heterologous coverage of clone libraries as a function of evolutionary distance for pairwise reciprocal comparisons (library A compared with library B and vice versa). Differences in coverage were considered significant at P values of < 0.05.

The phylogenetic analyses based on amino acid and nucleotide sequences of the ORUs and corresponding *cbbL* sequences from the database bank were performed by applying the neighbor-joining algorithm and drawing the trees using the Treeview software. The branching patterns of the constructed phylogenetic trees were confirmed by reconstruction of the phylogenies using two other methods of analysis, namely maximum-parsimony and maximum-likelihood, contained within the Phylip package.

The *cbbL* ORU sequences resulting from this study were deposited in the database bank under the accession numbers **AB201846** to **AB201894** for Fryer archaeal ORUs; **AB201895** to **AB201943** for Pika archaeal ORUs; **AB206404** to **AB206406** for Fryer form IA ORUs; **AB175492** to **AB175494**, **AB175813**, **AB176513** to **AB176515**, and **AB180250** to **AB180253** for Pika from IA ORUs; **AB175487** to **AB175491**, **AB175810** to **AB175812**, **AB180056** to **AB180057**, and **AB181171** to **AB181182** for Suiyo form IA ORUs; **AB181166** to **AB181170** for Pika form IC ORUs; **AB181162** to **AB181165** for Suiyo IC ORUs; **AB190992** to **AB190993** for Suiyo ID ORUs; **AB206044** to **AB206058** for Fryer form II ORUs; **AB174753** to **AB174761** for Pika form II ORUs; and **AB174747** to **AB174752** for Suiyo form II ORUs.

Results and discussion

Efficiency of the RuBisCO *cbbL* designed primers

This work aimed to cover the diversity of almost all known RuBisCO *cbbL* forms in the studied samples. The archaeal *cbbL* had no PCR primer set to amplify it from the environmental bulk microbial DNA. Also, other forms of RuBisCO *cbbL* need powerful modified PCR primers to improve the chance of amplification of these forms. Several considerations were involved in the design of an efficient primer set for amplification of archaeal *cbbL*. Only few archaeal *cbbLs* have been deposited in the database bank. Moreover, the archaeal *cbbLs* are highly diverse in sequence length, including the deletion of 1–14 codons in some species (Ezaki et al. 1999). This may result in a PCR product with different sizes if the PCR amplifies the variable *cbbL* sequence part from bulk microbial DNA, leading to misdetection of the target gene amplicon. To avoid this problem, the primers Arch-375f and Arch-891r were designed from the *cbbL* sequence parts that flank the codons of catalytic site amino acids Asparagine-120 and Arginine-292, respectively corresponding to those of *Synechococcus* sp. These primers could amplify a sequence part that does not include the major varieties of sequence deletions. This helped to generate a PCR product with an almost fixed size of 531–540 bp. This primer set amplified not only the *cbbL* from the common strain *M. jannaschii* (data not shown), but also a wide range of *cbbLs* that belong to different archaeal genera as presented in this work.

The codon regions of the *cbbL* catalytic site amino acid groups T(T) IKPKLG and T(V) KPKLG represented specific sequences for the design of primers IC-537f and ID-537f, respectively. The primers IC-1212r and ID-1212r were designed from the codons that encode the conserved IC/ID *cbbL* catalytic amino acids QFGGGTI. These primers differed from each other in seven nucleotides, sufficient to make each of the primers specific to its corresponding form (Table 1). These IC and ID primer sets provided *cbbL* product sizes longer than those produced by Nanba et al. (2004) and Paul et al. (2000), respectively. This gave an extended view to screen the diversity of these *cbbL* forms.

PCR amplification of the archaeal *cbbL*

Both Mariana Fryer and Pika samples showed amplification of the archaeal *cbbL* (Table 2). Attempts to amplify archaeal *cbbL* from Suiyo sample DNA failed, but PCR was achieved by mixing the Suiyo DNA with other DNAs of positive control *M. jannaschii* or Fryer and Pika samples. This indicated that the Suiyo DNA was free of any PCR inhibitor, and suggested that the Suiyo sample may lack RuBisCO-carrying archaea. This suggestion is supported by the predominance of two

Table 2 Scheme of PCR amplification of different forms of *cbbL* in the studied samples

Sample	RuBisCO form (amplified product size bp)					
	Archaea	Form IA	Form IB	Form IC	Form ID	Form II
Fryer	✓ **(531, 537)**	✓ **(813)**	×	×	×	✓ **(630, 636)**
Pika	✓ **(531, 537)**	✓ **(813)**	×	✓ **(700)**	×	✓ **(630)**
Suiyo	×	✓ **(813)**	×	✓ **(700)**	✓ **(700)**	✓ **(630)**

✓ Amplified, × Not amplified

Table 3 Nucleotide diversities and average pairwise differences $\theta(\pi)$ between ORUs within the clone libraries

Clone library	No. ORUs	No. of variable positions[a]	Nucleotide diversity	$\theta(\pi)$
Fryer (Archaea)	49	393	0.203 (0.098)	108.92 (47.55)
Pika (Archaea)	49	404	0.212 (0.103)	114.13 (49.81)
Fryer (Form IA)	3	169	0.092 (0.045)	52.39 (23.04)
Pika (Form IA)	11	229	0.156 (0.075)	88.68 (38.78)
Suiyo (Form IA)	22	274	0.180 (0.087)	102.43 (44.74)
Pika (Form IC)	5	200	0.036 (0.018)	25.29 (11.29)
Suiyo (Form IC)	4	146	0.034 (0.017)	23.91 (10.69)
Suiyo (Form ID)	2	23	0.018 (0.009)	11.27 (5.20)
Fryer (Form II)	15	374	0.245 (0.118)	156.12 (68.02)
Pika (Form II)	9	259	0.070 (0.034)	44.07 (19.43)
Suiyo (Form II)	6	282	0.132 (0.064)	83.32 (36.45)

[a]Variable positions represent the number of variable nucleotide sites (out of total recorded sequence lengths) within each library. Nucleotide diversity and average pairwise difference $\theta(\pi)$ are expressed as means (±SD) for each library

phylotypes of Epsilon- and Gammaproteobacteria in Suiyo vent plumes (Sunamura et al. 2004). The PCR primers Arch-375f and Arch-891r could amplify two different *cbbL* fragment lengths of 531 and 537 bp from both Fryer and Pika samples (Table 2). These amplification results indicated the possibility of bias in PCR amplification was minimized. This is because the PCR was tested using a range from 26 to 30 cycles, and then the amplicons were combined for cloning (Suzuki and Giovannoni 1996). Hence, the PCR could screen as much as possible of the actual composition of archaeal *cbbL* gene variants in the Mariana samples. Nevertheless, additional experiments are needed to ensure the complete avoidance of possible bias in PCR.

PCR amplification of bacterial and eukaryal *cbbL*

The studied samples showed the amplification of several bacterial *cbbL* forms and one form of eukaryal *cbbL* (Table 2). The bacterial form IA was amplified from all samples. In contrast, form IB was not detected in any sample. Bacterial form IC was amplified from the DNA of only Pika and Suiyo samples. Eukaryal form ID was detected only in the Suiyo sample (Table 2). Accordingly, the Suiyo sample was dominated by bacterial *cbbL*, but may be contaminated with eukaryal phytoplanktonic species that carry form ID. The possibility of the flux of surface water phytoplankton to the deep-sea exists, since form IB, which characterizes cyanobacteria, was detected in a hydrothermal vent plume (Elsaied and Naganuma 2001), in addition to the occurrence of a green sulfur anaerobic phytoplankton in a hydrothermal vent (Beatty et al. 2005). This sinking of surface water phototrophs into the deep-sea implies the possibility of genetic exchange between populations previously assumed to be genetically isolated, i.e., autotrophs in the surface water and those in the deep-sea (Turely and Mackie 1995). Nevertheless, forms IB and ID represent only a minor fraction of hydrothermal vent autotrophic communities compared with the dominant form IA (Elsaied and Naganuma 2001; Campbell and Cary 2004).

All samples showed the amplification of form II (Table 2). Also, this form was previously detected in a Mid-Okinawa Trough vent plume (Elsaied and Naganuma 2001). These features may distinguish free living autotrophic communities at Western Pacific arc hydrothermal vents from those which lack this form at certain sites of the Trans-Atlantic Geotraverse (TAG) and Loihi Seamounts (Elsaied and Naganuma 2001; Elsaied et al. 2002b).

Dominance of archaeal *cbbL* in Mariana samples and bacterial form IA *cbbL* in Suiyo sample based on statistical analyses of clone libraries

The fixed number of fifty clones from each clone library was screened by direct sequencing. Sequence analysis of 550 clones, representing all clone libraries, produced a total 175 ORUs. The number of ORUs in the clone libraries was used to measure the *cbbL* diversity in the studied samples (Table 3). Forty-nine archaeal *cbbL* ORUs, the highest recorded ORU number, were recovered from each of the Mariana samples. The Suiyo sample recorded 22 ORUs for bacterial form IA, a higher number than that for IC, ID and form II recorded in the same sample. These observations were reflected in the total expected diversity of *cbbL* in the samples (Fig. 1). The rarefaction curves of archaeal (Fig. 1a, b) and Suiyo bacterial form IA (Fig. 1c) *cbbLs* did not reach clear saturation, indicating that further sampling of these clone libraries would have revealed additional diversity. An underestimation of the diversity of Fryer and Pika archaeal *cbbL* and Suiyo form IA was expected, as the coverage of the libraries was estimated to be 79.5, 86.2 and 62.2%, respectively (Table 5). The ORU nucleotide diversities and average pairwise differences $\theta(\pi)$ within archaeal clone libraries were generally high compared with other *cbbL* clone libraries (Table 3). These results indicated the wide diversity of archaeal

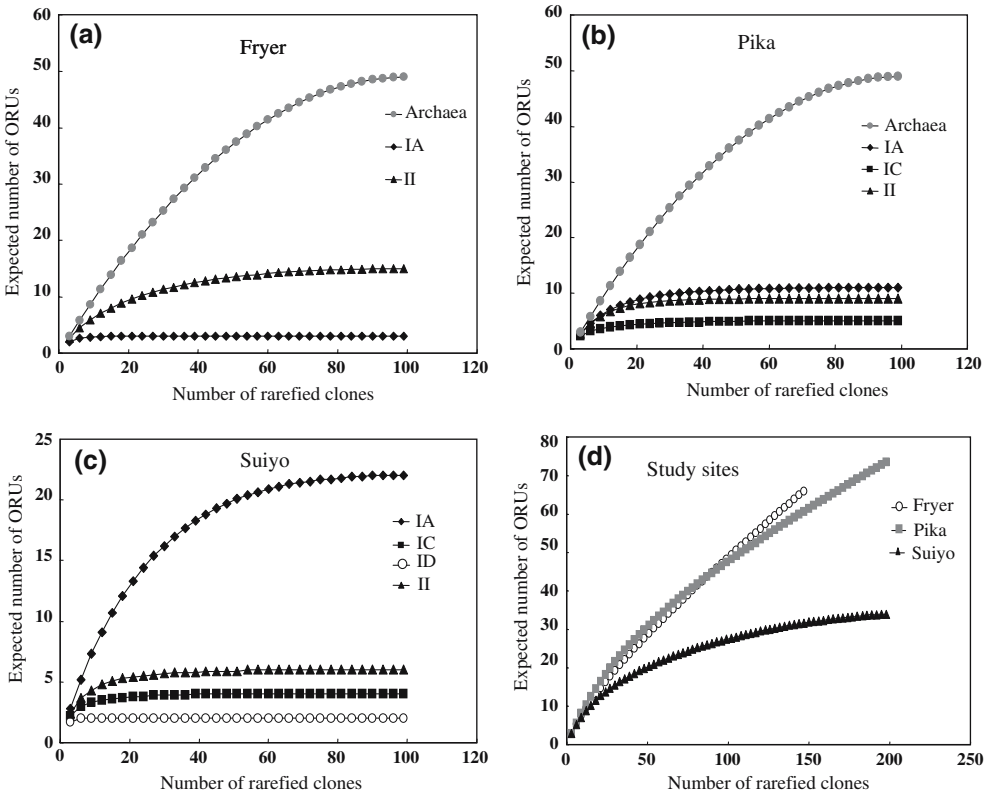

Fig. 1 Rarefaction curves for the expected number of ORUs for each RuBisCO form based upon grouping of clones that have 100% amino acid identity in each clone library, **a** Fryer, **b** Pika, and **c** Suiyo. **d** Rarefaction curves for the total expected number of ORUs for all forms of RuBisCO recorded in each sample

Table 4 Corrected average nucleotide pairwise differences $\theta(\pi)$ of ORUs and pairwise fixation indices (F_{ST}) between clone libraries

(RuBisCO form)	Site	$\theta(\pi)$ and F_{ST} for site		
		Fryer	Pika	Suiyo
Archaea	Fryer		82.44435	
	Pika	**0.42504***		
IA	Fryer		65.82530	61.16664
	Pika	**0.48273***		9.99216
	Suiyo	**0.44140***	**0.09467***	
IC	Pika			116.65311
	Suiyo		**0.82582***	
II	Fryer		54.39175	39.90566
	Pika	**0.35209***		5.25657
	Suiyo	**0.25000***	**0.07624***	

Corrected average, pairwise difference $\theta(\pi)$ = Average number of nucleotide pairwise differences for ORUs between two libraries − the average of sum of nucleotide pairwise differences for ORUs within the two libraries. The $\theta(\pi)$ values are shown above diagonal; F_{ST} values (bold) are shown below diagonal

*F_{ST} values show significance at a level $P < 0.05$

cbbL in Mariana plumes and suggested that archaea may play an efficient role in the primary production of organic carbon, while bacteria perform the same role in Suiyo. The dominance of bacterial form IA in the Suiyo sample paralleled with that recorded previously in other deep-sea hydrothermal vents (Campbell and Cary 2004). In addition, the Suiyo form IA ORU number was greater than those recovered from all previously studied hydrothermal vents (Elsaied and Naganuma 2001; Elsaied et al. 2002b; Campbell and Cary 2004), indicating that the Suiyo vent plume may harbor the most diverse form IA discovered to date.

Divergence of Fryer archaeal and bacterial *cbbL* sequences

Comparisons of the ORUs among libraries by AMOVA (Table 4) revealed that the F_{ST} values between Fryer and Pika or Suiyo were higher than those between Pika and Suiyo in the corresponding detected RuBisCO forms (archaea, IA and II) (Table 4). The results from LIBSHUFF analyses also confirmed population differentiation but provided somewhat different insights (Table 5). Paired reciprocal library comparisons showed that the Fryer archaeal and form II libraries differed significantly

from those of Pika and Suiyo but the form II Pika and Suiyo libraries did not differ from that of Fryer. One explanation for this pattern is that the Fryer archaeal sequences did not encompass the Pika sequences and vise versa, indicating the highly divergence between the two populations. On the other hand, the diversity of the Fryer form II library sequences encompassed and described some sequences in the Pika and Suiyo libraries, while they account for only a portion of the Fryer form II diversity. Both Pika and Suiyo showed high population coverage similarities in both form IA and form II libraries. On the other hand, both Pika and Suiyo form IA sequences encompassed those of Fryer, while the reverse did not hold true.

These statistical explanations were consistent with the phylogenetic distribution of sequences (Figs. 2, 3, 4). The recovered archaeal ORUs from Fryer and Pika formed three main clusters in the branches of hyperthermophilic anaerobic archaea (Fig. 2). Cluster III-1 included most of the recorded Fryer ORUs and seven Pika ORUs, occurring as two separated sequence groups that linked with the *cbbL* of thermophilic euryarchaeon *T. kodakaraensis* strain KOD1. The biggest fraction of Pika ORUs showed divergence with a group of five Fryer ORUs in the cluster III-2, which was affiliated with the sulfate reducer *A. fulgidus*. Cluster III-3 was represented by two divergent groups, each of seven ORUs, claded with *M. jannaschii*. The sequence identities between the Fryer and Pika ORUs within clusters III-1, III-2 and III-3 were 85–88%$_{aa}$ (amino acid identity), i.e. 81–82%$_{ND}$ (nucleotide identity), 83–85%$_{aa}$ (73–74%$_{ND}$) and 54–55%$_{aa}$ (53–55%$_{ND}$), respectively. This indicated that the cluster III-3 harbored the most divergent Fryer and Pika archaeal *cbbL* sequences. The divergence of Fryer ORUs also extended to those of forms IA and II (Figs. 3, 4). Both Pika and Suiyo form IA ORUs were distributed as closely related sequences distinct from those of Fryer (Fig. 3). Form IA ORUs were located within five clusters. The first cluster, IA-1, consisted of four Pika and nine Suiyo ORUs. This cluster was not affiliated with the known *cbbL*s, and represented additional new genotypes to the deep-sea RuBisCO form IA. The Pika form IA ORUs showed high identities of 95–97%$_{aa}$ (98%$_{ND}$), 93%$_{aa}$ (85%$_{ND}$), 97–98%$_{aa}$ (97%$_{ND}$) with those of Suiyo within clusters IA-1, IA-2 and IA-4, respectively. On the other hand, the Fryer ORUs IAF1 and IAF2 that form the unique cluster IA-5 exhibited the lowest identity values of 77–78%$_{aa}$ (77%$_{ND}$), 76–77%$_{aa}$ (72–75%$_{ND}$), 76–77%$_{aa}$ (72%$_{ND}$) and 80–82%$_{aa}$ (72–74%$_{ND}$) with Pika and Suiyo ORUs in the clusters IA-1, IA-2, IA-3 and IA-4, respectively. Also, the Fryer form II ORUs, which characterized cluster II-1 (Fig. 4), represented average identity values of 72.5%$_{aa}$ (68%$_{ND}$) and 83.6%$_{aa}$ (75.2%$_{ND}$) with Pika and Suiyo ORUs, which were concentrated in clusters II-3 and II-4, respectively. On the other hand, both Pika and Suiyo ORUs were distributed close to each other, reflecting higher amino acid and nucleotide identities of >95% within each of clusters II-3 and II-4. The ORUs IIF1 in cluster II-3, and IIF2, IIF13 and IIF15 in cluster II-4 showed an identity average value of 85.8%$_{aa}$ (79.2%$_{ND}$) with Pika and Suiyo ORUs located in the same clusters. Hence, the ORUs IIF1, IIF2, IIF13 and IIF15 may represent the Fryer form II library fraction that encompassed Pika and Suiyo form II sequences (Table 5). However, although Pika and Suiyo differed significantly from Fryer, additional sequences might yield different community structures. This can be realized in the case of archaeal and Suiyo form IA *cbbL*.

Table 5 LIBSHUFF comparison of clone libraries

Clone library	Cov_{hom} (%)	Cov_{het} (%)	*P*
Fryer (Archaea)	79.5	43.7	0.001
Pika (Archaea)	86.2	37.1	0.001
Fryer (Form IA)	73.3	60.0	0.425
Pika (Form IA)	68.7	35.8	0.010
Fryer (Form IA)		60.0	0.403
Suiyo (Form IA)	62.2	21.0	0.001
Pika (Form IA)		51.0	0.054
Suiyo (Form IA)		47.8	0.248
Pika (Form IC)	80.0	51.0	0.009
Suiyo (Form IC)	79.0	46.0	0.020
Fryer (Form II)	76.6	36.9	0.007
Pika (Form II)	69.8	46.0	0.214
Fryer (Form II)		38.7	0.010
Suiyo (Form II)	63.0	59.2	0.116
Pika (Form II)		48.1	0.452
Suiyo (Form II)		61.1	0.743

Homologous (Cov_{hom}) and heterologous (Cov_{het}) coverage percentages of libraries are given. Probability values (*P*) for the significance of differences between homologous and heterologous coverage in reciprocal comparisons as a function of evolutionary distance are also given

The current form IA and form II ORUs differed with those previously recorded in vent plumes at the Okinawa Trough (Elsaied and Naganuma 2001). This was clear in the branch of form IA (Fig. 3) and in cluster II-3 (Fig. 4). The identity average values between current ORUs and Okinawa *cbbL* sequences were 66%$_{aa}$ (65.8%$_{ND}$) in branch IA (Fig. 3) and 77%$_{aa}$ (70.5%$_{ND}$) in cluster II-3 (Fig. 4). This was expressed in the separated phyletic lineages of current ORUs from those of Okinawa. These results indicated that form IA- and/or II-carrying populations in the studied Western Pacific hydrothermal vent plumes might form three biogeographic groups, namely Fryer, Pika/Suiyo and Okinawa.

Differentiation of *cbbL* form IC between Pika and Suiyo

Although Pika and Suiyo ORUs showed phylogenetic similarity for form IA and II, the Pika form IC ORUs were clearly distinct from those of Suiyo, with F_{ST} value of 0.8 (Table 4). This statistical distinction was also visible in the significant differentiation between form IC libraries (Table 5). The sequence identity average between Pika and Suiyo form IC ORUs was 81.5%$_{aa}$

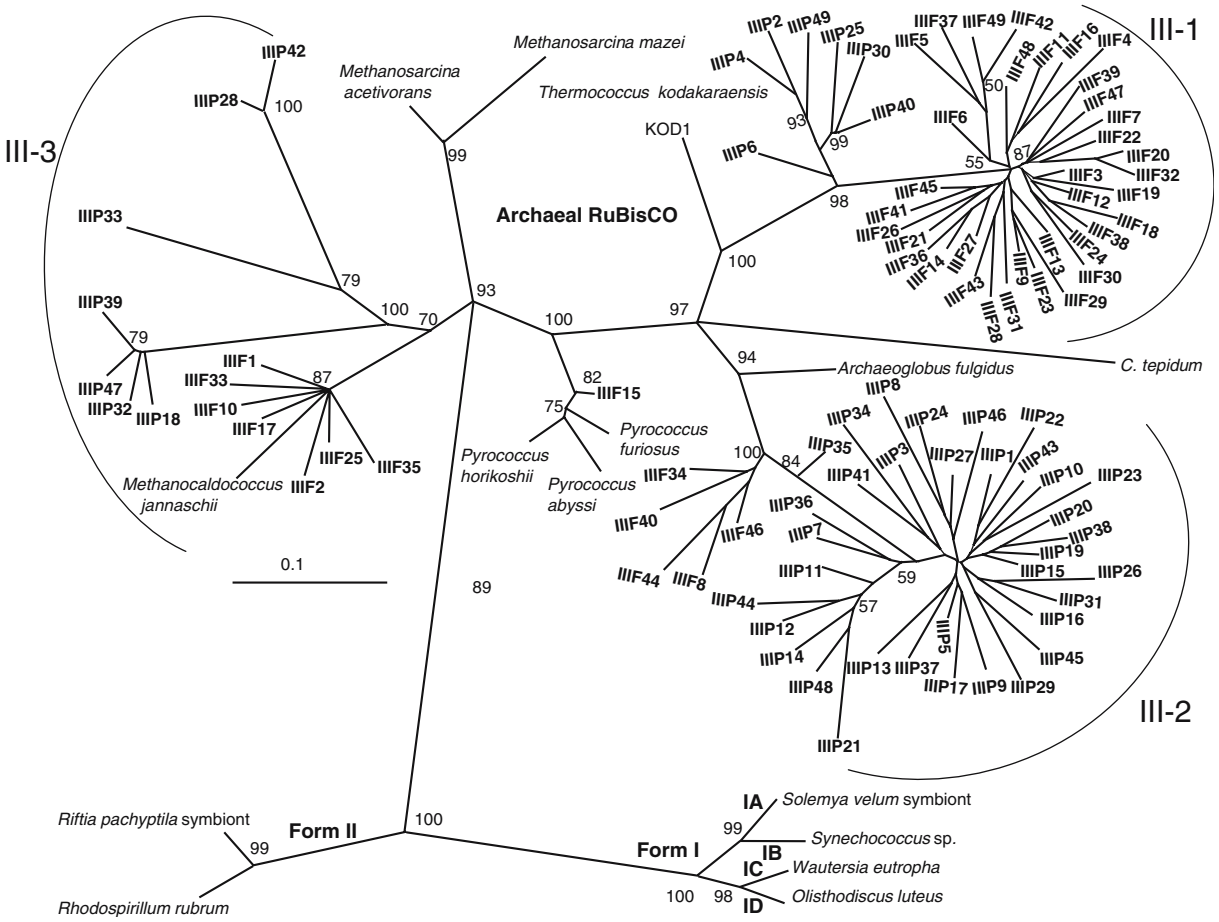

Fig. 2 Phylogenetic analysis of archaeal *cbbL* genes. A consensus tree was constructed by neighbor-joining analysis and confirmed by maximum-parsimony and maximum-likelihood methods. Bootstrap values of >50% are indicated at the nodes. ORUs are indicated in *bold*. Abbreviation for ORUs: IIIF, Fryer; IIIP, Pika. Clusters formed by ORUs are marked by arcs. The *bar* represents 0.1 changes per amino acid or nucleotide

(78%$_{ND}$) and this was expressed in their distant distribution within the branch IC (Fig. 3). Most Pika ORUs were rooted with uncultured clones from a volcanic deposit at the Kilauea Caldera, Hawaii (Nanba et al. 2004). The Suiyo ORUs formed two small clusters; one of them was claded with the ammonia-oxidizing Betaproteobacterium *Nitrosospira* sp. and the second of which consisted of the ORUs ICS2 and ICS3, which showed no affiliation to any known form IC sequence.

Generally, the diversity of form IC ORUs in Pika and Suiyo samples was less than those recorded in the Kilauea Caldera, a continental volcano, Hawaii, and agricultural soils (Nanba et al. 2004; Selesi et al. 2005). This is logically understandable as the deep-sea hydrothermal vents are isolated habitats with less population diversity, mainly derived from the lithosphere.

Both statistical and phylogenetic analyses suggested that the *cbbL* genetic content of Pika and Suiyo samples consists of two groups of genotypes. The first represents closely related form IA and form II genotypes and the second represents divergent form IC genotypes. It generally appears that several form IA genotypes are cosmopolitan, whereas similar *cbbL*s, especially those related to thiobacilli, have been recorded in widely distant hydrothermal vents (Elsaied and Naganuma 2001). In contrast, highly distinct form IC genotypes have been recorded in geographically close terrestrial volcanic sites (Nanba et al. 2004) and agricultural soils (Selesi et al. 2005). Hence, the genetic composition of hydrothermal vent form IC may be endemic to the vent habitat. However, the present paper provided the first record of form IC in deep-sea environments, and more data are needed to confirm this idea.

Impact of vent characteristics on the diversity of *cbbL* forms

The biogeographic composition of RuBisCO *cbbL* forms is strongly correlated with the vent physicochemical characteristics. The concentrations of erupted gases, which are essential for autotrophic growth in the vent plume of the Pika site (CO_2 45 mM/kg; CH_4 25 μM/kg; H_2S 7.48 mM/kg) were higher than those of Fryer (CO_2 12 mM/kg; CH_4 4 μM/kg; H_2S 0.4 mM/kg) and Suiyo (CO_2 40 mM/kg; CH_4 150 μM/kg; H_2S 2 mM/kg, cruise

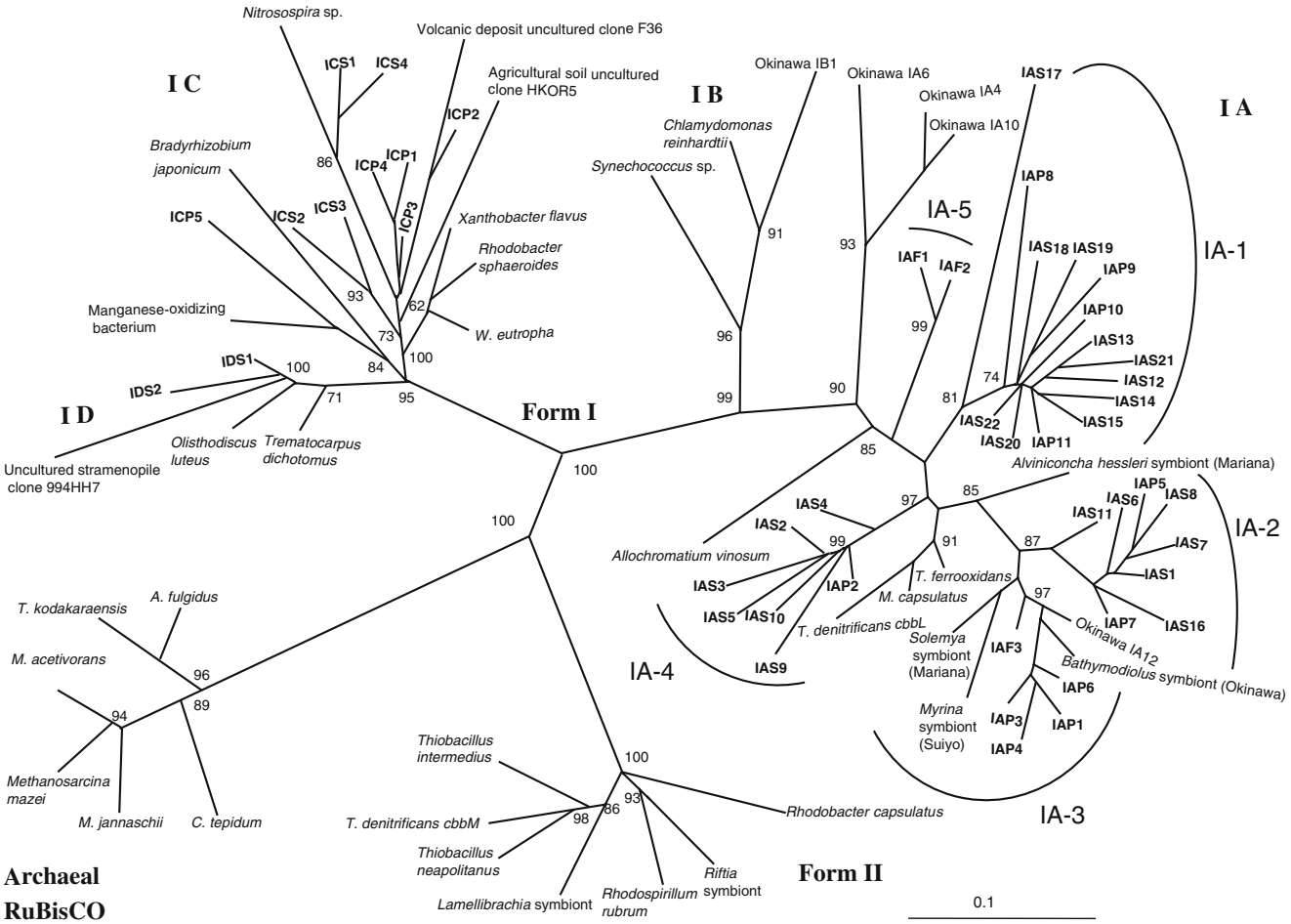

Fig. 3 Phylogenetic analysis of form I *cbbL* genes. A consensus tree was constructed by neighbor-joining analysis and confirmed by maximum-parsimony and maximum-likelihood methods. Bootstrap values of >50% are indicated at the nodes. ORUs are indicated in *bold*. Abbreviation for ORUs: IAF, form IA Fryer; IAP, form IA Pika; IAS, form IA Suiyo; ICP, form IC Pika; ICS, form IC Suiyo; IDS, form ID Suiyo. Clusters formed by form IA ORUs are marked by arcs. The *bar* represents 0.1 changes per amino acid or nucleotide

reports). These chemical characteristics were reflected in the highest diversity of RuBisCO-carrying communities recorded in the Pika sample (Fig. 1d). Unfortunately, hydrogen gas could not be measured during sampling cruises. However, most of the recorded ORUs were closely related to sulfur metabolizers (Fig. 3). On the other hand, hydrogen-oxidizing Epsilonproteobacteria that lack RuBisCO genes were recorded in some hydrothermal vents at the Mariana arc and Suiyo Seamount (Takai et al. 2005). Hence, the possibility for primary production of organic carbon at Mariana and Suiyo depends mainly on sulfur- and hydrogen-metabolizing phylogenetic taxa.

In the term of vent thermal activity, the old Fryer site is characterized by a significant decline in the temperature of the venting plume from 250 to 50°C over the course of five visits to the vent site between April 2003 and April 2004 (Urabe, unpublished data). Hence, the distinction of Fryer *cbbL*-carrying communities from those that inhabit the Pika vents is likely a reflection of difference in vent characteristics, i.e. old vents suffering from a decrease in temperature and gases; and newborn vents (Delaney et al. 1998). So, the oldest biologically complex site, Fryer, supports the most divergent *cbbL*-carrying communities. This divergence of Fryer communities could be a type of phylogenetic succession through adaptation to the decrease in vent temperature and changes in physicochemical characteristics. However, the dominance of RuBisCO sequences similar to those of thermophilic anaerobic archaea in Mariana vent plumes may suggest that the ORUs were derived from species living in the warmer sub-seafloor and were entrained in the event hydrothermal fluid that discharged into the vent surrounding plume, the site of sampling.

This study showed that the Mariana Spreading Center is the first discovered deep-sea house for *cbbL*-carrying archaea. At present, it is not possible to discuss the physiological or ecological functions of archaeal RuBisCO without expression studies. However, if the deep-sea archaeal *cbbL* gene is indeed expressed and the enzyme functions actively, this would represent a unique

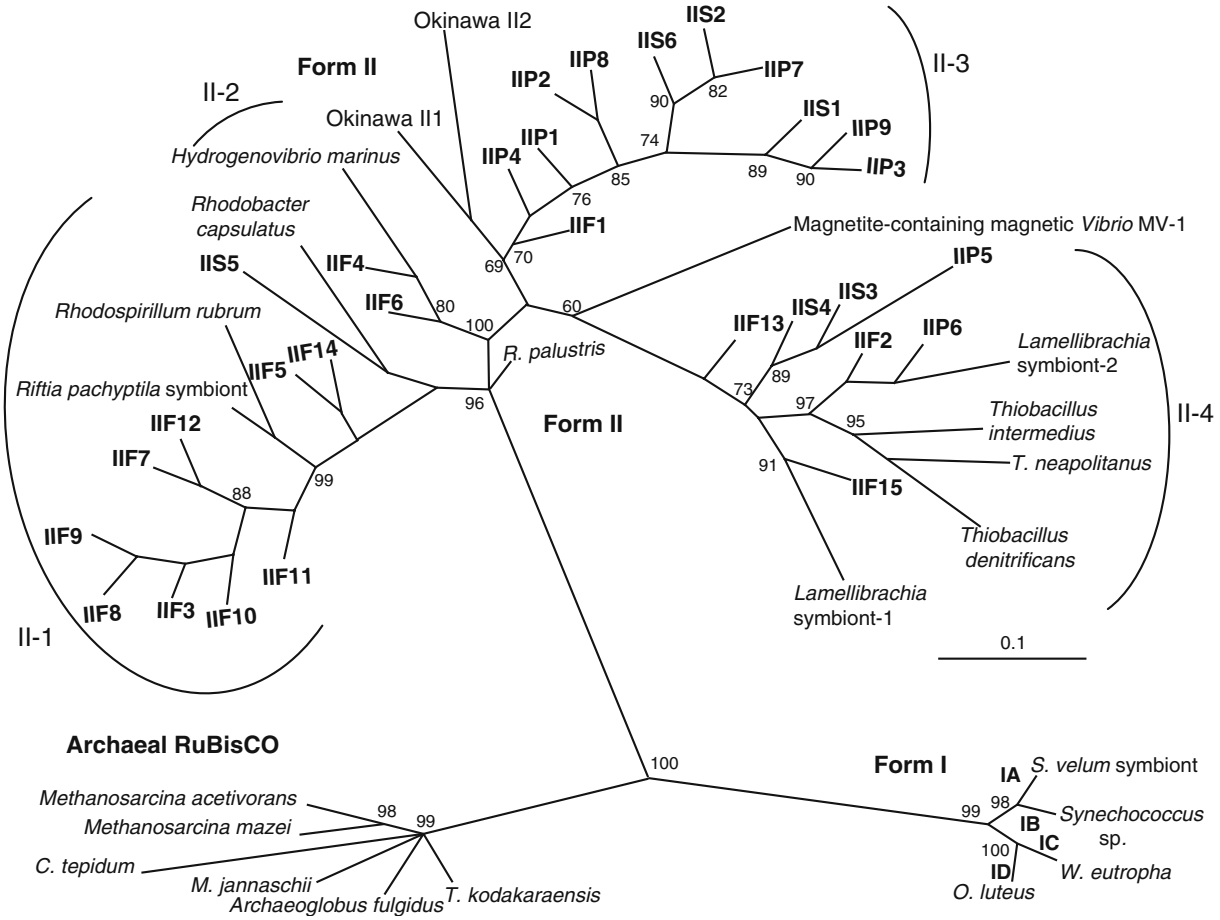

Fig. 4 Phylogenetic analysis of form II *cbbL* genes. A consensus tree was constructed by neighbor-joining analysis and confirmed by maximum-parsimony and maximum-likelihood methods. Bootstrap values of >50% are indicated at the nodes. ORUs are indicated in *bold*. Abbreviation for ORUs: IIF, form II Fryer; IIP, form II Pika; IIS, form II Suiyo. Clusters formed by ORUs are marked by arcs. The *bar* represents 0.1 changes per amino acid or nucleotide

trophic strategy, namely that archaea act as fundamental producers of organic carbon in the Mariana Spreading Center. In fact, archaeal *cbbL* activity has been shown to be expressed in anoxygenic photosynthetic bacteria (Finn and Tabita 2003). However, the detection of *cbbL*-carrying archaea in hydrothermal vent systems opened a new window on the phylogenetic diversity of RuBisCO *cbbL* in the deep-sea environment. Our future work will focus on studying the diversity and expression of archaeal *cbbL* in various other deep biosphere habitats, such as deep-sea cold seeps, deep subsurface, etc. in order to develop a complete concept for the phylogeny of RuBisCO *cbbL* in the deep microbial biosphere.

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References

Ashida H, Saito Y, Kojima C, Kobayashi K, Ogasawara N, Yokota A (2003) A functional link between RuBisCO-like protein of *Bacillus* and photosynthetic RuBisCO. Science 302:286–290

Beatty J, Overmann J, Lince MT, Manske AK, Lang AS, Blankenship RE, Van Dover CL, Martinson TA, Plumley FG (2005) An obligately photosynthetic bacterial anaerobe from a deep-sea hydrothermal vent. Proc Natl Acad Sci USA 102:9306–9310

Bult CJ, White O, Olsen GJ, Zhou L, Fleischmann RD, Sutton GG, Blake JA, FitzGerald LM, Clayton RA, Gocayne JD, Kerlavage AR, Dougherty BA, Tomb JF, Adams MD, Reich CI, Overbeek R, Kirkness EF, Weinstock KG, Merrick JM, Glodek A, Scott JL, Geoghagen NS, Venter JC (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science 273:1058–1073

Campbell BJ, Cary SC (2004) Abundance of reverse tricarboxylic acid cycle genes in free-living microorganisms at deep-Sea hydrothermal vents. Appl Environ Microbiol 70:6282–6289

Delaney J, Kelley D, Lilley M, Butterfield D, Baross J, Wilcock W, Embley R, Summit M (1998) The quantum event of oceanic crustal accretion: impacts of diking at Mid-Ocean Ridges. Science 281:222–230

Delwiche C, Palmer J (1996) Rampant horizontal transfer and duplication of RuBisCO genes in eubacteria and plastids. Mol Biol Evol 13:873–882

Deppenmeier U, Johann A, Hartsch T, Merkl R, Schmitz RA, Martinez-Arias R, Henne A, Wiezer A, Baumer S, Jacobi C, Bruggemann H, Lienard T, Christmann A, Bomeke M, Steckel S, Bhattacharyya A, Lykidis A, Overbeek R, Klenk HP, Gunsalus RP, Fritz HJ, Gottschalk G (2002) The genome of *Methanosarcina mazei*: evidence for lateral gene transfer between bacteria and archaea. J Mol Microbiol Biotechnol 4:453–461

Eisen JA, Nelson KE, Paulsen IT, Heidelberg JF, Wu M, Dodson RJ, Deboy R, Gwinn ML, Nelson WC, Haft DH, Hickey EK, Peterson JD, Durkin AS, Kolonay JL, Yang F, Holt I, Umayam LA, Mason T, Brenner M, Shea TP, Parksey D, Nierman WC, Feldblyum TV, Hansen CL, Craven MB, Radune D, Vamathevan J, Khouri H, White O, Gruber TM, Ketchum KA, Venter JC, Tettelin H, Bryant DA, Fraser CM (2002) The complete genome sequence of *Chlorobium tepidum* TLS, a photosynthetic, anaerobic, green-sulfur bacterium. Proc Natl Acad Sci USA 99:9509–9514

Elsaied H, Naganuma T (2001) Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) large subunit genes from deep-sea microorganisms. Appl Environ Microbiol 67:1751–1765

Elsaied H, Kimura H, Naganuma T (2002a) Molecular characterization and endosymbiotic localization of the gene encoding D-ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBisCO) form II in the deep-sea vestimentiferan trophosome. Microbiology 148:1947–1957

Elsaied H, Sato M, Naka J, Naganuma T (2002b) Analysis of 16S rRNA and RuBisCO large subunit genes from an abyssal low-temperature vent, Loihi Seamount, Hawaii. Cah Biol Mar 43:403–408

English RS, Williams CA, Lorbach SC, Shively JM (1992) Two forms of ribulose-1,5-bisphosphate carboxylase oxygenase from *Thiobacillus denitrificans*. FEMS Microbiol Lett 94:111–120

Ezaki S, Maeda N, Kishimoto T, Atomi H, Imanaka T (1999) Presence of a structurally novel type ribulose-bisphosphate caroboxylase/oxygenase in hyperthermophilic archaeon, *Pyrococcus kodakaraensis* KOD1. J Biol Chem 274:5078–5082

Finn M, Tabita F (2003) Synthesis of catalytically active form III ribulose 1,5-Bisphosphate carboxylase/oxygenase in archaea. J Bacteriol 185:3049–3059

Galagan JE, Nusbaum C, Roy A, Endrizzi MG, Macdonald P, FitzHugh W, Calvo S, Engels R, Smirnov S, Atnoor D, Brown A, Allen N, Naylor J, Stange-Thomann N, DeArellano K, Johnson R, Linton L, McEwan P, McKernan K, Talamas J, Tirrell A, Ye W, Zimmer A, Barber RD, Cann I, Graham DE, Grahame DA, Guss AM, Hedderich R, Ingram-Smith C, Kuettner HC, Krzycki JA, Leigh JA, LI W, Liu J, Mukhopadhyay B, Reeve JN, Smith K, Springer TA, Umayam LA, White O, White RH, Conway de Macario E, Ferry JG, Jarrell KF, Jing H, Macario AJ, Paulsen I, Pritchett M, Sowers KR, Swanson RV, Zinder SH, Lander E, Metcalf WW, Birren B (2002) The genome of *M. acetivorans* reveals extensive metabolic and physiological diversity. Genome Res 12:532–542

Hurlbert SH (1971) The nonconcept of species diversity: a critique and alternative parameters. Ecology 52:577–586

Kimura H, Sato M, Sasayama Y, Naganuma T (2003) Molecular characterization and in situ localization of endosymbiotic 16S ribosomal RNA and RuBisCO genes in the Pogonophoran tissue. Mar Biotech 5:261–269

Kitano K, Maeda N, Fukui T, Atomi H, Imanaka T, Miki K (2001) Crystal structure of a novel-type archaeal rubisco with pentagonal symmetry. Structure 9:473–481

Klenk H, Clayton R, Tomb J, White O, Nelson KE, Ketchum KA, Dodson RJ, Gwinn M, Hickey EK, Peterson JD, Richardson DL, Kerlavage AR, Graham DE, Kyrpides NC, Fleischmann RD, Quackenbush J, Lee NH, Sutton GG, Gill S, Kirkness EF, Dougherty BA, McKenney K, Adams MD, Loftus B, Venter JC (1997) The complete sequence of the hyperthermophilic sulfate-reducing archaeon *Archaeoglobus fulgidus*. Nature 390:364–370

Kusian B, Bowien B (1997) Organization and regulation of *cbb* CO_2 assimilation genes in autotrophic bacteria. FEMS Microbiol Rev 21:135–155

Li H, Sawaya M, Tabita F, Eisenberg D (2005) Crystal structure of a RuBisCO-like protein from the green sulfur bacterium *Chlorobium tepidum*. Structure 13:779–789

Nanba K, King G, Dunfield K (2004) Analysis of facultative lithotroph distribution and diversity on volcanic deposits by use of the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase. Appl Environ Microbiol 70:2245–2253

Paul JH, Alfreider A, Wawrik B (2000) Micro- and macrodiversity in *rbcL* sequences in ambient phytoplankton populations from the southeastern Gulf of Mexico. Mar Ecol Prog Ser 198:9–18

Rajagopalan R, Altekar W (1994) Characterization and purification of ribulose-bisphosphate carboxylase from heterotrophically grown halophilic archaebacterium, *Haloferax mediterranei*. Eur J Biochem 221:863–869

Robinson J, Stein J, Cavanaugh C (1998) Cloning and sequencing of a form II ribulose-1,5-bisphosphate carboxylase/oxygenase from the bacterial symbiont of the hydrothermal vent tubeworm *Riftia pachyptila*. J Bacteriol 180:1596–1599

Schneider S, Roesseli D, Excoffier L (2000) Arlequin version 2.000, a software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Geneva

Schwedock J, Harmer T, Scott K, Hektor H, Seitz A, Fontana M, Distel D, Cavanaugh C (2004) Characterization and expression of genes from the RubisCO gene cluster of the chemoautotrophic symbiont of *Solemya velum*: cbbLSQO. Arch Microbiol 182:18–29

Selesi D, Schmid M, Hartmann A (2005) Diversity of green-like and red-like ribulose-1,5-bisphosphate carboxylase/Oxygenase large-subunit genes (*cbbL*) in differently managed agricultural Soils. Appl Environ Microbiol 71:175–184

Shinozaki K, Yamada C, Takahata N, Sugiura M (1983) Molecular cloning and sequence analysis of the cyanobacterial gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Proc Natl Acad Sci USA 80:4050–4054

Simberloff D (1978) Use of rarefaction and related methods. In: Dickson KL et al (eds) Biological data in water pollution assessment quantitative and statistical analyses. American Society for Testing and Materials, Philadelphia, pp 150–165

Singleton D, Furlong M, Rathbun S, Whitman W (2001) Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl Environ Microbiol 67:4374–4376

Stein JL, Haygood M, Felbeck H (1990) Nucleotide sequence and expression of a deep-sea ribulose-1,5-bisphosphate carboxylase gene cloned from a chemoautotrophic bacterial endosymbiont. Proc Natl Acad Sci USA 87:8850–8854

Sunamura M, Higashi Y, Miyako C, Ishibashi J, Maruyama A (2004) Two bacterial phylotypes are predominant in the Suiyo Seamount hydrothermal plume. Appl Environ Microbiol 70:1190–1198

Suzuki M, Giovannoni S (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 62:625–630

Tabita F (1999) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: a different perspective. Photo Res 60:1–28

Takai K, Campbell B, Craig C, Suzuki M, Oida H, Nunoura T, Hirayama H, Nakagawa S, Suzuki Y, Inagaki F, Horikoshi K (2005) Enzymatic and genetic characterization of carbon and energy metabolisms by deep-sea hydrothermal chemolithoautotrophic isolates of *Epsilonproteobacteria*. Appl Environ Microbiol 71:7310–7320

Turely C, Mackie P (1995) Bacteria and cyanobacteria flux to the deep NE Atlantic on sedimenting particles. Deep-Sea Res Part I 42:1453–1474

Utsumi M, Nakamura K, Kakegawa T, Shitashima K, Kurusu Y, Yamanaka T, Takano Y, Kimura H, Higashi Y, Ishibashi J, Hirota A, Kaneko R, Minaba M, Kasai H, Settsu M (2004) First discovery of hydrothermal vent with black smoker (Pika

site) at the Southern Mariana region and its properties. In: Abstracts of Japan Earth and Planetary Science joint meeting B002-016, Chiba

Watson G, Tabita F (1997) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: a molecule for phylogenetic and enzymological investigation. FEMS Microbiol Lett 146:13–22

Watson F, Yu J, Tabita F (1999) Unusual ribulose 1,5-bisphosphate carboxylase/oxygenase of anoxic archaea. J Bacteriol 181:1569–1575