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16S rDNA-based phylogeny of sulphur-oxidising bacterial endosymbionts in marine bivalves from cold-seep habitats

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ABSTRACT: The phylogenetic relationship of sulphur-oxidising endosymbiotic bacteria from bivalves of the families Vesicomyidae (*Calyptogena* sp. C1, *Calyptogena* sp. C3), Solemyidae (*Acharax* sp.) and Thyasiridae (*Conchocele* sp.) from cold-seep habitats were determined by 16S rDNA nucleotide sequence analyses. The endosymbiotic bacteria form distinct groups within the gamma-Proteobacteria and are well separated from each other and from free-living sulphur-oxidising bacteria of the genera *Beggiatoa*, *Halothiobacillus* and *Thiomicrospira*. The endosymbiotic bacteria of *Acharax* sp. from cold seeps off Oregon, Indonesia and Pakistan have sequences highly similar to each other but quite distinct from other thiotrophic endosymbionts. This includes endosymbionts from *Solemya* spp., to which they are distantly related. Symbiotic bacteria of *Conchocele* sp. from a cold seep in the Sea of Okhotsk are similar to those of *Bathymodiolus thermophilus* and related species, as shown by their overall sequence similarity and by signature sequences. The endosymbiotic bacteria of *Calyptogena* spp. from cold seeps off Oregon and Pakistan are closely related to those of other vesicomyids. Endosymbiont species found off Oregon corresponded to 2 different clusters of *Calyptogena* spp. symbionts in the same samples. The results corroborate the hypothesis of a monophyletic origin of the symbionts in vesicomyid clams, and support the existence of deeply branching groups in solemyid symbionts and of divergent lines and distribution for thyasirid symbionts. The results also indicate that certain symbiont species cluster according to the depth distribution of their hosts, and that in consequence host species together with their symbionts may have undergone depth-specific adaptation and evolution.

KEY WORDS: Endosymbiotic sulphur bacteria · Bacterial phylogeny · Cold seeps · *Acharax* · *Conchocele* · *Calyptogena*

INTRODUCTION

A variety of marine invertebrate species live in symbiosis with thioautotrophic and/or methane-oxidising bacteria (Fisher 1990). These symbioses occur in sulphide- and methane-rich environments, at hydrothermal vents and cold seeps in the deep sea, but also in sulphidic sediments at shallow water depths. Symbionts are harboured by bivalves of the families Solemyidae (e.g. *Solemya* spp.), Mytilidae (e.g. *Bathymodiolus* sp.), Vesicomyidae (e.g. *Calyptogena* sp.), Lucinidae (e.g. *Lucina floridana* and *Codakia orbicularis*), and Thyasiridae (e.g. *Thyasira flexuosa*, *Maorithyas hadalis*) that live in such environments. Most of the symbiotic bacteria are sulphur-oxidising bacteria that have not yet been cultivated. Their properties have been inferred from enzymatic analyses of symbiont-bearing tissue and from phylogenetic relationships on the basis of 16S rDNA nucleotide sequences obtained directly from DNA isolated from host tissue (Distel et al. 1988, 1994, 1995, Distel & Wood 1992, Eisen et al. 1992, Distel & Cavanaugh 1994, Kim et al. 1995, Durand & Gros 1996, Durand et al. 1996, Krueger et al. 1996, Krueger & Cavanaugh 1997, Peek et al. 1998, Fujiwara et al. 2000a,b).

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Table 1. Origin of bacteria and sequences used in this study. Host species names are given for symbiotic bacteria. TVG: TV-guided grab

Host/Bacterium	Area	Type of habitat	Depth	Accession no. of 16S rDNA sequence	Source
Free-living bacteria					
Escherichia coli				K02555	Ehresmann et al. (1972)
Thiomicrospira thyasirae[a] DSM5322				AF016046	Distel & Wood (1992), Wood & Kelly (1989)
T. crunogena ATCC700270	Mid-Atlantic Ridge	Hydrothermal vent		AF069959	Wirsen et al. (1998)
T. crunogena ATCC35932		Hydrothermal vent		L40810	Muyzer et al. (1995)
T. crunogena XCL-2	Galápagos Rift	Hydrothermal vent		AF064545	Ahmad et al. (1999)
T. pelophila DSM1534		Hydrothermal vent		L40809	Muyzer et al. (1995)
Halothiobacillus hydrothermalis[b] r3				M90662	P. Durand et al. (unpubl.)
H. neapolitanus[b] DSM581				AF173169	Sievert et al. (2000)
Beggiatoa alba DSM 1416		Sulphidic sediment		L40994	Teske et al. (1995)
Beggiatoa sp.	Monterey Canyon	Cold seep		AF064543	Ahmad et al. (1999)
Beggiatoa sp.	Bay of Concepción			AF035956	A. P. Teske et al. (unpubl.)
Beggiatoa sp. 1401-13				L40997	Teske et al. (1995)
Vestimentifera					
Lamellibrachia sp.	Gulf of Mexico	Cold seep		U77479	Feldman et al. (1997)
Riftia pachyptila	East Pacific Rise	Hydrothermal vent		U77478	Feldman et al. (1997)
Solemyidae					
Solemya pusilla	Sagami Bay, Japan	Eelgrass beds	Subtidal	U62130	Krueger & Cavanaugh (1997)
S. occidentalis	Belize	Calcarous sands	Subtidal	U41049	Krueger et al. (1996)
S. reidi	Off California	Sewage outfall	150 m	L25709	Distel et al. (1994)
S. terraeregina	Lizard Island, Australia	Coral reef sediments	Subtidal	U62131	Krueger & Cavanaugh (1997)
S. velum	Off Massachusetts	Eelgrass beds	Subtidal	M90415	Eisen et al. (1992)
***Acharax* sp. A-71**	Off Oregon, TVG 71-2	Cold seep	780 m	AJ441185	This study
***Acharax* sp. A-40**	Off Oregon, TVG 40-2	Cold seep	910 m	AJ441187	This study
***Acharax* sp. A-56**	Off Oregon, TVG 56-1	Cold seep	780 m	AJ441197	This study
***Acharax* sp. 'Makran'**	Off Pakistan, TVG 330	Cold seep	2220 m	AJ441188	This study
***Acharax* sp. A-Java**	Off Indonesia, TVG 91	Cold seep	2940 m	AJ441189	This study
Thyasiridae					
Thyasira flexuosa	Plymouth Sound, England	Sulphidic sediment		L01575	Distel & Wood (1992)
Maorithyas hadalis	Japan Trench	Cold seep	7326 m		
Symbiont I				AB042413	Fujiwara et al. (2001)
Symbiont II				AB042414	Fujiwara et al. (2001)
***Conchocele* sp. Sakhakin1**	Sakhalin, Sea of Okhotsk, Trawl 25-1	Cold seep	700 m	AJ441190	This study
Lucinidae					
Lucina floridana	Florida	Eelgrass beds	Intertidal	L25707	Distel et al. (1994)
Codakia orbicularis	Guadeloupe	Seagrass beds	Subtidal	X84979	Durand & Gros (1996), Durand et al. (1996)

Table 1 (continued)

Bathymodiolidae					
Bathymodiolus thermophilus	Galápagos	Hydrothermal vent	2500 m	M99445	Distel & Felbeck (1988)
B. septemdierum	Izu-Bonin Arc	Hydrothermal vent	1289 m	AB036709	Fujiwara et al. (2000b)
B. puteoserpensis					
Symbiont MAR I (thiotrophic)	Mid-Atlantic Ridge	Hydrothermal vent	3476 m	U29163	Distel et al. (1995)
Vesicomyidae					
Calyptogena kilmeri	Off Oregon	Cold seep	795 m	AF035720	Peek et al. (1998)
C. phaseoliformis	Aleutian trench	Cold seep	6370 m	AF035724	Peek et al. (1998)
Calyptogena sp. 'Oregon'	Off Oregon	Cold seep	2000 m	L25710	Distel et al. (1994)
Calyptogena sp. 'Florida'	Florida	Cold seep	3313 m	AF035722	Peek et al. (1998)
C. pacifica	Juan de Fuca Ridge	Sediment-hosted hydrothermal vent	2200 m	AF035723	Peek et al. (1998)
C. elongata	Off California	Cold seep	500 m	AF035719	Peek et al. (1998)
C. elongata	Off California	Cold seep	500 m	L25719	Distel et al. (1994)
Vesicomya cordata	Gulf of Mexico	Cold seep	600 m	L25713	Distel et al. (1994)
V. gigas	Guaymas Basin	Cold seep	2000 m	AF035726	Peek et al. (1998)
V. lepta	Off California	Cold seep	600 m	AF035727	Peek et al. (1998)
***Calyptogena* sp. 'Makran'**	Off Pakistan, TVG 330	Cold seep	2334 m	AJ441191	This study
***Calyptogena* sp. C1-40**	Off Oregon, TVG 40-2	Cold seep	910 m	AJ441192	This study
***Calyptogena* sp. C1-68**	Off Oregon, TVG 68-1	Cold seep	950 m	AJ441193	This study
***Calyptogena* sp. C1-71**	Off Oregon, TVG 71-2	Cold seep	780 m	AJ441194	This study
***Calyptogena* sp. C3-40**	Off Oregon, TVG 40-2	Cold seep	910 m	AJ441186	This study
***Calyptogena* sp. C3-68**	Off Oregon, TVG 68-1	Cold seep	950 m	AJ441195	This study
***Calyptogena* sp. C3-71**	Off Oregon, TVG 71-2	Cold seep	780 m	AJ441196	This study

[a]Previously referred to as *Thiobacillus thyasiris* (Distel et al. 1994); [b]previously assigned to the genus *Thiobacillus* (Sievert et al. 2000)

We analysed the phylogenetic relationship of endosymbiotic sulphur bacteria from bivalves belonging to Vesicomyidae, Solemyidae and Thyasiridae recovered from deep-sea habitats in different geographical locations, and compared the phylogenetic relationship between free-living and symbiotic sulphur bacteria. Included in this investigation were host species that have not been studied so far, i.e. *Acharax* sp. (Solemyidae) and *Conchocele* sp. (Thyasiridae). In addition, knowledge on the phylogeny and distribution of vesicomyid clam symbionts (from *Calyptogena* spp.) has been extended.

MATERIALS AND METHODS

Habitats and bivalve collection. From different cold-seep areas, 5 morphologically distinct species (13 bivalve specimens) were collected (Table 1). Gill samples were excised aseptically from freshly collected bivalves and frozen immediately.

Specimens of a vesicomyid species and of *Acharax* sp. were collected at the Makran convergent margin (off Pakistan, northern Indian Ocean) by a TV-guided grab (TVG) 330 (24° 33.00′ N, 064° 15.64′ E) during RV 'Sonne' Cruise 130 (1998). Tectonically induced venting of fluids enriched in methane led to sulphide-rich environments at this location (von Rad et al. 2000). The vesicomyid clams (*Calyptogena* sp. 'Makran') were similar but not identical to *C. pacifica* (E. Krylova pers. comm.).

Specimens of *Acharax* sp. were collected at an anticline structure in the Sunda fore-arc basin (off Java, eastern Indian Ocean) by TVG 91 (07° 57.5′ S, 106° 17.7′ E) during RV 'Sonne' Cruise 139 (1999). At this location, methane-rich fluids were expelled and authigenic carbonates, bacterial mats, pogonophoran tubeworms (*Spirobrachia* sp.) and giant clams were observed (Wiedicke et al. 2002).

Specimens of *Calyptogena* spp. and *Acharax* sp. were collected at 3 different sites of the Cascadia convergent margin (off Oregon, NE Pacific) during RV 'Sonne' Cruise 143 (1999). Samples at the southern summit of Hydrate Ridge (44° 34.2′ N, 125° 08.8′ W) were taken by TVG 71-2 and TVG 56-1. Here, methane accumulates as gas hydrate near the sediment surface, creating a gradient of sulphide flux, in which free-living filamentous sulphur-

oxidising *Beggiatoa* spp., *Calyptogena* spp. and *Acharax* sp. have niches from higher to lower levels of sulphide fluxes (Sahling et al. 2002). Additional samples were collected at 2 sites of the second accretionary ridge with no apparent indication of the occurrence of gas hydrate: TVG 40-2 recovered bivalves at a feature named Mud Mount East (44° 40.2′ N, 125° 03.3′ W) and TVG 68-2 on the NW-Knoll (44° 43.9′ N, 125° 13.9′ W).

A living specimen of *Conchocele* sp. was recovered from gas seeps on the Sakhalin continental slope in the Sea of Okhotsk, NW Pacific, by Trawl 25-1 (54° 27′ N, 144° 05′ E) during MV 'Marshal Gelovany' Cruise 1 (1999). Video observation (by TV-sled) showed that *Conchocele* sp. was living in close proximity to vesicomyid clams and bacterial mats.

Identity of clam species. The solemyid bivalve specimens of this study were morphologically very similar to each other and compare well with *Acharax johnsoni*, the only deep-water *Acharax* species found off the west coast of northern America (Coan et al. 2000). We consider the specimens investigated in this study to be identical at the species level. However, due to the lack of a robust taxonomy of the genus *Acharax*, we will refer to them in the following as '*Acharax* sp.'.

Calyptogena sp. C1 corresponds well to the type specimen of *C. pacifica*. *Calyptogena* sp. C3 appeared similar, but not identical to *C. kilmeri*. *Calyptogena* sp. 'Makran' was similar but not identical to *C. pacifica* (all identifications by E. Krylova, Moscow, pers. comm.).

The thyasirid specimen in this study was a large-sized specimen morphologically very similar to *Conchocele disjuncta*, according to Bernard (1972). We refer to these clams as *Conchocele* sp., because the separation of the 2 known distinct species by Bernard (1972), i.e. *C. disjuncta* and *C. bisecta*, is under dispute and both species were considered to be identical by Coan et al. (2000).

DNA extraction. Gill tissues were thawed and resuspended in cold-autoclaved and sterile-filtered water and gently homogenised on ice in a glass-tissue grinder. Fractionation of gill homogenate was performed by differential centrifugation (Nelson et al. 1995). DNA from the bacterial fraction was extracted and purified by using the QIAGEN genomic DNeasy tissue kit. In order to prove the purity of DNA extracted from gill tissue homogenates, 16S rDNA fragments were amplified by universal eubacterial primers and separated by denaturing gradient gel electrophoresis (DGGE) according to Petri & Imhoff (2001). The DGGE separation yielded only a single band in all but 1 of the gill homogenates, indicating that just 1 eubacterium was present. Only the DGGE derived from gill homogenate of *Calyptogena* sp. C1-71 showed, in addition to the major band, a second minor band. Reamplification of the prominent band yielded a 16S rRNA sequence highly similar to that of symbionts of other specimens of *Calyptogena* sp. C1.

PCR amplification and sequencing. Polymerase chain reaction (PCR) amplification of 16S-rDNA was started with a combination of the following set of eubacterial primers: Primer 1: 5′-GTTTGATCCTGGCTCAG-3′ (Positions 17 to 27), Primer 2: 5′-GTCAATTCCTTTGAGTTT-3′ (Positions 907 to 924), Primer 3: 5′-CCCGGGAACGTATTCACCG-3′ (Positions 1368 to 1386) according to the *Escherichia coli* numbering of the International Union of Biochemistry. For sequence-analysis the amplified DNA fragments were purified using the SequiTherm sequencing kit (Biozym) based on the chain termination reaction (Sanger et al. 1977). Sequences were generated with an automated laser fluorescence sequencer (ABI310, Amersham Bioscience).

Sequence and phylogenetic analysis. The 16S rDNA sequences of bacteria listed in Table 1 were aligned using the ClustalW program (Thompson et al. 1994). This alignment was the basis for the phylogenetic analysis of the sequence data with different methods. Calculations were made (1) according to maximum likelihood methods (DNAML program of the PHYLIP program package Version 3.57; Felsenstein 1989) by applying a 'hidden Markov model', which infers different evolutionary rates at different sites (Hasegawa et al. 1985, Kishino & Hasegawa 1989, Felsenstein & Churchill 1996); (2) by using distance matrices (DNADIST, on the basis of the algorithm of Jukes & Cantor 1969) for least-squares methods (Fitch & Margoliash 1967; (3) by parsimony methods (DNAPARS program of the PHYLIP program package; Eck & Dayhoff 1966, Kluge & Farris 1969). Bootstrap analyses with 100 resamplings were performed to obtain estimates of phylogenetic tree topologies for all methods.

Because of the importance of the DNA sequence alignment, a second approach was employed, whereby DNA sequences were aligned using the ARB program and database. ARB uses additional information such as secondary structure and minimum energy calculations. The resulting alignment was treated in the same way as the ClustalW alignment. The calculated trees on the basis of the ARB alignment showed branching patterns highly similar to the ClustalW alignment-based trees. Therefore, the results presented for trees were calculated after alignment with ClustalW only. The sequences reported here were deposited in the EMBL database under the Accession nos. in Table 1.

RESULTS

Comparative analyses of the 16S rDNA nucleotide sequences place all symbiotic sulphur-oxidising bacteria into the gamma subclass of the Proteobacteria.

Sequence similarities of the species analysed in this study (see Table 1) were compared to other symbiotic sulphur bacteria as well as to selected free-living sulphur bacteria (Fig. 1). It is apparent that free-living sulphur bacteria of the genera *Beggiatoa* and *Halothiobacillus* form evolutionary lines distantly related to those of symbiotic sulphur bacteria. Also, *Thiomicrospira* species form a deeply branching line distant from other sulphur bacteria. In addition, the signatures of the 16S rDNA sequences support the separation of the *Thiomicrospira* cluster from all others considered here, and give no evidence of a close relationship between *Thiomicrospira* species and any symbiotic sulphur bacterium (Table 2).

The symbiont sequences obtained during this study belong to separate clusters of endosymbionts that are only distantly related to each other and to free-living sulphur bacteria. The symbionts of *Calyptogena* spp. fit well into the known cluster of vesicomyid symbionts, which is distantly related to the cluster including symbionts of *Conchocele* sp., *Bathymodiolus thermophilus* and related species (Fig. 1). Similarly, symbionts of *Acharax* sp. form a cluster clearly separated from all others and only distantly related to those of the mixed multispecies group including *Solemya* spp., *Thyasira flexuosa*, *Riftia pachyptila* and others. The phylogenetic trees obtained with both maximum likelihood and distance methods revealed identical or highly similar results which were also consistent with the grouping according to sequence signatures (Table 2). These signature sequences clearly support the existence of the clusters presented in Fig. 1, and specifically demonstrate the separation of the *Acharax* sp. cluster from all other groups. On the other hand, there is an obvious lack of common signatures within symbionts from the multispecies group with *Solemya* spp. and other host species, supporting the obviously distant relationship of these symbionts. Common signatures exist within the 2 subclusters of vesicomyid symbionts and the *Bathymodiolus*/*Conchocele* symbionts, but other signatures support the separation of these 3 as subclusters. These signatures give clear evidence for the inclusion of symbionts from both *Conchocele* and *Bathymodiolus* in the same cluster and for their relationship to vesicomyid symbionts.

It should be emphasised that the amplification of DNA extracted from the gill tissue of the investigated clams yielded a single amplification product, as witnessed by a single band observed after separation on DGGE gels (a highly sensitive method for the separation of similar DNA fragments). This was the case especially for the amplification products from *Acharax* sp. and *Conchocele* sp., ruling out the possibility that contamination by environmental bacteria did occur and that fragments of such bacteria had been amplified and sequenced. The clear and undisturbed signals during the sequencing further support the purity of the DNA fragments and give no indication of a significant contamination of the extracted DGGE bands. These results demonstrate that each of the host specimens carries a single symbiont strain.

Acharax sp. symbionts

High sequence similarity (>98.8% of 5 specimens) was found between the endosymbionts from different specimens of the *Acharax* sp. collected from cold seeps of geographically distant regions (off Oregon, Indonesia and Pakistan) and from various water depths (780 and 2940 m). These bacteria formed a new group of endosymbionts, not known to date (Fig. 1), that is separate from all other symbiotic sulphur bacteria but distantly related to symbiotic bacteria from *Solemya* spp. and others in the multispecies cluster of shallow-water solemyids, lucinacean bivalves and vestimentiferan tubeworms. Sequence similarities (e.g. to the *S. reidi* or *S. velum* endosymbionts) were <90%. In addition, a number of characteristic signature sequences quite specifically characterises this cluster (Table 2). Electron microscopic studies demonstrated the presence of bacteria within the gill tissue of *Acharax* sp. (data not shown). In addition, $\delta^{13}C$ values of −28 to −36‰$_{PDB}$ (Suess et al. 1998, H. Sahling unpubl.), which are in the typical range of chemoautotrophic sulphur bacteria (Fisher 1990), are indicative of a possibly sulphur-based chemoautotrophic mode of life of this *Acharax* species.

Conchocele sp. symbionts

The endosymbionts of *Conchocele* sp. (a thyasirid clam) are phylogenetically distant from those of other thyasirid species. The 16S rDNA nucleotide sequences are most similar to sulphur-oxidising symbionts of *Bathymodiolus thermophilus* and related species (Fig. 1) and also contain characteristic signature sequences of this group of endosymbionts (Table 2). These signatures clearly support the position of the corresponding sequence in the phylogenetic tree and their assignment to the cluster containing symbionts of *Bathymodiolus* spp. However, the sequence similarity of only 94.4 to 95.0% to symbionts from *Bathymodiolus* spp. indicates a clear separation from these bacteria at the species or even at the genus level.

Symbionts of vesicomyid clams

The endosymbiont sequences of *Calyptogena* spp. analysed during this study grouped into 2 related clus-

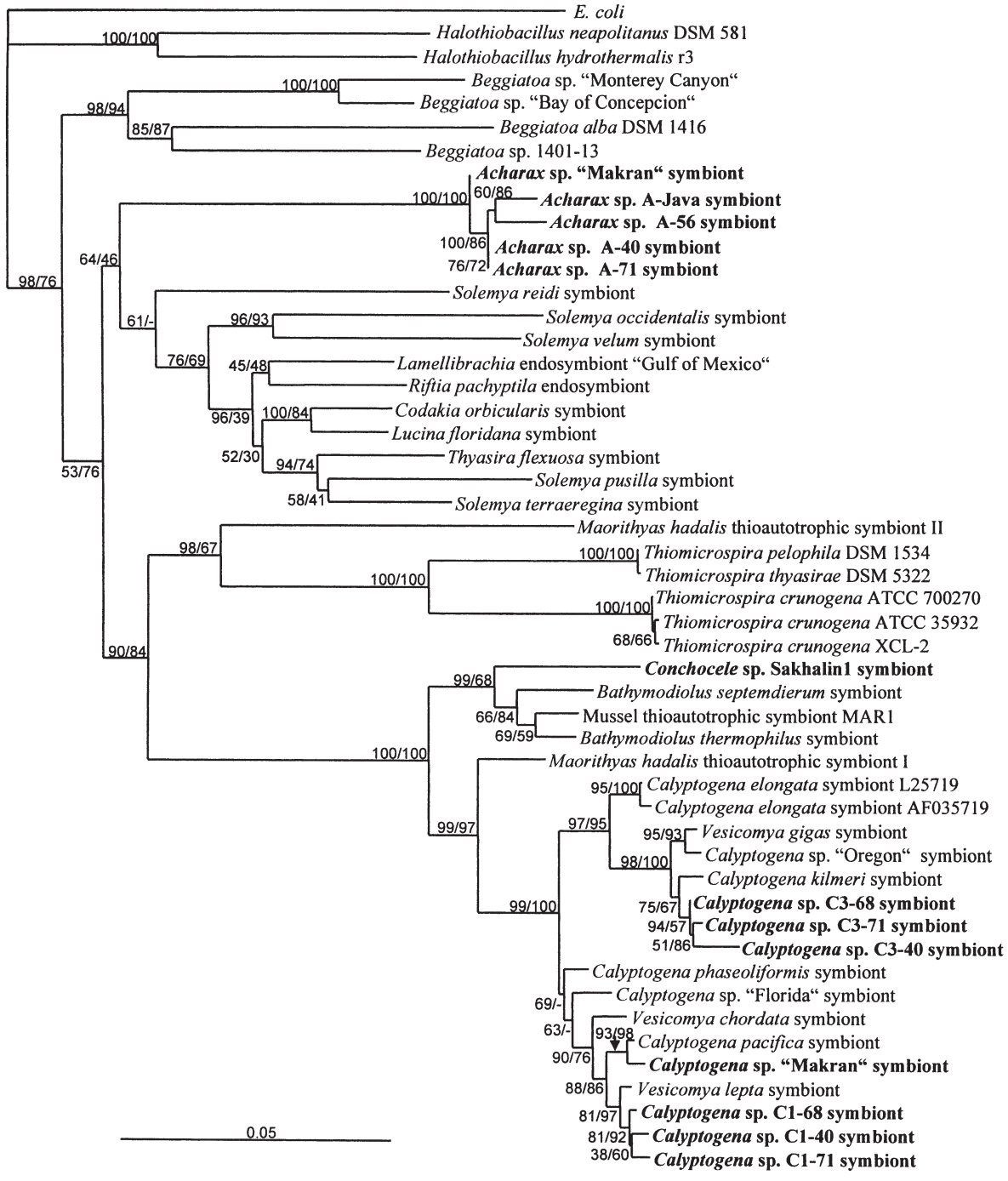

Fig. 1. Phylogenetic tree of free-living and symbiotic sulphur bacteria within the gamma-Proteobacteria demonstrating genetic relationships and grouping of these bacteria according to 16S rDNA sequence similarity. Information concerning sequences obtained during this study and reference sequences together with the deposition numbers are in Table 1; methods for sequence analysis and tree construction are in 'Materials and methods'. The consensus tree was constructed using distance matrix (DM) and maximum likelihood (ML) methods. Trees constructed with both methods showed identical or highly similar branching patterns that revealed symbiont clusters which could also be distinguished by the sequence signatures (Table 2). Values at the nodes correspond to bootstrap percentages of the 2 methods employed (DM/ML). Missing values indicate that the corresponding method did not place the preferred node at that position. The clear separation of *Acharax* sp. symbionts from other symbiont clusters is reflected in the sequence distance, and is supported by bootstrap values which, for the distance method, are 64% of the presented node position and 27% of a node position slightly more distant from the group of *Solemya* spp. symbionts. The corresponding node positions according to the maximum likelihood method have frequences of 46 and 28% respectively. The scale bar corresponds to 5% estimated sequence divergence. Symbiont sequences determined during this study are in bold-face

ters (Fig. 1): (1) The endosymbiotic bacteria of the host species identified as *Calyptogena* sp. C1 have sequence similarities >99.2% (of 3 specimens) and are closely related to endosymbiotic bacteria of a clam referred to as '*Vesicomya lepta*' (>99% sequence similarity). All these clam specimens were recovered from similar depths around 600 to 910 m on the NW coast of the USA. According to signatures, symbionts of the *Calyptogena* sp. 'Makran' are also included in this group, although the overall sequence similarity demonstrates a small distance from the symbionts of *Calyptogena* sp. C1. This specimen was collected from cold seeps at the Makran accretionary ridge off Pakistan, and its sequence is most similar to a symbiont sequence of *C. pacifica* obtained from Juan de Fuca Ridge. Both bivalve specimens were recovered from depths around 2000 to 2300 m, and their symbionts have 16S rDNA sequence similarities of 99.5%. (2) The symbiont sequences of *Calyptogena* sp. C3 share a sequence similarity of >99.0% (of 3 specimens) and cluster with those of a specimen assigned to *C. kilmeri* collected at the Cascadia margin (Peek et al. 1998). These 4 specimens were collected at a depth of 780 to 910 m, and the phylogenetic cluster formed by their endosymbionts is distinct from that of '*Vesicomya gigas*'/*Calyptogena* 'Oregon' (specimens recovered from depth around 2000 m), as shown in Fig. 1.

DISCUSSION

Comparison of the 16S rDNA nucleotide sequences by maximum likelihood and distance methods as well as the presence of characteristic signatures within the sequences that are typical for each of the bacterial groups considered here yielded an analogous picture in regard to the relationship of the chemotrophic sulphur bacteria belonging to the gamma-Proteobacteria. It is evident that separate groups are represented by the free-living sulphur bacteria belonging to the genera *Halothiobacillus*, *Beggiatoa* and *Thiomicrospira* and by the symbiotic bacteria of *Solemya* sp. (including the 'mixed multispecies group' of *Lamellibrachia*, *Riftia*, *Thyasira*, *Lucina* and *Codakia* sp.), *Acharax* sp., *Calyptogena* spp. and related vesicomyid clams, and *Bathymodiolus* spp. (including *Conchocele* sp.). The 2 symbionts with *Maorithyas hadalis* spp. do not fit into any of the existing symbiont clusters but represent separate phylogenetic lines distantly associated with *Thiomicrospira* spp. and with the vesicomyid symbiont cluster respectively (Fujiwara et al. 2001; present Fig. 1). Because none of the symbiont strains and none of the groups of symbionts showed a close relationship to free-living sulphur bacteria, it can be concluded that phylogenetic lines of symbiotic and free-living sulphur bacteria have undergone a long period of evolution separately. This also holds for *Thiomicrospira* spp., which apparently have some ancient ancestor in common with symbiotic sulphur bacteria. Comparison of the age of the symbionts' hosts by means of fossil records revealed that the hosts' phylogenetic lines are similar in age to those of their endosymbionts and suggests a common evolutionary history, at least in some of the host/symbiont partnerships.

Acharax sp.

The host species harbouring endosymbiotic bacteria belong to families that are well known from the fossil record. Among the oldest families known from as early as the Lower Ordivician (~440 million years ago) are the Solemyidae. This family comprises living genera, *Acharax* and *Solemya*. The genus *Acharax* is a cosmopolitan deep-water genus and is distinguished from the genus *Solemya* by a ligament positioned on a nymph, a property well preserved in the fossil record (Coan et al. 2000). Both genera of this family are quite ancient. The *Acharax* lineage is known since the Permian or perhaps the middle Devonian, the genus *Solemya* since the Jurassic (Coan et al. 2000). Apparently, *Acharax* sp. is a deep-water solemyid species living at reducing environments such as cold seeps, while *Solemya* spp. are restricted to shallow water depths. In this context, the identification of *Solemya* spp. at deep-water cold seeps in various publications (see review in Sibuet & Olu 1998) should be treated with caution, because of the similar appearance of *Solemya* and *Acharax* spp. and the possibility of misidentification. The *Acharax* specimens in this study were recovered from 3 cold seeps in the NE Pacific and the Indian Ocean at depths between 780 and 2940 m, although this species may occur in deeper as well as in somewhat shallower waters.

It is striking that symbionts of the 2 solemyid genera *Solemya* and *Acharax* have a particularly deep branching point compared to other clusters of symbiotic bacteria (Fig. 1), but nevertheless form distantly related groups. The large phylogenetic distances between symbionts of different *Solemya* species support their ancient evolutionary history. In fact, this cluster not only contains symbionts from *Solemya* but in addition branches with non-solemyid symbionts: (1) a branch of vestimentiferan symbionts (e.g. of *Lamellibrachia* sp. and *Riftia pachyptila*), (2) a branch of Lucinidae symbionts (e.g. of *Lucina floridana* and *Codakia orbicularis*), and (3) the symbiont of *Thyasira flexuosa* (thyasirid clams). The phylogenetic relationships in Fig. 1 indicate that *Solemya* and *Acharax* have the most ancient ancestor of symbiotic sulphur bacteria

Table 2. Selected signatures of 16S rDNA sequences. Nucleotides that are highly specific for 1 or more clusters

Bacterium	89–91	136–43	152–57	162–68	185–193	222–27
Codakia orbicularis symbiont	TCG	TAGTAGTG	CTCGGG	CTCGAGC	CTAC–GGGGG	ATTAGA
Lucina floridana symbiont	TCG	TAGTAGTG	CTCGGG	CTCGAGC	CTAC–GGGGG	ATTAGA
Riftia pachyptila symbiont	TCT	CAGTAGTG	CTCGGA	TCCGAGC	CTAC–GGGGG	ATTGGA
Lamellibrachia sp. 'Gulf of Mexico' symbiont	TCC	TAGTAGTG	CTCGGA	TCCGAGC	CTAC–GGGGG	ATTAGA
Thyasira flexuosa symbiont	TGT	TATTAGTG	CTCGGA	TCCGAGC	CTAC–GGGGG	AATAGA
Solemya pusilla symbiont	TCT	TATTAGTG	CTTCCG	CGGAAGC	CTAC–GGGGG	ATTAGA
S. terraeregina symbiont	TCC	TAGTAGTG	CTTCCG	CGGAAGC	CTAC–GGGGG	ATTAGA
S. velum symbiont	ATG	TAGTAGTG	CTACCG	CGGTAGC	CTAC–GGGGG	ATTAGA
S. occidentalis symbiont	CCG	CAGTAGTG	CTTCGG	CCGAAGC	CTTA–GGGAG	GTTGGA
S. reidi symbiont	TGC	ATATAGTG	CT–GGG	CCCAGGC	CTAC–GGAGG	ATTTGA
Beggiatoa sp. 1401-13	– – –	TTGTAGTG	CTTGGG	CTCAAGC	CTAC–GGGTG	ATAAGA
B. alba DSM 1416	– – A	TGATAGTG	CCTAGG	CTTAGGC	– – – – –GGGGG	AACAGA
Beggiatoa sp. 'Bay of Concepcion' symbiont	GGA	CAGTAGTA	CCTGAG	CTCAGGC	CTAC–GGGAG	ATTGGA
Beggiatoa sp. 'Monterey Canyon' symbiont	GGA	TAGTAGTG	CTTGGG	CTCAAGC	CTAT–GGGAG	ATTAGA
Acharax sp. A-71 symbiont	CTG	TAGTAGTG	CC**TGGT**	**ACC**AGGC	CTAC–GG**ATG**	**ATTAGA**
Acharax sp. A-40 symbiont	CTG	TAGTAGTG	CC**TGGT**	**ACC**AGGC	CTACAGG**ATG**	**ATTAGA**
Acharax sp. A-56 symbiont	CTG	TAGTAGTG	CC**TGGT**	**ACC**AGGC	CTAC–GG**ATG**	**ATTAGA**
Acharax sp. A-Java symbiont	CTG	TAGTAGTG	CC**TGGT**	**ACC**AGGC	CTAC–GG**ATG**	**ATTAGA**
Acharax sp. 'Makran' symbiont	CTG	TAGTAGTG	CC**TGGT**	**ACC**AGGC	CTAC–GG**ATG**	**ATTAGA**
Thiomicrospira crunogena ATCC 700270	TTT	**CTTTAGTT**	CA**TATG**	**CGTA**TGC	CTAC–GGAGT	**AAAGGA**
T. crunogena ATCC 700270	TTT	**CTTTAGTT**	CA**TATG**	**CGTA**TGC	CTAC–GGAGT	**AAAGGA**
T. crunogena ATCC 700270	TTT	**CTTTAGTT**	CA**TATG**	**CGTA**TGC	CTAC–GGAGT	**AAAGGA**
T. thyasirae DSM 5322	CCT	**CTTTAGTT**	CA**TATG**	**CGTA**CGT	CTAC–GGATG	**AAAGGA**
T. pelophila DSM 1534	CCT	**CTTTAGTT**	CA**TATG**	**CGTA**CGT	CTAC–GGATG	**AAAGGA**
Bathymodiolus thermophilus symbiont	CTT	TAGTAGTG	**CCCAGA**	**TCTGGAT**	CTAT GGATT	ACTAGA
Mussel thioautotrophic symbiont MAR1	CTT	TGATAGTG	**CC AGA**	**TCTGGAT**	CTAT GGAGT	ATCAGA
Conchocele sp. Sakhalin1 symbiont	CTT	TGATAGTG	**CCCAGA**	**TCTGGAT**	CTAT GGATT	ATCACA
B. septemdierum symbiont	CTT	TAGTAGTG	**CCCAGA**	**TCTGGAT**	CTAT GGATT	ACTAGA
Calyptogena sp. C1-40 symbiont	**A T**	TGATAGTG	**CCCGGA**	**TCCGGAT**	CTAC GGAGT	ATCAGA
Calyptogena sp. C1-68 symbiont	**AGT**	TGATAGTG	**CCCGGA**	**TCCGGAT**	CTAC GGAGT	ATCAGA
Vesicomya lepta symbiont	**AGT**	TGATAGTG	**CCCGGA**	**TCCGGAT**	CTAC GGAGT	ATCAGA
Calyptogena sp. C1-71 symbiont				**CCGGAA**	CTAC GGAGT	ATCAGA
Calyptogena sp. 'Makran' symbiont	**AGT**	TGATAGTG	**CCGGGA**	**TCCGGAT**	CTAC GGAGT	ATCAGA
V. chordata symbiont	**AGT**	TGATAGTG	**CCCGGA**	**TCCGGAT**	CTAC GGAGT	ATCAGA
Calyptogena pacifica symbiont	**AGT**	TGATAGTG	**CCCGGA**	**TCCGGAT**	CTAC GGAGT	ATCAGA
Calyptogena sp. 'Florida' symbiont	**AGT**	TGATAGTG	**CCCGGA**	**TCCGGAT**	CTAC GGAGT	ATCAGA
C. phaseoliformis symbiont	**AGT**	TGATAGTG	**CCCGGA**	**TCCGGAT**	CTAC GGAGT	ATCAGA
Calyptogena sp. C3-68 symbiont	**AGT**	TGATAGTG	**CCCGGA**	**TCCGGAT**	**TGAT AA**AGT	ATCAAA
Calyptogena sp. C3-71 symbiont	**AGT**	TGATAGTG	**CCCGGA**	**TCCGGAT**	**TGAT AA**AGT	ATCAA
Calyptogena sp. C3-40 symbiont	**AGT**	TGATAGTN	**CCCGGA**	**TCCGGAT**	**TGAT AA**AGT	ATCAAA
C. kilmeri symbiont	**AGT**	TGATAGTG	**CCCGGA**	**TCCGGAT**	**TGAT AA**AGT	ATCAAA
C. elongata symbiont AF035719	**AGT**	TGATAGTG	**CCCGGA**	**TCCGGAT**	**TGAT AA**AGT	ATCAGA
Calyptogena sp. 'Oregon' symbiont	**AGT**	TGATAGTG	**CCCGGA**	**TCCGGAT**	**TGAT AA**AGT	ATCAAA
V. gigas symbiont	**AGT**	TGATAGTG	**CCCGGA**	**TCCGGAT**	**TGAT AA**AGT	ATCAAA

of the bacteria considered (as defined in Fig. 1) are shown in bold-face. Species grouped into clusters shown in Fig. 1

234–240	453–461	468–479	488–91	513	536	598–601	612–14	636–39	646–49	853–55
TGCGTTG	AGCTTGAGG	CCCTCG–AGTCTT	TTT	C	G	CAGA	CTG	TGAT	CCTCA	GGC
TGCGTTG	AGCCTAGGG	ACCCTG–GGTCTT	TTT	C	G	CAGA	CCG	TGAA	CTTGG	GGC
TGCGTTG	AGCCTGAGG	CCCTTG–GGTCTT	TTT	C	G	CAGA	CTG	TGAT	GCAGG	GGC
TGCGTCG	AGCCTAGAG	GCTCTG–GGTCTT	TTT	C	G	CAGA	CTG	TGAT	CCTGA	GGC
TGCGTTG	AGTTTGAGG	ACCTCG–AATCTT	TTT	C	G	CAGA	CTG	TGAT	CTGAG	GGC
TGCGTTG	AGCTGAAGG	ACCTTT–GTCTTT	TTT		T	CAGA	CTG	TGAT	CCAAG	GGC
TGCGTTG	AGCTTAGGG	CCCCTG–AGTCTT	TTT	C	G	CAGA	CTG	TGAT	CTTGG	GGC
TGCGTCG	AGCCTAAAG	ACTTTG–GGTCTT	TAC	C	G	CAGA	CTG	TGAT	CCTGG	GGC
TGCGTCG	AATCTGAGG	CCCTTG–GATCTT	TAC	C	G	CAGA	CTG	TGAT	CTAAG	GGC
CATGTCG	A– – – – – – – –	– – – – – T – – –	CTC	C	G	TGGA	CCG	CAAA	TTGGG	GGC
TATGTCT	AGCTGTGTG	CCACAT–AGTGTT	**AAC**	**T**	**A**	CAGA	TCG	TGAA	CTTAG	**ATC**
CATGTCG	AAGCACATG	CCATGT–GAAATT	**AAC**	**T**	**A**	CAGA	CTG	TGAT	GTTGA	**ATC**
TATGTCG	AGCTTTAGG	CTCTAA–AGTCTT	**AAC**	**T**	**A**	CGGA	CAA	CGAT	CTTAG	**ATC**
TACGTCG	AGCATTAGG	TCCTAA–TGTGTT	**AAC**	**T**	**A**	CGGA	CAA	CGAT	CTTAG	**ATC**
TATGTCA	**AGTTTAAGG**	**CCCTTG–AACCGT**	**ATT**	C	G	**TGGA**	**CAG**	**CAAA**	**CAAGA**	GAC
TATGTCA	**AGTTTAAGG**	**CCCTTG–AACCGT**	**ATT**	C	G	**TGGA**	**CAG**	**CAAA**	**CAAGA**	GAC
TATGTCA	**AGTTTAAGG**	**CCCTTG–AACCGT**	**ATT**	C	G	**TGGA**	**CAG**	**CAAA**	**CAAGA**	
TATGTCA	**AGTTTAAGG**	**CCCTTG–AACCGT**	**ATT**	C	G	**TGGA**	**CAG**	**CAAA**	**CAAGA**	GAC
TATGTCA	**AGTTTAAGG**	**CCCTTG–AACCGT**	**ATT**	C	G	**TGGA**	**CAG**	**CAAA**	**CAAGA**	GAC
CGCGTTA	ATTAAGTAG	CC**TGCT**–TAGTTT	TTT	**T**	**A**	CGGT	CTG	CGAT	GCAAT	AGC
CGCGTTA	ATTAAGTAG	CC**TGCT**–TAGTTT	TTT	**T**	**A**	CGGT	CTG	CGAT	GCAAT	AGC
CGCGTTA	ATTAAGTAG	CC**TGCT**–TAGTTT	TTT	**T**	**A**	CGGT	CTG	CGAT	GCAAT	AGC
CATGTTA	GGTTAGTAG	CC**TGCT**–AGCTGT	CAC	**T**	**A**	CAGT	CTG	TGAA	GCAGT	AGC
CATGTTA	GGTTAGTAG	CC**TGCT**–AGCTGT	CAC	**T**	**A**	CAGT	CTG	TGAA	GCAGT	AGC
TGCGTAA	AGCTAATGG	CCCATT **AGCCTT**	TTT	C	G	CAGA	CTG	TGAA	GCAAA	GGC
TGCGTAA	AGCTAATGG	CCCATT **AGCCTT**	TTT		G	CAGA	CTG	TGAA	GCAAA	GGC
TGCGTAA	AGCTAGTAG	CTTATT **AGCCTT**	TTT	C	G	CAGA	CCG	TGAA	GTAAA	GGC
TGCGTAA	AGCTAGCAG	CCTGTT **AGCCTT**	TTT	C	G	CAGA	CCG	TGAA	GCAAA	GGC
TGCGTAA	AACTAATGG	CCCAATTAGTCTT	TTT	C	G	CAGA	CCG	TGAA	GCAAA	GGT
TGCGTAA	AACTAATGG	CCCAATTAGTCTT	TTT	C	G	CAGA	CCG	TGAA	GCAAA	GGT
TGCGTAA	AACTAATGG	CCCAATTAGTCTT	TTT	C	G	CAGA	CCG	TGAA	GCAAA	GGT
TGCGTAA	AACTAATGG	CCCAATTAGTCTT	TTT	C	G	CAGA	CCG	TGAA	GCAAA	GGT
TGCGTAA	AACTAGTGG	CCCAATTAGTCTT	TTT	C	G	CAGA	CCG	TGAA	GCAAA	GGT
TGCGTAA	AACTAATGG	CCTA TTAGTCTT	TTT	C	G	CAGA	CCG	TGAA	GCAAA	GGC
TGCGTAA	AACTAGTGG	CCCAATTAGTCTT	TTT	C	G	CAGA	CCG	TGAA	GCAAA	GGT
TGCGTAA	AACTAATGG	CTCA TTAGTCTT	TTT	C	G	CAGA	CCG	TGAA	GCAAA	GGC
TGCGTAA	AATTAATGG	CCCA TTAGTCTT	TTT	C	G	CAGA	CCG	TGAA	GCAAA	GGC
TGCGTAA	AAATAATGG	TCTATT ATTTTT	TTT	C	G	CAGA	CTG	TGAA	GAAAA	GGC
TGCGTAA	AAATAATGG	TCTATT **ATTTTT**	TTT	C	G	CAGA	CTG	TGAA	GAAAA	GGC
TGCGTAA	AAATAATGG	TCTATT **ATTTTT**	TTT	C	G	CAGA	CTG	TGAA	GAAAA	GGC
TGCGTAA	AAATAATGG	TCTATT **ATTTTT**	TTT	C	G	CAGA	CTG	TGAA	GAAAA	GGC
TGCGTAA	AACTAATGG	CCCATTTAGTCTT	TTT	C	G	CAGA	CTG	TGAA	GCAAA	GGC
TGCGTAA	AAATAATGG	TCTATT **ATTTTT**	TTT	C	G	CAGA	CTG	TGAA	GAAAA	GGC
TGCGTAA	AAATAATGG	TCAATT **ATTTTT**	TTT	C	G	CAGA	CTG	TGAA	GAAAA	GGC

and that vestimentiferan and lucinidan symbionts evolved later, possibly from symbionts harboured by ancestors of present-day *Solemya* spp. The assumption of a common symbiotic ancestor in solemyid clams is congruent with both the age of these symbioses, as demonstrated by the fossil records, and the symbiont phylogeny.

In this context, the occurrence of the same symbiont species (according to highly similar nucleotide sequences of the 16S rDNA) in specimens of *Acharax* sp. from very distant localities is quite remarkable. (Note that we consider also the *Acharax* specimens analysed during this work as belonging to the same species.) It indicates that the symbionts of our *Acharax* sp. are a relatively modern species of a much more ancient phylogenetic line.

Calyptogena sp.

The family Vesicomyidae has appeared more recently and dates back to the late Cretaceous (~65 million years; Kanie et al. 1993). Among symbiont-bearing clams, it is one of the most recent developments. Symbionts of Vesicomyidae display much smaller phylogenetic distances than other clam symbionts, form a coherent phylogenetic group, and were proposed to be of monophyletic origin (Distel et al. 1994, Peek et al. 1998). Quite obviously, the phylogenetic distance between symbionts from hosts of ancient history (solemyid clams) is much greater than that of more recent developments (vesicomyid clams). A cospeciation of the endosymbiotic sulphur bacteria and their vesicomyid clam hosts has been suggested based on the comparison of 16S rDNA phylogeny of the symbionts with those of host mitochondria (and a mitochondrial cytochrome oxidase gene; Peek et al. 1998). Mitochondrial DNA sequences revealed that the species '*Vesicomya gigas*'/*Calyptogena kilmeri* and *C. pacifica*/'*Vesicomya lepta*' comprise 2 species complexes (Peek et al. 1997). Taking into account this proposed coevolution of vesicomyid hosts and their symbionts, the phylogenetic relationship of the symbiotic bacteria should also reflect the relationship of the host clams. Indeed, the 2 host species complexes can be recognised by their symbiont phylogeny (Fig. 1). The sequences of symbionts from our study cluster with those of both of the vesicomyid species complexes: symbionts of *Calyptogena* sp. C1 and *Calyptogena* sp. 'Makran' with those of the cluster containing *C. pacifica* and symbionts of *Calyptogena* sp. C3 with those of the cluster containing *C. kilmeri*. Apparently, species of both clusters (*Calyptogena* sp. C1 and *Calyptogena* sp. C3) occur together at the same habitats in western Pacific waters off Oregon.

Conchocele sp.

Conchocele disjuncta is a thyasirid clam found at seeps in Japanese waters (Fujikura et al. 1999) and in the Sea of Okhotsk (Zonenshayn et al. 1987). It is also well documented in the fossil cold-seep record (Callender & Powell 1999). Several observations suggest that the nutrition of *C. disjuncta* could be mixotrophic. It can grow chemoautotrophically due to thiotrophic and methanotrophic endosymbiotic bacteria, but in addition may feed by filtration (chemoheterotrophically). This was concluded by Kuznetsov et al. (1991) because of the presence of this species at methane seeps, the existence of organic matter in the digestive system, the greatly enlarged gills, transmission electron microscopy of gill tissue (Kuznetsov et al. 1991), methane oxidation of gill homogenates (Galchenko et al. 1988) and $\delta^{13}C$ values in the range of −33 to −37 $‰_{PDB}$ (Strizhkov et al. 1990, Kuznetsov et al. 1991). However, evidence given for the presence of methanotrophic endosymbionts was weak, since the bacteria in the electron microscopic pictures lacked the stacked internal membranes typical for Type I methanotrophic bacteria (Kuznetsov et al. 1991), and methane oxidation was only slightly elevated compared to control measurements (Galchenko et al. 1988).

The 16S rDNA from gill tissue of the *Conchocele* sp. specimen available in the present study was analysed in order to confirm the presence of 1 or more endosymbiotic bacteria and to reveal their phylogenetic relationships. We found no evidence for methanotrophic symbionts in this clam. The phylogenetic position of the symbionts from *Conchocele* sp. close to other thiotrophic symbionts suggests that chemoautotrophic oxidation of reduced sulphur compounds is possible. Symbionts of *Conchocele* sp. are, however, related to those of mytilid species but not to those of other thyasirid clams. This adds a new facet to the picture known for other thyasirid symbionts, which are found in different clusters of symbiotic bacteria, but do not form a common coherent group.

Depth-dependent distribution

Symbionts from *Calyptogena* sp. 'Makran' and *C. pacifica* from Juan de Fuca Ridge (both clams recovered from 2000 to 2300 m depth) are separated in the phylogenetic trees from those of *Calyptogena* sp. C1 and '*Vesicomya lepta*' (recovered from 600 to 900 m depth). In the second cluster of vesicomyid clams, symbionts from '*V. gigas*' and *Calyptogena* sp. Oregon (both recovered from approx. 2000 m depth) are separated from those of *Calyptogena* sp. C3 and *C. kilmeri* (recovered from 780 to 910 m depth). This agrees well

with an apparent depth-related distribution pattern of vesicomyid species observed by Fujikura et al. (2000). In consequence, the host species together with their symbionts may have shown depth-specific adaptation and evolution. Also, *Acharax* and *Solemya* spp. clearly show depth-dependent distribution, and their symbionts display quite significantly large phylogenetic distance. In this case, however, the early phylogenetic divergence certainly is of primary importance and overshadows any possible depth-specific relationship between *Acharax* sp. (specimens recovered from 780 to 2940 m) and *Solemya* spp. (found in shallow-water habitats).

Conclusions

As revealed by their phylogenetic relationships (Fig. 1, Table 2), a much greater phylogenetic distance between the symbionts from *Acharax* and *Solemya* species, compared to those from vesicomyid clams, is involved. Nonetheless, symbionts and hosts within this group may have a common evolutionary history, although this is certainly more difficult to define than in the vesicomyid clams. However, we do not see any indication for or necessity to assume a multiple phylogenetic origin of the solemyid symbionts, as proposed by Krueger & Cavanaugh (1997), one of whose arguments was that high symbiont diversity found within a single host genus, *Solemya*, implies that the hosts are phylogenetically much younger than their symbionts. Because we know from the fossil record that *Solemya* is among the oldest clam genera known to carry symbionts, host species of this genus are expected to be genetically much more diverse than more modern genera and than would be concluded from their morphological similarity. Because information on host phylogeny within *Solemya* spp. is insufficient, it is premature to deny parallel diversification of hosts and symbionts in this group. Because the phylogenetic analyses suggest that all solemyid symbionts, including the genus *Acharax*, are derived from a common, although ancient ancestor, which is of approximately the same age as these clams according to the fossil records, such a parallel development could have occurred. The mean sequence difference of approx. 13% between symbionts of *Acharax* spp. and different *Solemya* species is in the range expected for bacteria which have had a separate evolutionary history for approx. 400 to 500 million years if a rate of sequence variation of 1.5% per 50 million years is assumed. This is near the time of the first fossil records of solemyid clams approx. 440 million years ago.

One aspect that has been used to support the hypothesis of parallel evolution of symbionts and hosts in vesicomyid clams is the existence of vertical transmission mechanisms of the symbionts from one generation to the next. Vertical transmission was demonstrated in *Calyptogena* sp. by amplification of symbiont 16S rDNA sequences from ovarial tissue (Cary & Giovannoni 1993). Such a mechanism of symbiont transfer has also been indicated in *Solemya* sp., in *S. velum* (Krueger et al. 1996) and *S. reidi* (Cary 1994). This is certainly an additional strong argument in favour of a co-evolution of symbionts and hosts in solemyid clams also. However, evidence of a parallel host and symbiont evolution on the basis of genetic relationships to support this hypothesis is so far lacking.

The fact that symbiont sequences of hosts other than solemyid clams cluster within this group does not disprove the idea of a common ancestor of symbionts of *Solemya* and *Acharax* spp. Rather, non-solemyid symbionts enclosed within this cluster are considered to be descendants of solemyid symbionts. The relatively close relationship of solemyid gill-associated symbionts and e.g. trophosom-associated symbionts of vestimentiferan tube worms (e.g. *Riftia pachyptila*) is quite remarkable. The mechanism by which the tubeworms have acquired these symbionts remains unexplained and, unless one assumes the free occurrence of the symbionts in the environment, is difficult to explain. So far, free-living close relatives of endosymbiotic sulphur bacteria have not been encountered, not even in the environments inhabited by the symbioses, and, as depicted in Fig. 1, all known free-living sulphur bacteria are distantly related to symbiotic sulphur bacteria.

The situation in the thyasirid clams is quite different: 4 different symbiont sequences known to date occur in 4 different major phylogenetic lines of endosymbionts (Fig. 1). The symbiont of *Thyasira flexuosa* is related to symbionts of *Solemya* sp., the symbiont of *Conchocele* sp. is related to those of *Bathymodiolus* sp., and the 2 symbionts of *Maorithyas hadalis* are not clearly assigned to any of the known clusters (Fig. 1). If a monophyletic origin of the host species is assumed, symbionts in species of this family must have become established independently of each other. Although the data are indicative of the transfer of symbionts from one host species to another, there is currently no evidence to support this.

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