



Analyses of 16S rRNA and RuBisCO large subunit genes from an abyssal low-temperature vent, Loihi Seamount, Hawaii

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Introduction

Loihi Seamount is an active, submarine, hotspot volcano and potentially the next Hawaiian Island. Loihi Seamount has none of the luxuriant biological communities of macrobenthos including vestimentiferan tubeworms and vesicomyid clams that harbor bacterial endosymbionts (Karl et al., 1988). The absence of macrofaunal communities may be related to chemical features of the hydrothermal fluids such as the absence or extremely low levels of sulphide, the low pH and the high levels of dissolved CH₄ and iron at active Loihi vents (Karl et al., 1988).

The lavas around the Loihi vent fields are mantled with red-orange nontronite deposits, which contain various amounts of iron oxides, silica and iron-rich smectite (Karl et al., 1988). The acidic vent fluid and diffuse flow are the controlling factors in the formation of abundant iron-rich oxide and silicate nontronite deposits, as well as of the extensive bacterial mats (Moyer et al., 1995). The microflora associated with mineral deposits is mainly composed of metal-oxidizers that may contribute to chemoautotrophic CO₂ fixation. Phylogenetic analysis of the 16S rDNA from the northern vent field (Pele's vent) in the crater of Loihi Seamount revealed a diversity of microflora (Moyer et al., 1995). This study used sequences from the gene for the CO₂-fixing enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) to examine the diversity of the chemoautotrophic microflora at a vent site located on the lower flank of Loihi Seamount, at 4772 m depth.

The RuBisCO enzyme is one of the most abundant proteins on Earth and has attracted much phylogenetic

attention. RuBisCO catalyzes the assimilation of carbon dioxide to organic carbon via the Calvin-Benson cycle. Generally, RuBisCO has two distinct forms. Form I consists of large and small subunits encoded by the genes *cbbL* and *cbbM*, respectively. Form II consists only of large subunits encoded by the gene *cbbM*, and shows 25-30% amino acid identity with Form I (Kellogg & Juliano, 1997). The site responsible for carbon fixation is in the large subunit (Kellogg & Juliano, 1997). Form I is found in the vast majority of eukaryotic and prokaryotic autotrophs. Form II is commonly found in anoxygenic phototrophic bacteria such as the α -Proteobacterium, *Rhodospirillum rubrum* (Watson & Tabita, 1997). Chemoautotrophic endosymbionts of deep-sea mollusks usually have Form I, while the endosymbionts of vestimentiferans tubeworms and the epibionts of the vent polychaete *Alvinella* sp. and the vent shrimp *Rimicaris exoculata* have Form II (Robinson & Cavanaugh, 1995). Thus, RuBisCO genes occur in diverse groups of prokaryotes and may serve as a phylogenetic window to describe the autotrophic microbial communities.

We have studied the diversity of the RuBisCO genes (*cbbL* and *cbbM*) from various deep-sea hydrothermal vent habitats including mid-ocean ridge vents and back-arc basin vents (Elsaied & Naganuma, 2001). To add new information to the deep-sea RuBisCO data set, we analysed RuBisCO genes as well as 16S rDNA from a 4772 m deep vent in the south rift zone of Loihi Seamount (Naka et al., 2000). This is the first report of both the structural and functional diversity of chemoautotrophic bacteria responsible at abyssal depths of the non-divergent, non-convergent intra-plate region.

Material and methods

Sample collection

A sediment sample containing nontronite deposit was collected from a low-temperature (6.9 °C) hydrothermal vent site (18°46.121'N, 155°07.891'W) at 4772 m deep in the south rift zone of Loihi Seamount during the 517th dive of the manned submersible *Shinkai 6500*, Japan Marine Science and Technology Center (Naka et al., 2000). For the microbiological approach, yellowish nontronite deposit was scooped from inside of the push-core sampler about 1 to 5 cm below the top of the core and kept frozen at -80 °C until DNA extraction.

DNA extraction

Bulk genomic DNA was extracted from the sample according to Elsaied & Naganuma (2001). Ten g of nontronite was suspended in 10 ml of 250 mM NaCl and 100 mM EDTA (pH 8.0) in a sterile 50 ml propylene tube. The negative control was a tube containing only the solutions used for DNA extraction procedure. Sample suspensions were inoculated with 500 µl of lysozyme solution (50 mg ml⁻¹), and incubated at 37 °C with continuous shaking for 1 hr. Then, the sample suspension was augmented with 10 ml of denaturing solution (250 mM NaCl, 100 mM EDTA, 4% SDS) and 2 ml of 5 M guanidine thiocyanate, and incubated at 65 °C with continuous shaking for 2 hrs, followed by centrifugation at 14,000 xg for 15 min at 4 °C to precipitate the remnants. The supernatant crude lysate was transferred into a sterile clean tube. The bulk genomic DNA was purified from the potentially metal-rich crude lysate using the Easy Nucleic Acid Isolation kit (Omega Biotek, USA) according to manufacturer's instructions and checked by agarose gel electrophoresis.

Amplification and Cloning of 16S rRNA and RuBisCO genes

The 16S rDNA was amplified from bulk extracted DNA using well-known primers Eubac27F and Eubac1492R (DeLong, 1992). The primer sets for the amplification of RuBisCO Form I and Form II genes (*cbbL* and *cbbM*) were designed and used previously by Elsaied & Naganuma (2001). The *cbbL* forward and reverse primers were 5'-GAC TTC ACC AAA GAC GAC GA-3' and 5'-TCG AAC TTG ATT TCT TTC CA-3', respectively, and would yield approximately 800-bp amplified product. The *cbbM* forward and reverse primers were designed as 5'-ATC ATC AAR CCS AAR CTS GGC CTG CGT CCC-3' and 5'-MGA GGT GAC SGC RCC GTG RCC RGC MCG RTG-3', respectively, to yield PCR product of about 400-bp.

The PCR conditions for amplifying 16S rDNA and *cbbL/cbbM* were set according to DeLong (1992) and Elsaied & Naganuma (2001), respectively. The PCR products were cloned using TA cloning kit, (Invitrogen, USA) according to the manufacturer's instructions. From each of 16S rDNA and RuBisCO gene clone libraries, 50 clones having the target size were randomly selected and analysed directly by DNA sequencing, using an ABI model 373 automated DNA sequencer.

Sequencing analysis of 16S rRNA and RuBisCO genes

Sequences of ca. 500 bp from the 5' end of either the 16S

rDNA or RuBisCO genes were the maximum accurate sequence regions for alignment of both 16S rDNA and RuBisCO gene sequences. These sequences were homology-searched and chimera-checked through the Ribosomal Database Project (RDP; <http://rdp.cme.msu.edu/html>) and the DNA Data Bank of Japan (DDBJ; <http://www.ddbj.nig.ac.jp>) using the programs FASTA (Lipman & Person, 1985) and BLAST (Altschul et al., 1990). The 16S rDNA sequences sharing >97% nucleotide identity were grouped into the same operational taxonomic unit (OTU). The RuBisCO nucleotide sequences were translated into amino acids using the program Protein Engine at European Bioinformatics Institute (EBI; <http://www.ebi.ac.uk/>). A set of the RuBisCO gene sequences sharing 100% amino acid identity was grouped into an operational RuBisCO unit (ORU). Two phylogenetic trees were constructed based on the multiple-alignments of 16S rDNA nucleotide sequences and RuBisCO deduced amino acid sequences with other corresponding sequences from databases using the program ClustalW and software Tree View. The sequences of 16S rDNA and RuBisCO genes obtained in this study were registered in the DNA databases under the accession numbers shown in Table 1.

Table 1. Distribution of OTUs / ORUs within the 16S rDNA and *cbbL* clone libraries

Clone library	OTU / ORU	Accession	No. of clones	% Nucleotide divergence ^a	Frequency of OTU/ORU (%) ^b
16S rDNA	Loihi-16s-1	AB049741	3	0.9	6
	Loihi-16s-2	AB049742	1	0	2
	Loihi-16s-3	AB049743	1	0	2
	Loihi-16s-4	AB049744	5	1.4	10
	Loihi-16s-5	AB049746	14	3.9	28
	Loihi-16s-6	AB049747	4	0.2	8
	Loihi-16s-7	AB049749	6	3.4	12
	Loihi-16s-8	AB058672	7	3.2	14
	Loihi-16s-9	AB058673	6	1.2	12
	Loihi-16s-10	AB058674	3	0.7	6
Total			50		
<i>cbbL</i>	Loihi- <i>cbbL</i> -1	AB046931	1	0	2
	Loihi- <i>cbbL</i> -2	AB046932	1	0	2
	Loihi- <i>cbbL</i> -3	AB046933	9	4.2	18
	Loihi- <i>cbbL</i> -4	AB046934	1	0	2
	Loihi- <i>cbbL</i> -5	AB046935	20	8.6	40
	Loihi- <i>cbbL</i> -6	AB046936	18	3.8	36
Total			50		

^a % Nucleotide sequence divergence is the percentage of mismatched nucleotides between the clones within the OTU or ORU compared with the total length of analyzed nucleotide sequences (500 bp) for 16S rDNA and *cbbL*. However, the *cbbL* clones have > 90% nucleotide identity showed 100% amino acid identity.

^b Frequency of the OTU or ORU compared with the total number of clones analyzed in the clone library.

Results and Discussion

Phylogenetic analysis of 16S rDNA

The 50 analyzed 16S rDNA clones were grouped into 10 OTUs. These OTUs were abbreviated as Loihi-16S-1 to -10 (Table 1). The phylogenetic analysis of 16S rDNA sequences suggested that 6 OTUs were related to the metal-oxidizing species of α -, β -, and γ -*Proteobacteria* (Fig. 1). Possibly chemolithoautotrophic, ϵ -*Proteobacteria* were reported to be the dominant species at the northern Loihi Pele's vents (Moyer et al., 1995). The phylogenetic diversity of metal-oxidizing bacteria at Loihi vents may imply that the variability of vent chemistry affects the species compositions of vent microflora. The OTUs Loihi-16S-8, -9, and -10 were located in the clusters of sulphur-/iron-oxidizing bacteria related to the classes of α -, β -, and δ -*Proteobacteria*, respectively. The OTUs Loihi-16S-1, -6 and -7 formed the monophyletic and paraphyletic groups with the species of the *Pseudomonase* group I of γ -*Proteobacteria*. The group I *Pseudomonas* contains well-studied manganese- and iron-oxidizing/reducing species, which are characterized by production of fluorescent pigments involved in iron transport (Hersman et al., 1996). The presence of OTUs related to different metal-oxidizing bacteria implies that these bacteria may be involved in the formation of nontronite deposit as previously detected at a low-temperature hydrothermal vent in the Southern Explorer Ridge, in the northeast Pacific Ocean (Fortin et al., 1998). The formation of metal oxides, which are the main constituents of nontronite, may be initiated by direct binding of soluble metal to reactive sites like carboxyl, phosphate and hydroxyl groups present in the bacterial cell wall (Fortin et al., 1998).

Also of interest were the OTUs Loihi-16S-2 and -3 in the cluster of *Methylophaga* species, which are unable to grow on methane (Janvier & Grimont, 1995). Moreover, the interchangeability between methylotrophy and thiotrophy has been indicated previously in this genus (de Zwart & Kuenen, 1997). Hence, this may represent biological evidence for the absence or low concentration of methane at the deep water site where the sample was collected. This contrasts with the high methane concentration (ca. 7.2 μ M) at Pele's vent on the Loihi summit (Sedwick et al., 1992).

The OTU Loihi-16S-4 and -5 were grouped in the cluster of halotolerants. The phylogenetic similarity between the halotolerants in different temperate waters including the deep Pacific, and Antarctic waters and terrains suggests that deep-sea bacterial distribution and evolution have been mediated by global deep-ocean circulation linked to the sinking of cooled water in the polar regions (Okamoto et al., 2001).

It should be noted that most of the OTUs were closely related to known pelagic bacterial species; for example, the OTUs Loihi-16S-1, -4, -5, -6 and -7, accounting for 64% of total OTU frequency, were related to the common pelagic groups of marinobacters, halomonads and pseudomonads. Since these OTUs were recovered from a low-temperature hydrothermal vent deposit, the pelagic species may have originated from the convective accumulation of sea water flowing through a nontronitic filter over a long period of time.

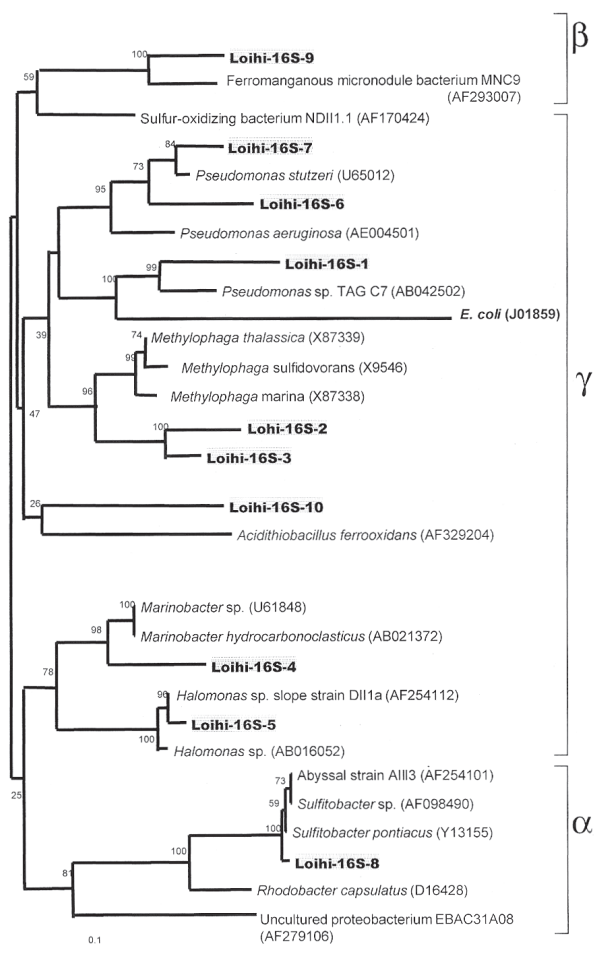


Figure 1. Phylogenetic tree based on 16S rDNA nucleotide sequences of the Loihi OTUs (shaded) and those from known species in the databases. Tree topography and evolutionary distance are given by the neighbor-joining method with Kimura distances. Bootstrap values, calculated from 1,000 replicates, are expressed as percentages and are shown at the roots of the tree. Symbols α , β , and γ illustrate the different classes of *Proteobacteria*. Scale bar, 0.1 substitutions per site.

Occurrence of a single RuBisCO gene in the nontronite deposit

Although the PCR primers used in this study targeted the large subunit genes of the green-like RuBisCO form I and form II (Elsaied & Naganuma, 2001), only the RuBisCO form I gene (*cbbL*) was amplified by PCR, while the form II gene (*cbbM*) was not amplified from the extracted bulk DNA. The absence of *cbbM* was previously observed in the sulphide-anhydrite-rich chimney fragments from the TAG hydrothermal mound, Mid-Atlantic Ridge (Elsaied & Naganuma, 2001). The absence of *cbbM* in the Loihi and TAG vent autotrophs is explained by the aerobic and anaerobic nature of the RuBisCO form I and form II, respectively (Watson & Tabita, 1997). Anhydrite chimney

and nontronite deposit are formed by aerobic oxidation of sulphide, ferrous and other ions which may favor the dominance of the aerobic RuBisCO form I. This explanation is supported by the observation that all the Loihi 16S rDNA OTUs were affiliated to the aerobic species of *Proteobacteria*. Another observation relates the distribution of RuBisCO forms within the deep-sea autotrophs to the carbon isotope values of the organic matter assimilated by different RuBisCO forms. Autotrophy using the aerobic Form I produced organic matter with $\delta^{13}\text{C}$ values of -30‰ or less, while autotrophy using the Form II assimilates organic carbon with higher $\delta^{13}\text{C}$ value of -11‰ (Robinson & Cavanaugh, 1995). Thus, the variation in the carbon isotopic ratio may reflect the difference in the CO_2 sources and consequently in the use of RuBisCO forms. However, since no $\delta^{13}\text{C}$ values were determined for the Loihi and TAG samples, this question cannot be definitively resolved.

Analysis of the RuBisCO *cbbL* sequences

The phylogenetic analysis of RuBisCO *cbbL* based on deduced amino acid sequences often shows clearer phylogenetic relationships than those based on nucleotide sequences (Watson & Tabita, 1997). The analysis of the 50 *cbbL* sequences resulted in 6 ORUs abbreviated as Loihi-*cbbL*-1 to -6 based on the deduced amino acid sequences. The range of multiple alignment of the Loihi *cbbL* amino acid sequences (Fig. 2) corresponded to the position 195 to 367 of the *cbbL* of *Synechococcus* sp. strain PCC6301. Most of the Loihi *cbbL* ORUs showed conservation in the catalytic amino acid residues. The conserved region flank Lys-198 (Fig. 2) is considered as the site of CO_2 -binding and carbamate formation during enzyme activation (Watson & Tabita, 1997). Other catalytic amino acid residues including His-291, Arg-292, His-324, Lys-331, and Leu-332 were conserved in the current ORUs except Loihi-*cbbL*-4 and -5 in which some of these catalytic amino acid residues were replaced by conserved or dissimilar amino acid residues. Substitution of absolutely conserved catalytic amino acid residues by dissimilar or even conserved substitutions leads to inhibition of the RuBisCO activity (Kellogg & Juliano, 1997). Whether or not the ORUs Loihi-*cbbL*-4 and -5 represent inactive deep-sea RuBisCOs cannot be confirmed without further physiological studies. However, RuBisCO sequences from other deep-sea hydrothermal vents have been found to have the same mutations in the catalytic site amino acids (Elsaied & Naganuma, 2001).

All the Loihi-*cbbL* ORUs were related to the green-like RuBisCOs type IA which is found in the most of chemosynthetic β - and γ -

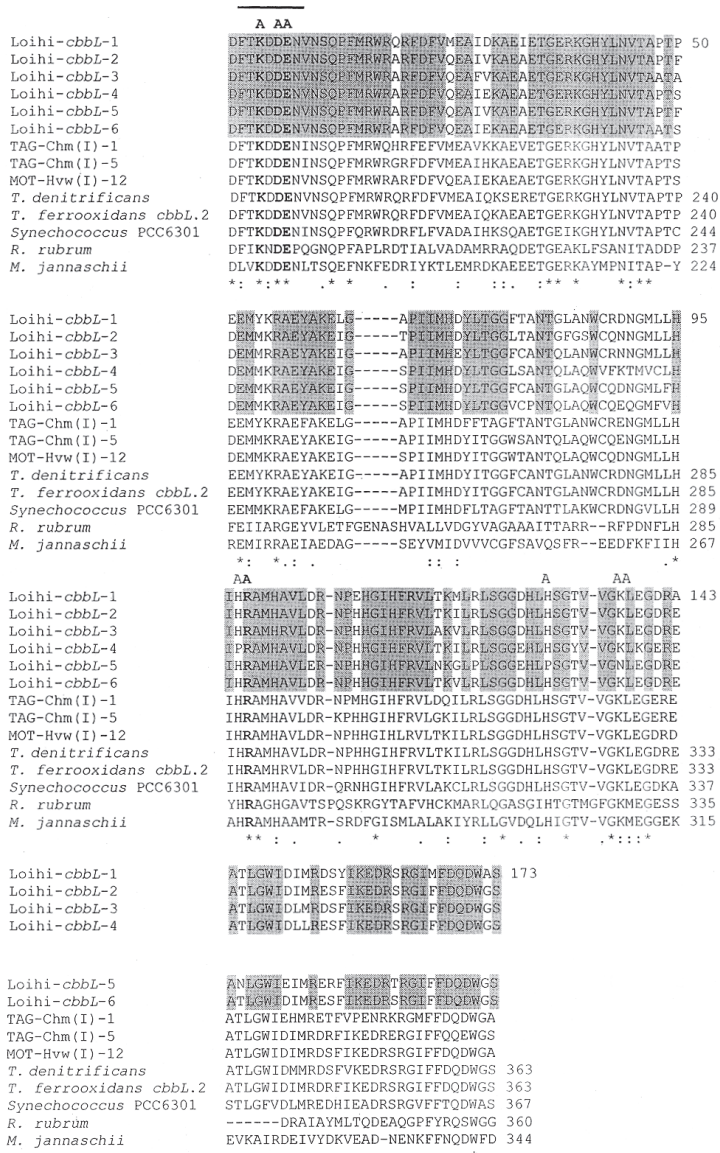


Figure 2. Deduced amino acid partial sequence alignment of Loihi *cbbL* ORUs with those from different RuBisCO types of form I and representatives of form II and archaeal RuBisCOs from *Rhodospirillum rubrum* and *Methanococcus jannaschii*, respectively. Multiple sequence alignments were performed by using ClustalW. The accession numbers corresponding to each large subunit sequence are as follow: TAG-Chm(I)-1, AB038680; TAG-Chm(I)-5, AB038684; MOT-Hvw(I)-12, AB038635; *Thiobacillus ferrooxidans* *cbbL*2, X70355; *Thiobacillus denitrificans*, LA2940; *Synechococcus* sp. PCC6301, X03220; *Rhodospirillum rubrum*, X00286; *Methanococcus jannaschii*, U67564. The residue identities in all alignment sequences are marked with asterisks, conserved substitutions are marked with colons, and semiconserved substitutions are marked with periods. The shaded regions represent the identical amino acid residues in the Loihi *cbbL* ORUs. The characteristic RuBisCO amino acid motif sequence DFTKDDEN is over lined. Known active-site residues are labeled A. Positions of the identical active-site residues in all sequences are typed in boldface. The numbers of aligned *cbbL* amino acid positions of the Loihi ORUs and those of other species are shown on the right side.

Proteobacteria (Fig. 3) (Watson & Tabita, 1997). These Loihi ORUs showed high amino acid identities with those from other hydrothermal vents such as TAG-Chm(I)-1, -5 and MOT-Hvw(I)-12 (Table 2) from the TAG site at Mid Atlantic Ridge and Mid-Okinawa Trough, Japan, respectively (Elsaied & Naganuma, 2001). These phylogenetic similarities imply the conservation of the aerobic RuBisCO gene *cbbL* among widely distributed hydrothermal vents. Moreover, the higher amino acid identities between the Loihi ORUs and the thiobacillic *cbbL* (Table 2, Fig. 3) may reflect the high iron-low pH nature of the Loihi vent (Karl et al., 1988). This coincides with the optimum conditions for certain thiobacillic species such as *Acidithiobacillus ferrooxidans*. The presence of thiobacillic *cbbL*-like ORUs suggest that thiobacilli are responsible for both primary production of organic carbon and formation of Fe-oxide which is the major constituent of Loihi nontronite deposits (Karl et al., 1988).

Table 2. The percentage of amino acids identity between the abyssal Loihi *cbbL* ORUs and the nearest neighbor species from database.

	% identity												
	2	3	4	5	6	7	8	9	10	11	12	13	
1. Loihi- <i>cbbL</i> -1	87	86	80	82	84	80	83	87	91	92	32	39	
2. Loihi- <i>cbbL</i> -2		89	87	89	90	78	87	91	88	89	32	38	
3. Loihi- <i>cbbL</i> -3			84	85	89	78	85	88	88	90	31	38	
4. Loihi- <i>cbbL</i> -4				83	88	72	84	87	82	83	27	39	
5. Loihi- <i>cbbL</i> -5					87	75	84	87	84	86	26	36	
6. Loihi- <i>cbbL</i> -6						76	87	91	86	87	31	39	
7. TAG-Chm(I)-1.							78	77	82	82	30	38	
8. TAG-Chm(I)-5.								90	86	89	32	39	
9. MOT-Hvw(I)-12									89	90	32	40	
10. <i>T. denitrificans</i>										96	32	40	
11. <i>T. ferrooxidans</i> <i>cbbL.2</i>											32	39	
12. <i>R. rubrum</i> ^a												27	
13. <i>M. jannaschii</i> ^a													

^a The species *Rhodospirillum rubrum*, and *Methanococcus Jannaschii* carrying the form II (*cbbM*) and the archaeal RuBisCO gene, respectively, were used as out groups.

Implications from 16S rDNA and RuBisCO phylogenetic trees

Both the 16S rDNA and RuBisCO sequence analyses indicated that chemoautotrophic bacteria are present and may serve as the main primary producers of organic carbon at the studied vent. Methanotrophy may not be a major energy source at this site, as implied by the occurrence of 16S rDNA OTUs closely related to the genus *Methylophaga* whose known species are unable to grow on methane. In addition, some methylophaga species such as *Methylophaga sulfidovorans* draw metabolic energy for CO₂-fixation from the oxidation of sulphur compounds (de Zwart & Kuenen, 1997). The diversity of the chemoautotrophic microflora at this site may reflect the variety of metals that contribute to the *cbbL*-mediated

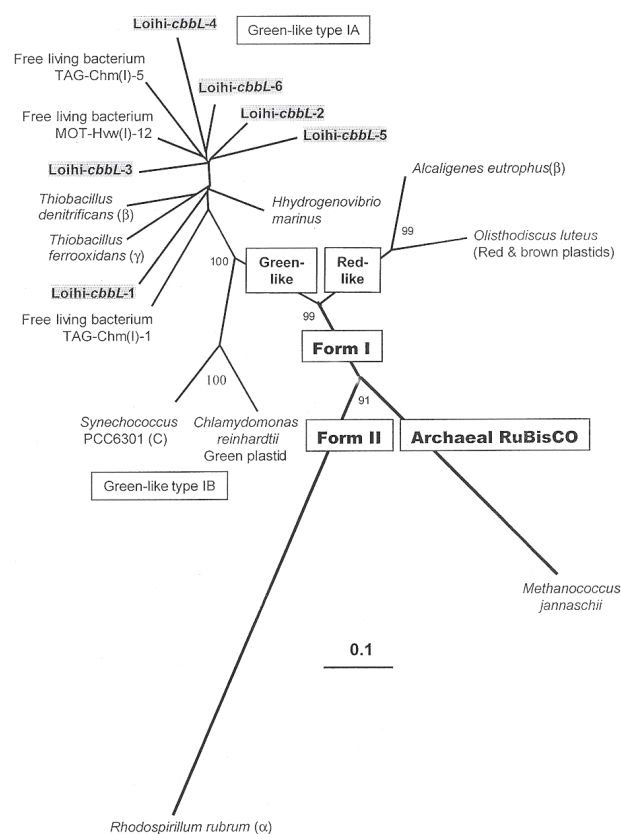


Figure 3. Molecular phylogenetic tree based on the RuBisCO large subunit amino acid sequences of the Loihi ORUs (shaded) and those from database. Tree topology and evolutionary distance are given by the neighbor-joining method with Kimura distances. This tree is unrooted. Bootstrap values, calculated from 1000 replicates, are indicated only at major nodes of the tree and are expressed as percentages. The letters in parentheses represent the expected classification from 16S rDNA or other studies: α , α -Proteobacterium; β , β -Proteobacterium; γ , γ -Proteobacterium; C, cyanobacterium. Scale bar 0.1 substitutions per site.

CO₂-fixation as electron donors, as well as the variability of the vent fluid chemistry (Hilton et al., 1998) and consequently, different pathways of microbial-metal interaction.

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