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Two classes of metabolites from *Theonella swinhoei* are localized in distinct populations of bacterial symbionts

C. A. Bewley, N. D. Holland and D. J. Faulkner*

Scripps Institution of Oceanography, University of California, San Diego, La Jolla (California 92093, USA)
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Abstract. The marine sponge *Theonella swinhoei* (lithistid Family Theonellidae, Order Astrophorida) has yielded many important, bioactive natural products, most of which share structural features with bacterial natural products. The presence of microbial symbionts in *T. swinhoei* has been reported, and it was originally suggested that the cytotoxic macrolide swinholide A and many of the bioactive cyclic peptides from *T. swinhoei* were all produced by symbiotic cyanobacteria. By transmission electron microscopy, we found four distinct cell populations to be consistently present in *T. swinhoei*: eukaryotic sponge cells, unicellular heterotrophic bacteria, unicellular cyanobacteria and filamentous heterotrophic bacteria. Purification and chemical analyses of each cell type showed the macrolide swinholide A to be limited to the mixed population of unicellular heterotrophic bacteria, and an anti-fungal cyclic peptide occurred only in the filamentous heterotrophic bacteria. Contrary to prior speculation, no major metabolites were located in the cyanobacteria or sponge cells.
Key words. *Aphanocapsa feldmanni*; *Beggiatoa*; cyanobacteria; lithistid sponge; *Theonella swinhoei*; secondary metabolites; swinholide A; symbiosis.

Natural products are generally assumed to be produced by the organism from which they are extracted. This assumption, which provides the basis for chemotaxonomy, is not always justified since marine invertebrates can accumulate bioactive metabolites from their diet[1] or from microbial symbionts[2]. However, despite considerable speculation, it is rare to find the major metabolites of an invertebrate located exclusively in associated microorganisms, as we have demonstrated to be the case for the lithistid sponge *Theonella swinhoei* from Palau. Sponges thought to be *T. swinhoei* are common in most tropical and sub-tropical oceans, and typically contain macrolides such as swinholide A (fig. 1, **1**)[3], cyclic peptides such as theonegramide (**2**)[4], and at least two distinct types of symbiotic microorganisms[3-6]. Our interest in the origins of natural products from marine invertebrates that contain bacterial symbionts led us to investigate Palauan specimens of *T. swinhoei* that contain swinholide A and a new cyclic peptide, referred to as P951 (**3**). P951 differs from theonegramide by the presence of a hexose rather than a pentose sugar, and its structure will be reported elsewhere.

Swinholide A (**1**) is a symmetrical dimeric dilactone that is cytotoxic to a variety of cancer cell lines[7]. The total synthesis of swinholide A[8], and a possible mechanism of action for its cytotoxicity[9], were recently reported. The macrolide scytophycin C (**4**), from the filamentous cyanobacterium *Scytonema pseudohofmanni*, is strikingly similar in structure and biological activity to swinholide A (see fig. 1)[10]. Based on a scanning electron micrograph, the presence of filamentous bacteria, which the authors suggested were cyanobacteria, were reported for the Okinawan collection of *T. swinhoei* that contained **1**, and it was suggested that these microorganisms might produce swinholide[3].

Theonegramide (**2**), from a Philippine specimen of *T. swinhoei*, is an anti-fungal bicyclic glycopeptide that contains the unusual β-amino acid AHMP (fig. 1)[4]. AHMP (3-amino-4-hydroxy-6-methyl-8-phenylocta-5,7-dienoic acid) is structurally similar to the β-amino acid 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4,6-dienoic acid (ADDA) (fig. 1)[11], a constant component of hepatotoxic cyclic peptides, such as microcystin YR (**5**)[12], that are responsible for the hepatotoxicity associated with cyanobacterial blooms[13]. To date, lithistid sponges and cyanobacteria are the only known sources of peptides containing these unique β-amino acids, which led to the suggestion of a 'blue-green algal' origin for the peptides[14].

The sponge *Theonella swinhoei* contains four distinct cell populations: sponge cells, unicellular heterotrophic bacteria, unicellular cyanobacteria and filamentous heterotrophic bacteria. All populations of associated bacteria are located extracellularly, and only the unicellular cyanobacterium *Aphanocapsa feldmanni* has been identified to species based on ultrastructural studies[5]. *A. feldmanni* is found only in the outermost tissues (ectosome) of the sponge, and specimens of *T. swinhoei* growing in completely dark environments are devoid of these ectosomal cyanobacteria. The filamentous bacterial symbionts, which we have observed consistently in specimens of *T. swinhoei* from all locations, are found in large numbers and only in the interior (endosome) of the sponge. The collective group of unicellular hetero-

* Corresponding author.

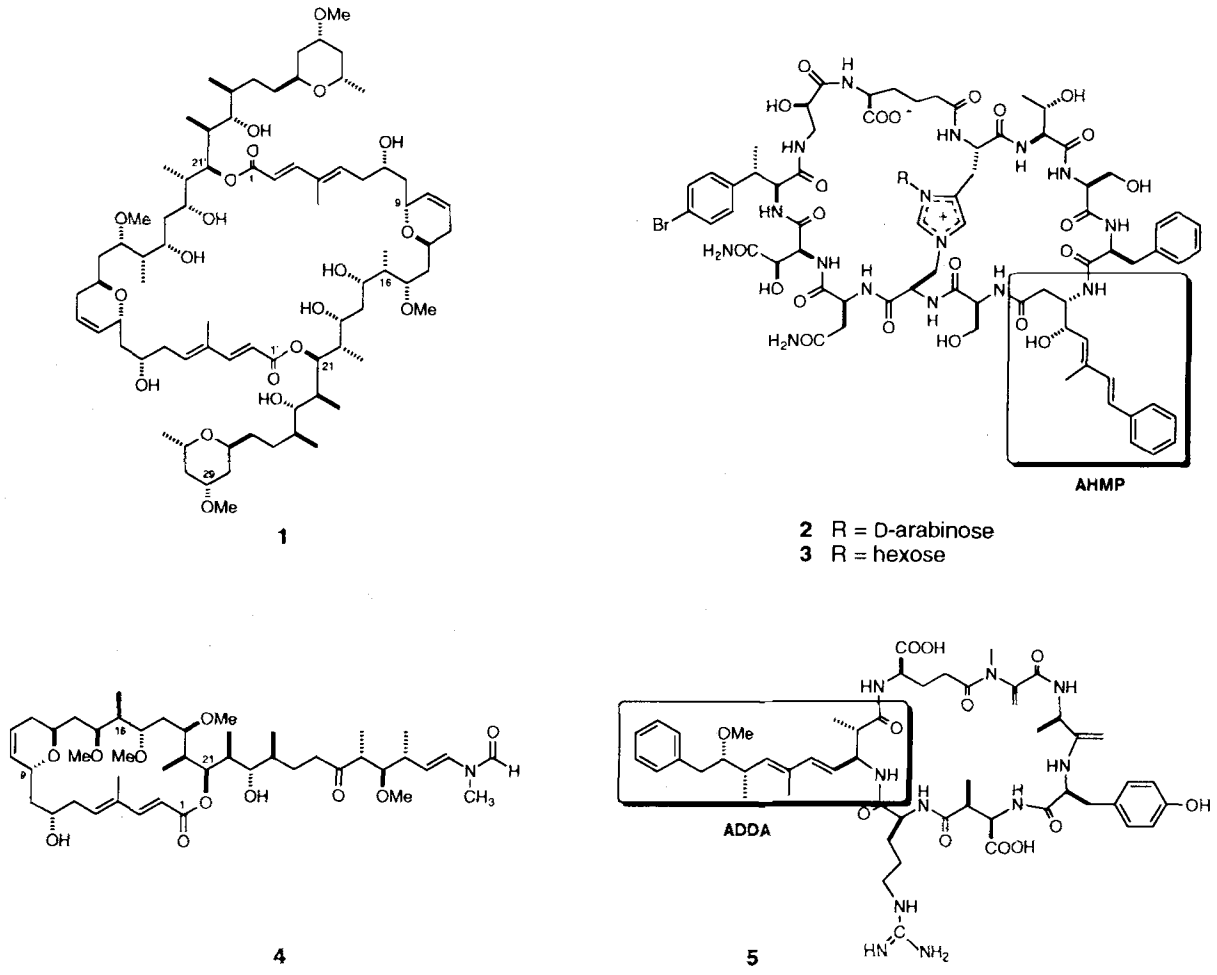

Figure 1. Structures of the sponge metabolites swinholide A (**1**), theonegramide (**2**) and P951 (**3**) from the lithistid sponge *T. swinhoei*, and the cyanobacterial metabolites scytophycin C (**4**) and microcystin YR (**5**), where Y and R refer to tyrosine and arginine, from *Scytonema pseudohofmanni* and *Microcystis aeruginosa*, respectively. The β-amino acids AHMP of **2** and **3**, and ADDA of **4**, are highlighted for comparison.

trophic bacteria are found throughout the sponge in both ectosomal and endosomal tissues and presumably include symbionts as well as non-specifically associated bacteria ingested as food from surrounding waters.

Others have reported difficulties in culturing symbiotic microorganisms from marine invertebrates[15,16], and have observed that a bacterial isolate may not necessarily produce the metabolite of interest when grown in culture[17]. We avoided these problems by separating the four cell populations found in *T. swinhoei* by centrifugation and analyzing the organic extracts of each cell pellet. We propose that metabolites found exclusively in one cell type are produced therein, since complete transport of a large secondary metabolite from one cell type to another is unlikely.

Materials and methods

General experimental procedures. All solvents used were spectrophotometric grade (Fisher Optima). Column chromatography was carried out on Merck silica gel 60 (70–230 mesh) and Si-gel and reversed-phase C-18 Sep-paks (Millipore-Waters). ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity 500 spectrometer; samples were dissolved in deuterated chloroform ($CDCl_3$) or dimethylsulfoxide (DMSO-d_6) (Isotec). High-resolution mass spectra were run at the Mass Spectrometry Facility, University of California at Riverside. A Perkin-Elmer high-performance liquid chromatograph and diode-array detector were used for analytical HPLC experiments. A Zeiss transmission electron microscope was used for transmission electron micrographs.

Collection. Eight specimens of *T. swinhoei* (Order Astrophorida, Family Theonellidae), a fleshy, evert tube-shaped sponge with a red exterior (attributable to the cyanobacteria) and beige interior, were collected by hand using scuba at depths ranging from 10 to 33 m at three different locations in the Republic of Palau, Western Caroline Islands. Specimens were transported in seawater to the laboratory and transferred to aquaria.

One specimen (400 g wet weight), to be used for total extraction of secondary metabolites, was frozen immediately after collection at −20 °C and shipped frozen back to La Jolla, California. Examination of the sponges and their spicules by light microscopy confirmed the identity of our specimens with *T. swinhoei* by Sollas[18].

Cell separations. Since the cyanobacterium *A. feldmanni* is found only in the ectosome of *T. swinhoei*, and the filamentous heterotrophic bacteria are found only in the endosome, separation of these two types of bacteria was accomplished by gross dissection of intact specimens of *T. swinhoei*. The distinct red ectosome was completely removed from three sponges to yield ectosomal tissue and cyanobacteria-free endosomal tissue. These samples were processed separately by dissociating within an Omega 1000 juicer. The tissue collected on the filter was immediately fixed by suspending in 1.25% glutaraldehyde/1.9% formaldehyde in 0.2 μM-filtered seawater (300 ml, pH 8.15). The supernatant cell suspensions were filtered (42 μM nylon mesh), and three replicate cell pellets, each 1 ml in volume, of the four cell types were prepared by repeated centrifugation of the filtrate. The filamentous bacteria pelleted at 200 × g (5 min), and the sponge cells pelleted at 600 × g (5 min). The remaining supernatants were shown by light microscopy to contain >95% unicellular heterotrophic bacteria which were pelleted by centrifugation of 4500 × g (15 min). The unicellular cyanobacteria obtained from the dissociated ectosomal tissue were purified by centrifugation at 1000 × g (7 min). Each cell pellet was washed three times by resuspending in sterile filtered seawater followed by centrifugation.

TEM. Aliquots of glutaraldehyde-fixed cells were embedded in agar, post-fixed for 2 h at 4 °C in 1% OsO_4 in 0.2 μM-filtered seawater, and dehydrated through an ethanol series. The specimens were embedded in Spurr's resin, and thin sections were stained in lead nitrate/acetate/citrate[19] before observation in the transmission electron microscope.

Extraction and analysis by HPLC. Each washed cell pellet was frozen in liquid nitrogen (N_2) and lyophilized overnight. The average dry weight for each type of cell pellet (n = 3) was as follows: sponge cells, 81.6 mg; unicellular cyanobacteria, 102.7 mg; filamentous heterotrophic bacteria, 98.2 mg; unicellular heterotrophic bacteria, 117.2 mg. The freeze-dried cells were extracted with MeOH (2 ml). 100 μl of each extract was removed for analysis by reversed-phase HPLC. Peaks were detected and UV spectra were recorded by diode array detection. Conditions: Vydac C-18 analytical column, 25–80% acetonitrile in 0.05% TFA in 50 min, flow rate = 1.5 ml/min. The UV spectra (not shown) of the peptide P951 (R_f 12.56 min) and the major peak (R_f 12.62 min) in chromatogram (c) were identical, as were the UV spectra of swinholide A (R_f 42.62 min) and the major peak (R_f 42.11 min) in chromatogram (d). When coinjected, the major components of extracts from the filamentous bacteria and the unicellular heterotropic bacteria coeluted with P951 (R_f 12.28) and swinholide A (42.56 min), respectively.

^{1}H NMR. The MeOH extracts of each cell pellet were dried under a stream of N_2 and dissolved in $CDCl_3$ (800 μl) for analysis by ^{1}H NMR. Extracts of sponge cells and cyanobacteria were completely soluble in $CDCl_3$. Extracts of the unicellular heterotrophic bacteria and the filamentous bacteria were incompletely soluble in $CDCl_3$, and the $CDCl_3$ insolubles of these extracts were dried under a stream of N_2 and dissolved in DMSO-d_6 (800 μl) for analysis by ^{1}H NMR. A 500 MHz ^{1}H NMR spectrum was acquired for each extract (12 in total–3 replicates for each of 4 cell types). All spectra were acquired with the samples not spinning and consisted of 612 scans; FIDs were processed in absolute intensity mode and therefore indicate the quantities of metabolites present in each extract. Proton NMR spectra for swinholide A (**1**) and P951 (**3**) were acquired on 1 mM solutions in $CDCl_3$ and DMSO-d_6, respectively. The FIDs of these standards consisted of 128 scans and were processed in absolute intensity mode.

Extraction of *Theonella swinhoei* and purification of secondary metabolites. One frozen specimen of *T. swinhoei* was cut into slices 3 × 3 × 1 cm and lyophilized overnight. The freeze-dried sample (60 g dry weight) was extracted exhaustively with hexanes, ethyl acetate (EtOAc) and 50% aqueous acetonitrile[4]. NMR showed the EtOAc extract to contain swinholide A (**1**), and the aqueous acetonitrile extracts were shown by ^{1}H NMR, thin-layer chromatography (TLC) and bioassays against *Candida albicans* to contain an antifungal peptide, P951 (**3**), similar in structure to theonegramide (**2**).

Swinholide A (1). 200 mg of the ethyl acetate extract was chromatographed on Si-gel by flash-column chromatography (1.5 × 20 cm) eluting with a solvent gradient from 50% EtOAc in hexanes to 100% EtOAc for elution. Fractions that contained swinholide A were combined and re-chromatographed on a Si-gel Sep-pak (Millipore-Waters, 3 cc), and 50% EtOAc in hexanes was used as the eluent to yield 2.2 mg of pure swinholide A; positive ESI (electrospray), observed *m/z* 1390.2 $(M + H)^+$, $C_{78}H_{133}O_{20}$ requires *m/z* 1389.94.

Peptide P951 (3). The peptide was isolated using the procedure described for the isolation of theonegramide (2)[4]. Ultraviolet (UV) and infrared (IR) spectral data were identical to theonegramide, and ^{1}H NMR spectra for **2** and **3** in DMSO-d_6 are indistinguishable. Fast atom bombardment mass spectroscopy (FABMS), observed *m/z* 1749.6 $(M + H)^+$, $C_{76}H_{100}N_{16}O_{27}{}^{81}Br$ requires *m/z* 1749.6107; high-resolution FABMS, observed *m/z* 1771.5663 $(M + Na)^-$, $C_{76}H_{99}N_{16}O_{27}Na^{81}Br$ requires *m/z* 1771.6212 (−5.7 ppm). The distribution of peaks

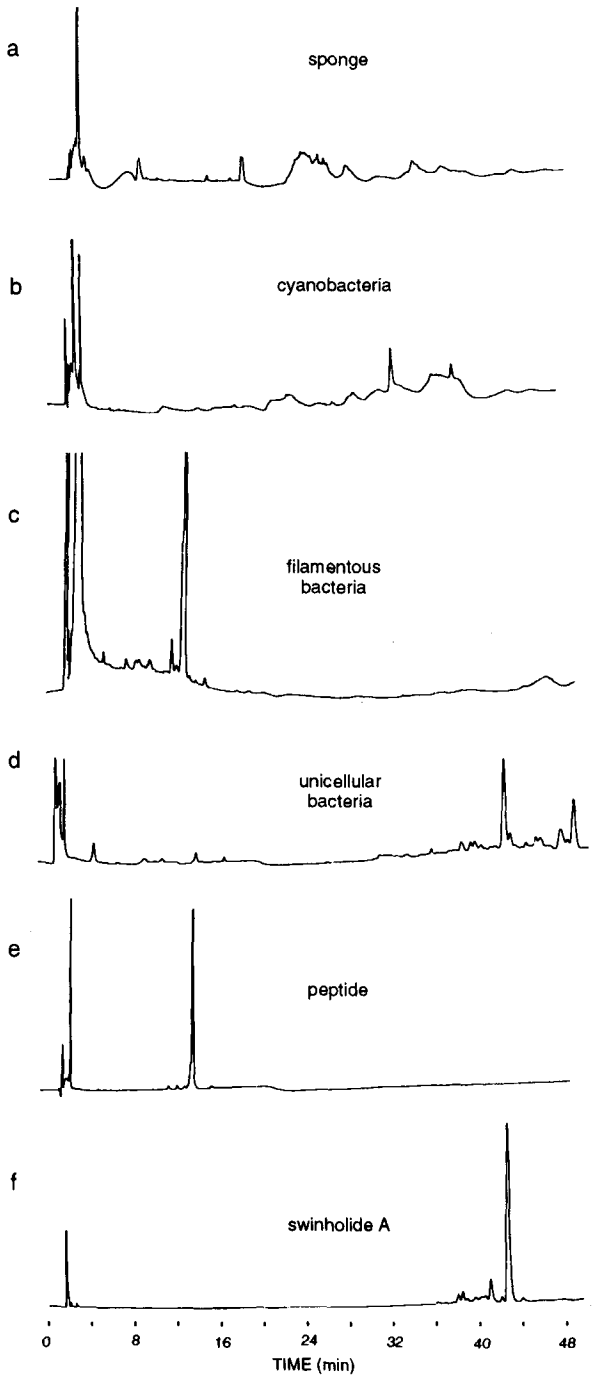

Figure 2. HPLC chromatograms of the extracts of the four cell types found in *T. swinhoei*, namely, (*a*) sponge cells, (*b*) unicellular cyanobacteria (*A. feldmanni*), (*c*) filamentous heterotrophic bacteria and (*d*) unicellular heterotrophic bacteria, and standards of (*e*) P951 and (*f*) swinholide A. When coinjected, the major components of the extracts of the filamentous bacteria and the unicellular heterotrophic bacteria coeluted with standards of the peptide P951 and swinholide A, respectively.

around the molecular ion was identical to the FAB mass spectrum of **2** and indicates the presence of one bromine atom; thus, theonegramide (**2**) and P951 (**3**) differ in their sugar units.

Results

Two major secondary metabolites of unrelated chemical classes, namely, swinholide A (**1**) and a peptide P951 (**3**) that differs from theonegramide (**2**) in the sugar moeity, were isolated from an intact specimen of *T. swinhoei*. Proton NMR (fig. 3) and mass spectra of swinholide A were consistent with published data. Differential centrifugation of cell suspensions from the ectosome and endosome of *T. swinhoei* yielded at least three replicate cell pellets (1 ml in volume) of each of the four cell types. Cell counts (four replicates for each of three pellets; $n = 12$) showed the sponge cell fractions to consist of 76% sponge cells, 17% unicellular bacteria and 7% filamentous bacteria. Each of the three bacterial fractions, that is, the unicellular cyanobacteria, the unicellular heterotrophic bacteria and the filamentous heterotrophic bacteria, was greater than 90% homogeneous. (See reference 21 for light micrographs of purified cell preparations.)

Cell pellets were lyophilized and extracted with methanol, and each of the extracts was analyzed by HPLC (fig. 2) and UV and ^{1}H NMR (fig. 3) spectroscopies. The results were unequivocal; swinholide A (**1**) was found in extracts of the unicellular bacteria, and the peptide P951 (**3**) was found in extracts of the filamentous bacteria. The cyanobacteria (*A. feldmanni*) and the sponge cells were devoid of these natural products. Transmission electron microscopy (TEM) showed that the structural integrity of all cell types was maintained throughout the separation procedure (fig. 4); thus, it is unlikely that the metabolites in question were leached from a particular cell type. TEM also confirmed that the cyanobacterium *A. feldmanni* (fig. 4c) is limited to the ectosome, and the complete absence of thylakoids in the filamentous bacteria (fig. 4d) revealed that they are not cyanobacteria (see below).

Discussion

Our findings that swinholide A is limited to a mixed population of heterotrophic unicellular bacteria, that the filamentous bacterial symbiont is not a cyanobacterium and that the cyanobacterium *A. feldmanni* does not contain either class of metabolite contrast with previous proposals regarding the organismal origins of macrolides and β-amino acid-containing peptides. However, all of these findings concur with our previous reports of the non-photosynthetic nature of the filamentous bacteria found in some lithistid sponges[4,20], and may explain other observations made in our laboratory. For example, a white specimen of *T. swinhoei* that was collected from the back of a submarine cave was devoid of ectosomal populations of *Aphanocapsa*, yet still contained swinholide A and a similar peptide in yields typical of specimens harboring dense populations of

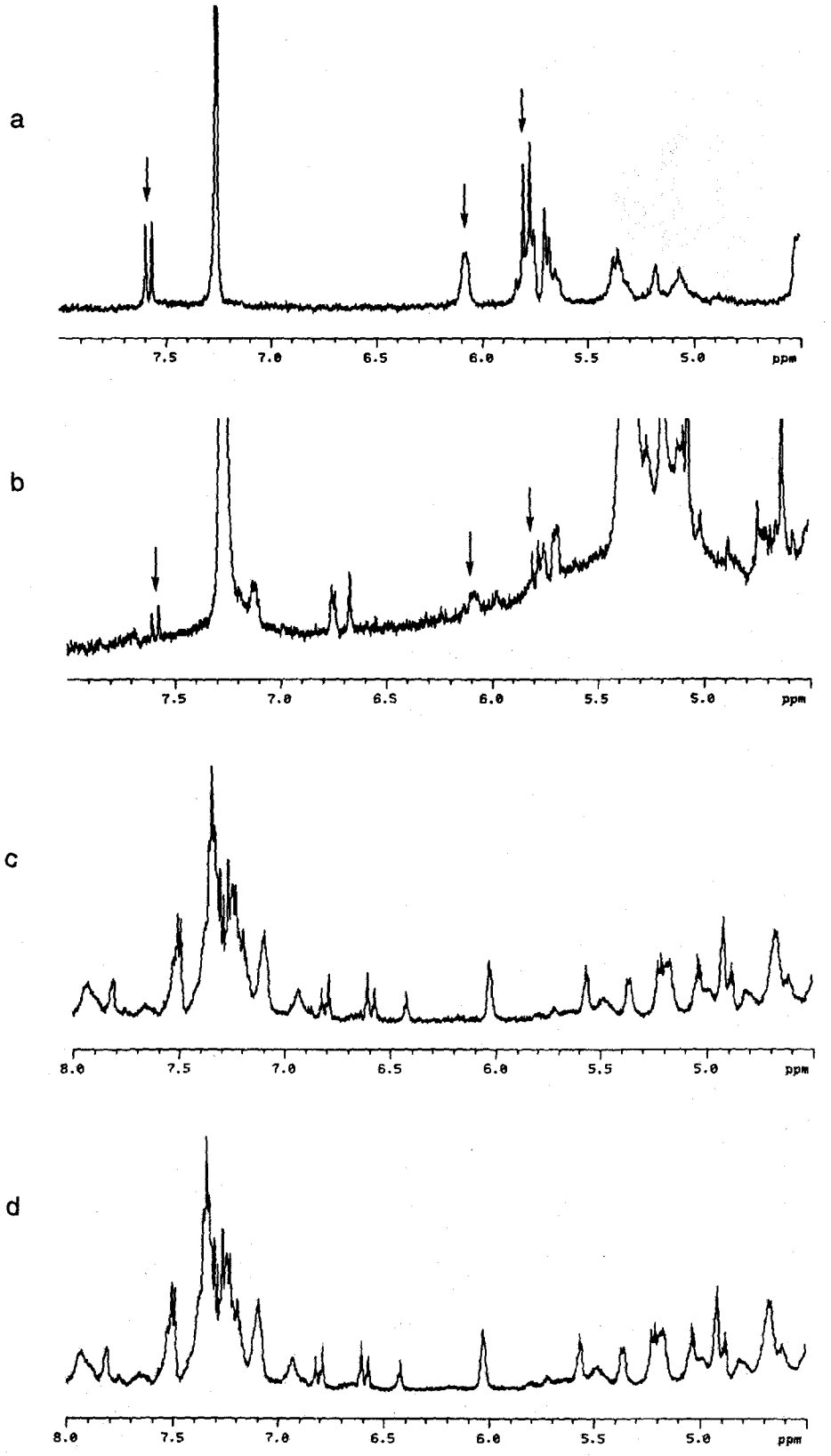

Figure 3. 500 MHz ^{1}H NMR spectra of (*a*) 1 mM swinholide A in $CDCl_3$, (*b*) unicellular bacterial extracts in $CDCl_3$, (*c*) 1 mM P951 in DMSO-d_6 and (*d*) filamentous bacterial extracts in DMSO-d_6. The region between 4.5 and 8.0 ppm that contains characteristic signals of the macrolides and β-amino acid-containing peptides is shown. The signals of δ 5.79 (*d*, $J = 15.8$ Hz), 7.58 (*d*, $J = 15.8$ Hz) and 6.08 (m) in spectra (*a*) and (*b*) are indicated by arrows and correspond, respectively, to H-2, H-3 and H-5 of swinholide A.

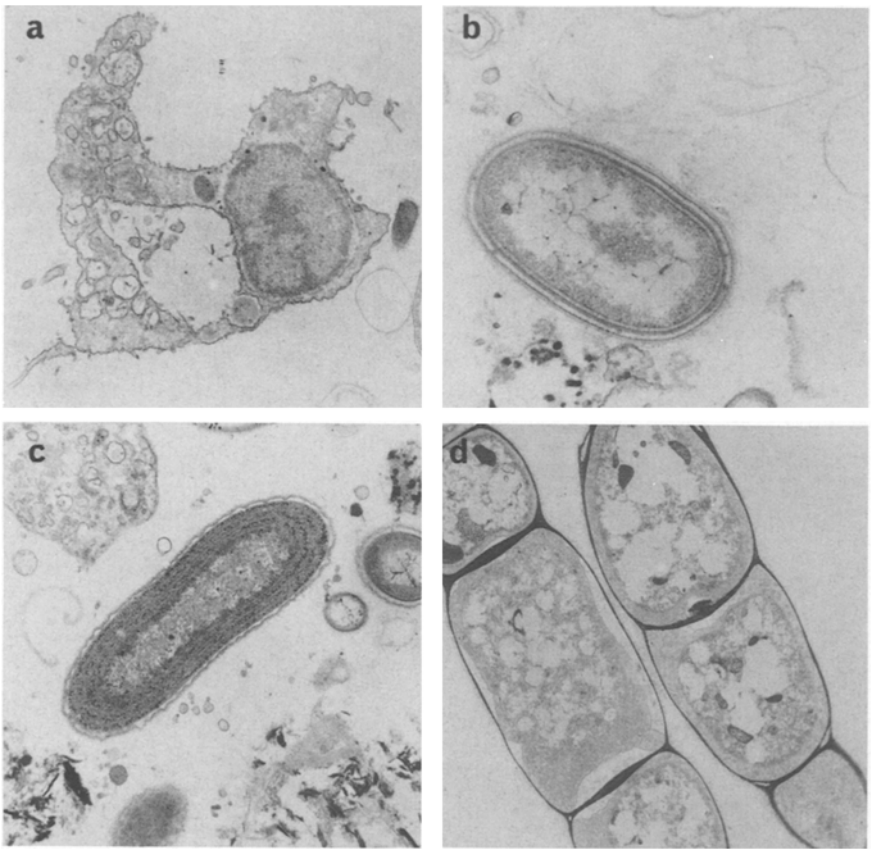

Figure 4. Electron micrographs of the four types of cells found in *T. swinhoei*: (*a*) sponge cell, ×10,000; (*b*) unicellular heterotrophic bacterium, ×25,000; (*c*) unicellular cyanobacterium *A. feldmanni*, ×12,500 (*d*) filamentous heterotrophic bacteria, ×8,000. The bacterium shown in (*b*) is just one example of the many types of bacteria present in the mixed population of unicellular heterotrophic bacteria, all of which appear to be gram-negative based on cell wall structure. The bacterium shown in (*b*) is surrounded by a capsule or sheath peripheral to the cell wall. The micrograph of the unicellular cyanobacterium (*c*) contains conspicuous and well-preserved thylakoids in the peripheral cytoplasm, while the filamentous bacterial symbionts (*d*) lack thylakoids but have numerous lucent vacuoles and electron-dense inclusions in the cytoplasm.

Aphanocapsa in the ectosome. In addition, swinholide A has been isolated from a Philippine specimen of *Ircinia* sp. and a South African specimen of *Theonella conica*[21]. Other macrolides, namely, sphinxolides B-D[22] and reidispongiolides A and B[23], that are strikingly similar to scytophycin C, were isolated from the deep-water lithistids *Neosiphonia superstes* and *Reidispongia coerulea*, respectively. Analysis of these four sponges by light and epi-fluorescence microscopy revealed that none contained cyanobacteria or filamentous bacteria[21].

Our finding that the filamentous bacteria in these and other lithistid sponges are not photosynthetic, and therefore are not technically cyanobacteria, raises interesting evolutionary questions. Ultrastructurally, these symbionts resemble filamentous, non-photosynthetic gliding bacteria[24,25] of the Family Beggiatoaceae, examples of which include the genera *Beggiatoa* and *Thiploca*; yet morphologically and chemically they resemble filamentous cyanobacteria of the subgroups Oscillatoriales and Nostocales[26]. It has been shown that these groups of filamentous cyanobacteria are phylogenetically distant from the beggiatoas[27,28]. Thus, if the filamentous symbionts are more closely related to the beggiatoas, the occurrence of related secondary pathways in phylogenetically distant bacteria – that is the cyanobacteria and beggiatoas – suggests that the secondary pathways were either present before the groups diverged or they evolved independently, whether convergently or by horizontal gene transfer. If closer to the filamentous cyanobacteria, it is remarkable that the symbiont has dispensed with the structures necessary for photosynthesis. Similar questions apply to the unicellular heterotropic bacterium responsible for the production of swinholide A, since similar macrolides are known from the unrelated cyanobacteria[29].

Our discovery that two different bacterial symbionts are responsible for the production of two different classes of sponge metabolites underlines the complexity of associations in certain organisms from which marine natural products are obtained. We have demonstrated that it is not always possible to predict the correct source of marine natural products simply by comparing the structure of the chemical with those produced by microorganisms. Sessile marine invertebrates are an important

but finite source of potential pharmaceuticals, some of which are in clinical development.[30] If, as circumstantial evidence suggests, some of these valuable compounds are produced by associated microorganisms, culturing the producing microorganisms would provide valuable and predictable sources of potential pharmaceutical agents. However, in such cases it seems illogical to attempt the random culture of microorganisms from marine invertebrates, the majority of which are considered difficult or even impossible to grow on standard media, without first establishing a microbial source for the desired compound. In cases where the associated microorganisms cannot be cultured, the knowledge that a valuable compound is found in a symbiont rather than the host should encourage research aimed at transferring the genes responsible for biosynthesis into a more tractable bacterium. Our findings therefore presage a current goal of marine biotechnology – to produce compounds of biomedical value from cultured microorganisms, either natural or engineered.

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