BIPHYTANES AS BIOMARKERS FOR SPONGE-ASSOCIATED ARCHAEA

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ABSTRACT

Various sponges of the classes Hexactinellida and Demospongiae were studied for the presence of archaeal biomarkers. In several species four ether-bound isoprenoids of the biphytane series (C_{40}), which are known as abundant lipid constituents of Archaea, were detected. The lack of these biomarkers in some sponges implies that a prominent influence of dietary plankton can be excluded, and gives evidence that associations of Archaea and specific sponge taxa are more common than previously thought. The highest concentrations of biphytanes were found in the hexactinellid *Aulosaccus* cf. *mitsukuri* and the demosponge *Phakellia ventilabrum*. The distributions of the biphytanes imply that these compounds are of crenarchaeotal and euryarchaeotal origin.

KEYWORDS

Biphytanes, Crenarchaeota, Hexactinellida, Demospongiae, biomarker.

INTRODUCTION

Sponges (phylum Porifera) are the most primitive metazoans whose origins date back to Precambrian times. Numerous taxa are known to maintain diverse prokaryotic communities (WEBSTER *et al.,* 2001b; HENTSCHEL *et al.,* 2002; and refs. cited in either). These prokaryote-eukaryote relationships may have formed an integral part of metazoan evolution (WILKINSON, 1984; BRUNTON & DIXON, 1994).

In general, sponge-associated microorganisms are considered to be involved in essential ecological processes, like nutrient acquisition (VACELET *et al.,* 1995), the supply of precursor lipids incorporated into sponge cell membranes (HAHN *et al.,* 1988), or the production of secondary metabolites (for refs. see *e.g.* WEBSTER & HILL, 2001). In particular, for the Demospongiae numerous studies have shown that their associated microbiota comprise a variety of phylogenetic groups of the domain Bacteria (WEBSTER *et al.,* 2001b; HENTSCHEL *et al.,* 2002; among others). Nevertheless, by employing diverse techniques it has been shown, that most sponge-specific Bacteria elude traditional cultivation methods. Therefore, the ecological functions of most sponge-associated Bacteria are still unclear.

Although frequently attempted by applying different methodological approaches, indications for sponge-associated Archaea have been rarely reported (PRESTON *et al.,*

1996; FUERST *et al.,* 1999; WEBSTER *et al.*, 2001a; MARGOT *et al.,* 2002). Up to now, members of the *Crenarchaeota* and *Euryarchaeota* subgroup have unambiguously been identified only in halichondrid and dictyoceratid demosponges. As indicated by phylogenetic surveys, sponge-specific *Crenarchaeota* are close affiliates of marine benthic or pelagic crenarchaeotes (PRESTON *et al.,* 1996; WEBSTER *et al.,* 2001a; MARGOT *et al.,* 2002). Euryarchaeotal gene sequences in sponge tissue placed within the Methanomicrobiales (WEBSTER *et al.*, 2001a). However, representatives of sponge-associated Archea have resisted isolation in pure culture thus, their general metabolic capabilities remain unknown.

In a recent comprehensive lipid study of numerous sponges from different geographical settings, we frequently identified isoprenoid hydrocarbons diagnostic for Archaea (THIEL *et al.,* 2002). These findings prompted us to focus on the presence of biomarkers highly specific for archaeal subgroups in selected sponges. Further, the biphytane biomarker concentrations were applied for an estimation of archaeal cells in the sponge tissues.

MATERIALS AND METHODS

The sponges analysed for lipid biomarkers are listed in Tab. I. The specimens were sampled by manned submersibles or by triangular dredge and kept at -20°C immediately upon collection. The tissues examined were drawn from the internal parts of the mesohyl and lyophilizised prior to analysis.

Tab. I. Taxonomy, sampling locations of sponges, and occurrence of biphytanes.

Species	***Order***	**Sampling location**	**Water depth [m]**	**Abundance [Σ C_{40}]**
Hexactinellida				
Aulosaccus cf. *mitsukuri* (IJIMA, 1898)	*Lyssakinosida*	Isla Bartholome, Galapagos	440	**high**
Iphiteon panicea (BOWERBANK, 1869)	*Hexactinosida*	Turks & Caicos, Caribbean	480	**high**
Hyalonema (?) sp. 1 (GRAY, 1832)	*Amphidiscosida*	Negril, Jamaica	375	**tr.**
Hyalonema sp. 2 (GRAY, 1832)	*Amphidiscosida*	Long Island, Bahamas	800	**n.d.**
Demospongiae				
Geodia barretti (BOWERBANK, 1858)	*Astrophorida*	Sula-Ridge, Norway	320	**n.d.**
Pachymatisma johnstonia (BOWERBANK, 1842)	*Astrophorida*	Korsfjord, Norway	100-200	**tr.**
Mycale lingua (BOWERBANK, 1866)	*Poecilosclerida*	Sula-Ridge, Norway	290	**low**
Phakellia ventilabrum (LINNEAUS, 1767)	*Halichondrida*	Sula-Ridge, Norway	290	**high**
Phakellia robusta (BOWERBANK, 1866)	*Halichondrida*	Korsfjord, Norway	100-200	**moderate**

tr. = trace, n.d. = not detected.

One sample (2 to 4 g dry weight) of each sponge was extracted and lipids were separated as previously described (MICHAELIS *et al.,* 2002). Briefly, neutral lipids were extracted and saponified by refluxing in a mixture of KOH in CH_3OH, followed by liquid/liquid extraction with *n*-hexane. Polar lipids were separated from the neutral lipid fraction by thin layer chromatography. The polar lipid fraction, comprising glycerol dibiphytanyl glycerol tetraethers (GDGTs), was subjected to cleavage of ether bonds (HI) and subsequent reduction ($LiAlH_4$) of the alkyliodides to hydrocarbons. Hydrocarbons were analysed by capillary gas chromatography (GC) and combined gas chromatography-mass spectrometry (GC-MS) as described elsewhere (MICHAELIS *et al.,* 2002).

Identifications of organic compounds were based on their relative GC retention times, on coinjection with authentic standards prepared from the hyperthermophilic crenarchaeote *Sulfolobus solfataricus* (DE ROSA *et al.,* 1986), and on comparison of their mass spectra with those of published data (SCHOUTEN *et al.,* 1998). Concentrations were determined by GC using 5α(H)-cholestane as an internal standard.

RESULTS

The polar lipids of nine species of the Hexactinellida and the Demospongiae were analysed for the occurrence of archaeal biomarkers. Four archaeal C_{40} isoprenoids (biphytanes), deriving from GDGTs, were found in seven species (Tab. I) of six orders after ether bond cleavage. Highest amounts of biphytanes in total were observed in the hexactinellid *A.* cf. *mitsukuri* (2.4 μg g^{-1} dry wt.) and the demosponge *P. ventilabrum* (0.8 μg g^{-1} dry wt.).

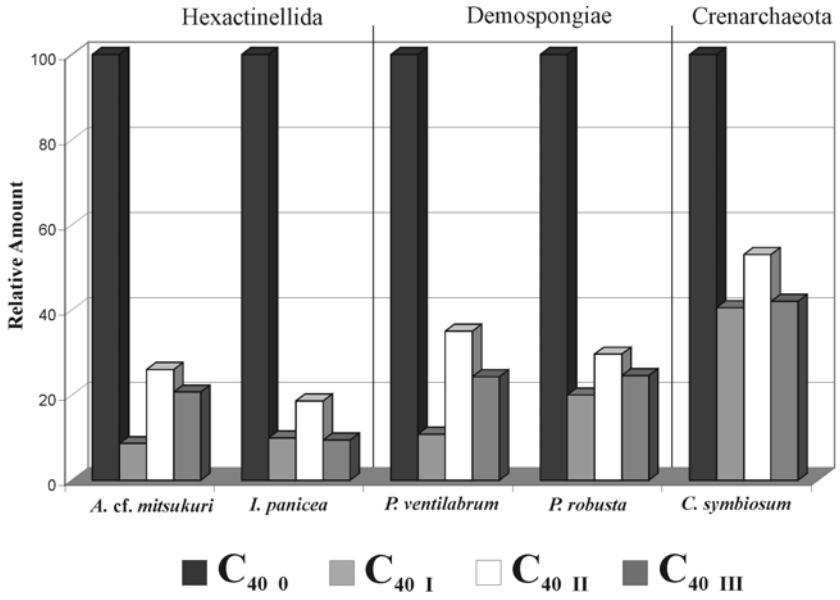

Fig. 1. Abundance of $C_{40\ 0}$, $C_{40\ I}$, $C_{40\ II}$ and $C_{40\ III}$ (normalized to $C_{40\ 0}$) in selected sponges. Abundance in the crenarchaeotal sponge symbiont *C. symbiosum* is shown for comparison (data adapted from DELONG *et al.,* 1998).

The biphytanes in sponges possessed no to three cycloalkyl rings ($C_{40\ 0}$, $C_{40\ I}$, $C_{40\ II}$ and $C_{40\ III}$; Figs 1 and 2). Our experiments indicated that the acyclic $C_{40\ 0}$ and the cyclopentyl ring-containing $C_{40\ I}$ and $C_{40\ II}$ in sponges are structurally identical to those identified in *S. solfataricus*. In contrast, the retention time and mass spectral characteristics of the $C_{40\ III}$ compounds in sponges are different to those found in *S. solfataricus*, suggesting that they are structural isomers (HOEFS *et al.,* 1997; DELONG *et al.,* 1998; SINNINGHE DAMSTÉ *et al.,* 2002b).

Considering the diverse settings, the distributions of biphytanes varied slightly in each sponge, with the acyclic $C_{40\ 0}$ always being the main component followed by the $C_{40\ II}$ homologue (18.6 to 35.0 % relative to the abundance of $C_{40\ 0}$). With one exception (*I. panicea*) the lowest relative abundances were observed for the $C_{40\ I}$ (7.7 to 20 %). Therefore, the C_{40} isoprenoid distributions were fairly similar in sponges of different origins and water depths.

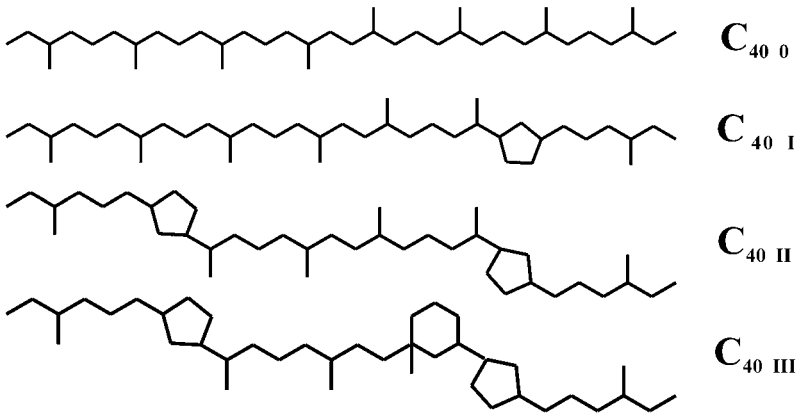

Fig. 2. Structures of GDGT derived biphytanes found in sponges. Shown are the acyclic $C_{40\ 0}$ and the internally cyclized $C_{40\ I}$, $C_{40\ II}$ and $C_{40\ III}$.

DISCUSSION AND CONCLUSIONS

We discovered for the first time ether-derived acyclic ($C_{40\ 0}$) and internally cyclized ($C_{40\ I}$ to $_{III}$) biphytanes indicative for Archaea in various sponge orders (Tab. I). These compounds were absent in the sponges *Hyalonema* sp. 2 and *Geodia barretti*, suggesting that the biphytanes originated from sponge-specific Archaea. With regard to our observations, it is remarkable that, so far, archaeal symbionts have been discovered only in halichondrid and dictyoceratid demosponges (PRESTON *et al.,* 1996; WEBSTER *et al.*, 2001a; MARGOT *et al.,* 2002).

The biosynthesis of biphytanes with two and three cycloalkyl rings accompanied by the acyclic homologue seems to be diagnostic for the *Crenarchaeota* subgroup (PEARSON *et al.,* 2001). Indeed, the distribution patterns of biphytanes in the sponges are very similar to those observed in enrichments of the crenarchaeotal sponge symbiont *Cenarcheum symbiosum* (Fig. 1). However, in comparison to *C. symbiosum*, a

higher relative abundance of the $C_{40\ 0}$ was found in the sponges studied, suggesting an additional contribution by euryarcheaotes (TORNABENE & LANGWORTHY, 1978; KOGA *et al.,* 1993).

Very recently SINNINGHE DAMSTÉ *et al.* (2002b) determined by two-dimensional high resolution NMR studies the structure of a GDGT termed crenarchaeol, which is thought to be indicative of nonthermophilic crenarchaeotes. Crenarchaeol comprises a $C_{40\ II}$ and a $C_{40\ III}$, the latter possessing two cyclopentyl and one cyclohexyl ring (Fig. 2). The mass spectra of the $C_{40\ III}$ in sponges are similar to those published previously for crenarchaeol derived $C_{40\ III}$ (SCHOUTEN *et al.,* 1998), suggesting that $C_{40\ III}$ in our samples emanated from crenarchaeol.

Based on the total amounts of biphytanes, the minimum portion of archaeal biomass in sponges can be roughly estimated as follows: it is assumed, that one cell of marine Archaea contains 1.0 x 10^{-15} g GDGTs (SINNINGHE DAMSTÉ *et al.,* 2002a). Considering the quantitative distributions of biphytanes in the sponges examined, one archaeal cell would contain, on average, 0.9 x 10^{-15} g biphytanes bound in GDGT. For *P. ventilabrum* 0.8 x 10^{-6} g biphytanes per gram sponge tissue (dry wt.) were measured, which corresponds to about 0.9 x 10^{9} archaeal cells g^{-1}. Given the cell weight observed for methanogenic Archaea (one cell corresponds to 1.7 x 10^{-13} g (dry wt.); WHITE *et al.,* 1979), the portion of archaeal biomass in *P. ventilabrum* may be estimated to be 0.2 mg g^{-1} (dry wt.) sponge tissue.

In comparison, using DAPI staining and microscopic counting, bacterial cells in homogenates of *P. ventilabrum* were calculated to be 2.4 x 10^{11} g^{-1} (dry wt.) (*pers. comm.* I. Graeber). Assuming that cells of sponge-associated Bacteria on average are similar in size and weight to those of the bacterium *Escherichia coli* (one dried cell is equivalent to 1.7 x 10^{-13} g; MANCUSO *et al.,* 1990), *P. ventilabrum* would contain about 40.8 mg bacterial biomass g^{-1} (dry wt). Thus, it may be concluded that Archaea - though most probably associated to various sponge taxa - in *P. ventilabrum* constitute a comparatively small fraction of sponge-associated microorganims.

Biomarker analysis in a number of studies have proven to be an effective and specific tool to document the composition and metabolic capabilities of microorganisms in fossil and recent sample sets. Employing this methodological approach to examine the microbial communities in sponges, our work established for the first time that:

- Archaea, as evidenced by the presence of biphytanes, are more common constituents of sponge-associated microorganisms than previously thought;
- the specific presence of biphytanes in sponges reveals sponge-associated cren- and euryarchaeotal populations rather than a contribution of pelagic Archaea.

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