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Identification of the antifungal peptide-containing symbiont of the marine sponge *Theonella swinhoei* as a novel δ-proteobacterium, “*Candidatus Entotheonella palauensis*”

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**Abstract** The Palauan sponge *Theonella swinhoei* (class Demospongiae, order Lithistida, family Theonellidae) harbors filamentous bacterial symbions that contain thepalauamid, an antifungal, bicyclic glycopeptide. In this study, the filamentous symbions were shown to be novel bacteria belonging to the δ-subdivision of proteobacteria. The 16S rRNA gene sequence was determined using a combination of denaturing gradient-gel electrophoresis (DGGE) and specific polymerase chain-reaction (PCR) primers, and its source was confirmed by in situ hybridization. In a series of culture experiments, the filamentous bacteria were propagated in a mixed culture on agar plates. Related 16S rRNA gene sequences were isolated from related sponges with slightly different chemistry. The taxonomic status “*Candidatus Entotheonella palauensis*” is proposed for the thepalauamid-containing filamentous bacteria from *T. swinhoei*.

**Introduction**

Sponges belonging to the order Lithistida commonly contain bioactive and chemically interesting natural products (Bewley and Faulkner 1998). Within this order, members of the genus *Theonella* are particularly noteworthy for the diversity and potency of their peptide and polyketide secondary metabolites. Many theonellid metabolites have been hypothesized to originate in symbiotic microbes because of close structural similarities to microbial metabolites (Fusetani and Matsunaga 1993; Kobayashi and Ishibashi 1993). Sponge compounds in general are often ascribed a microbial origin (Faulkner et al. 1993), but few metabolites have been definitively localized in bacterial cells (Unson et al. 1994). In lithistid sponges, all but one of the microbial-origin hypotheses remain conjecture, but they gained some experimental weight when Bewley et al. (1996) separated two bacterial cell fractions from the lithistid sponge *T. swinhoei* (Fig. 1). One cell fraction was composed of a mixture of unicellular bacteria, and the macrocyclic polyketide cytotoxin swinholide A (Kitagawa et al. 1990) was found solely associated with these cells. The other fraction was enriched in a single morphotype of filamentous bacteria that contained the bicyclic glycopeptide, thepalauamide (Schmidt et al. 1998). Bewley (1995) also described the symbiosis between the filamentous bacteria and their host sponges and compared the presence of filamentous symbions in lithistid sponges to the presence of modified peptide metabolites.

The filamentous bacteria containing thepalauamide have a distinct morphology and can be clearly recognized under light microscopy (Bewley 1995), but despite their discovery in *Theonella swinhoei* in 1888 (Sollas) the identity of the symbions remained unknown. The association between these distinctive filamentous bacteria and *T. swinhoei* appears to be a specific association, rather than the result of indiscriminate filter-feeding. The filaments have been observed in *T. swinhoei* throughout the Indo-Pacific and do not occur in other sponges found in the same locations (Bewley 1995). The bacteria are found in the mesohyl, not in the choanocyte chambers where digestion occurs, and no phagocytosis of the bacteria has been observed (Bewley 1995). Although they could exist in other habitats and may be recruited from seawater, these bacteria appear to be selectively retained and supported by *T. swinhoei* relative to other sponges. Based on gross morphology, some workers proposed that the bacteria were cyanobacteria (Kobayashi and Ishibashi 1993), even though the
bacteria do not contain photosynthetic pigments. Based on more detailed morphological studies using transmission electron microscopy, Bewley et al. (1996) suggested that the bacteria might belong to the family Beggioaaceae. However, because the bacteria had not been cultivated, despite several attempts (Kobayashi and Kitagawa 1998), no molecular or metabolic data were available to confirm these proposed identities.

The lack of culture conditions for the isolation of filamentous *Theonella swinhoei* symbionts is not surprising, since sponge symbionts are generally difficult to isolate. For example, in two studies of the same sponge species, major populations of cultured bacteria were wholly different than those detected by molecular methods (Müller et al. 1981; Althoff et al. 1998). From the viewpoint of biotechnology, culturing the symbionts could help to solve the recognized supply problem for sponge-derived drugs. Even excellent drug candidates from sponges are often not developed because sponges are rare, difficult to collect, or both (Faulkner 2000). If some compounds are derived from symbionts, culturing these bacteria could provide an improved source of bioactive compounds.

Finally, little is known about the extent and specificity of the symbiosis. Some sponge symbioses may have originated before the divergence of modern sponge orders, approximately 500 million years ago (Wilkinson 1987). As evidence of the long-term co-existence of sponges and microbes, fossil reefs from the Cambrian Era include sponges in intimate association with mats of filamentous photosynthetic and heterotrophic bacteria (Brunton and Dixon 1994). Filamentous, non-photosynthetic microorganisms are often found in lithistid sponges (Bewley 1995), and many of these sponges are not closely related to each other (Kelly-Borges and Pomponi 1994). These data are intriguing, but because of the polyphyly of Lithistida, general conclusions will be difficult to obtain without extensive comparison of chemistry, symbiont 16S rDNA sequence, and molecular taxonomy of sponge hosts. However, some preliminary hypotheses about the origin and extent of certain symbioses can be gleaned by comparing symbionts within a more tractable group of sponges, such as the theonellids, with those from other sponges. The filamentous symbionts of *Theonella swinhoei* provide a good starting point because the symbiosis has a readily identifiable chemical marker, the marine natural product, theopalaumamide, in addition to the unique morphology of the filaments. In this study, sponges were selected for symbiont characterization based on their chemistry to see if there was a correlation between the type of symbionts and the secondary metabolites. The goals of this study were to determine the phylogeny of the filamentous symbionts using 16S rDNA analysis, to culture the symbionts, and to develop methodology for further studies of sponge-bacteria symbiosis.

**Materials and methods**

Collection of sponges

Samples of *Theonella swinhoei* (Demospongiae: Lithistida: Theonellidae) were collected at 10 to 30 m depth in the Republic of Palau, Western Caroline Islands, in 1997 and 1998, and at 5 to 20 m depth near Boracay Island, Philippines, in 1998. Samples ($n = 12$) were analyzed in the field for the presence of theopalaumamide and related compounds by thin-layer chromatography and for filamentous microbes by microscopy. Once symbiont-specific PCR primers were in hand, they were applied to a number of sponge samples that had been collected for chemical research and stored in methanol at 4 °C for several years. While these samples could be used for analysis of chemistry, 16S rRNA sequence, and the presence of filamentous bacteria, they were unsuitable for cell separation, cultivation, or in situ hybridization studies because of their age and storage conditions.

Preparation of samples for microbiological and molecular biological experiments

Enriched filamentous bacterial preparations and fresh sponge tissue served as inocula for various microbiological media and were used...