# BACTERIAL DIVERSITY AND ANTIBIOTIC ACTIVITY IN TEMPERATE AUSTRALIAN MARINE SPONGES

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## KEY WORDS

Symbiosis, sponge, bacteria, biodiversity, marine.

## ABSTRACT

The occurrence of permanent sponge-bacterial associations in five temperate marine sponges from Western Australia (*Ircinia* sp., *Chondrilla australiensis*, *Echinodictyum clathrioides*, *Tethya ingalli* and *Coelosphaera* sp.) was investigated using culturing techniques, facilitating both bacterial characterisation and subsequent screening for the presence of antimicrobial activity. Based on biochemical tests, most of the 136 bacterial isolates obtained from the sponges and surrounding water column on several occasions were tentatively identified as members of the *Vibrionaceae*. Fatty acid methyl ester (FAME) analysis indicated that the isolates represented a broad diversity of 32 distinct bacterial groupings. Most bacterial groups (18) were only ever found associated with sponge tissues, whilst others were found only in the water (6) or were present in the water and one or more sponges (8). For only three bacterial groups was there evidence, based on their presence in host tissues on every sampling occasion and absence from the surrounding water, of a permanent relationship with sponge hosts. One of these groups occurred consistently in three of the sponges species studied (*C. australiensis*, *T. ingalli* and *Coelosphaera* sp.), while the other two were consistently associated with only a single sponge species (*T. ingalli* and *Coelosphaera* sp.). Crude extracts of *Coelosphaera* sp. were not inhibitory to *Staphylococcus aureus*, but a bacterium from this sponge produced a heat labile, non-dialysable inhibitor of *S. aureus*. Crude extracts of three other sponge species (*Ircinia* sp., *E. clathrioides* and *T. ingalli*) inhibited *S. aureus*, but culture supernatants of the bacteria from these sponges did not. These results are discussed in terms of their contribution to understanding sponge microbial diversity and the potential for discovery of new pharmacologically active chemicals.

## INTRODUCTION

In outlining the importance of exploration and conservation of microbial diversity, COLWELL (1997) emphasised several key factors: firstly, microbes play fundamental and irreplaceable ecological roles; secondly, knowledge of their activity, diversity and distribution is rudimentary, and; thirdly, the characteristics of microbes from natural habitats offer many unique biotechnological opportunities. The study

of microbes associated with marine sponges provides the opportunity to advance understanding in all three of these areas.

Marine sponges have proved to be a rich source of novel chemicals, many with potential use in industrial applications and for treatment of human disease (MUNRO *et al.,* 1999; FAULKNER, 2001; LEE *et al.,* 2001). Concurrent with these discoveries has come mounting speculation (*e.g.* JENSEN & FENICAL, 1996) and evidence (STIERLE *et al.,* 1988; UNSON & FAULKNER, 1993; OCLARIT *et al.,* 1994; UNSON *et al.,* 1994; BEWLEY *et al.,* 1996; SCHMIDT *et al.,* 2000) that many of the novel metabolites found in sponges are microbial in origin and result from the activities of microbes associated with tissues of the sponge. As a group, marine sponges have been found to contain a broad suite of microbes ranging from autotrophic, heterotrophic and non-culturable prokaryotes to eukaryotic organisms including microalgae and protozoans (see LEE *et al.,* 2001). Studies of sponge species including *Theonella swinhoei* (BEWLEY *et al.,* 1996), *Aplysina* sp. (HENTSCHEL *et al.,* 2001) and *Rhopaloeides odorabile* (WEBSTER *et al.,* 2001) demonstrate that individual sponges can support an enormous diversity of microbes, particularly prokaryotes. However, there is less evidence concerning the specificity and permanence of particular sponge-microbe associations. As sponges filter suspended particulate matter (including microbes) from the water column (REISWIG, 1975), it is probable that many microbes reported from sponges are water column inhabitants and occur in the sponge as transients or as a concentrated food source.

Most convincing evidence of mutualistic sponge-microbe associations has come from studies indicating consistent association of particular microbes with a sponge host, particularly where there is no evidence of co-occurrence in surrounding waters. Such associations have been demonstrated between archaea and *Axinella mexicana* from the Pacific (PRESTON *et al.,* 1996; SCHLEPER *et al.,* 1997, 1998) and *A. damicornis, A. verrucosa* and *Axinella* sp. from the Mediterranean (MARGOT *et al.,* 2002); between cyanobacteria and sponges of the genus *Chondrilla* worldwide (USHER *et al.,* in press); and between an α-proteobacterium and *Rhopaloeides odorabile* (WEBSTER & HILL, 2001a) from the Great Barrier Reef. With the exception of the latter, these studies have been based on molecular evidence, as the microbes involved were non-culturable. While molecular techniques allow a broader description of microbial community diversity in sponges (*e.g.* LOPEZ *et al.,* 1999), culture methods remain useful in comparative assessment. In addition, the ability to culture sponge symbionts presents a number of advantages. Cultures facilitate detailed studies of the biology and ecology of these symbioses, enable investigations of the role of symbiotic microbes in production of novel metabolites derived from sponges (FAULKNER *et al.,* 1999) and provide opportunities to apply fermentation biotechnology in the identification and development of microbial metabolites with potential for commercial applications (JENSEN & FENICAL, 1996; WEBSTER & HILL, 2001b).

In this study we describe the diversity and host associations of culturable heterotrophic bacteria in 5 species of co-occurring sponges from temperate coastal marine waters of Western Australian (WA). We also report the occurrence of antimicrobial activity in sponge tissues and culturable sponge-associated bacteria.

## MATERIALS AND METHODS

Collection of sponges

Representatives of the sponges *Chondrilla australiensis, Tethya ingalli*, *Echinodictyum clathrioides*, *Coelosphaera* sp. and *Ircinia* sp. were chosen for the study, representing marine sponges common to the Perth area, WA. Sponges were collected on SCUBA from 7 m depth at South Mole, Fremantle (32°03' S, 115°45' E) during early autumn (March) and late winter (August) 2001. Seawater samples from the same location were collected in sterile bottles on the same occasions. Sponge specimens were transported in seawater from the field site to flow-through ambient seawater aquaria (Fremantle Institute of TAFE). Tissue samples for bacterial isolation were removed from the sponges (see below) within 2 days and returned to the laboratory for analysis. In addition to the above, a specimen of *C. australiensis*, held for 18 months in a flow-through seawater aquarium at the Department of Fisheries Marine Research Laboratories (Waterman, Perth, WA) was collected in June and analysed as above. Although not definitive, the association of the same bacteria with both naturally occurring sponges and aquarium held specimens would indicate a strong host-microbe relationship. Additional samples of each sponge collected during the August sampling were stored at -20° C until tested for the presence of antimicrobial activity.

Media

All media were sterilised by autoclaving at 121° C for 15 min and stored at 4° C until used, and all processes associated with bacterial isolation from sponges and subsequent manipulation were carried out using aseptic technique. Artificial seawater (ASW), and marine Basal Medium (BM) were prepared as described by SUTTON & BESANT (1994). Luminous Medium (LM) was prepared by the addition of 3 ml glycerol, 5 g yeast extract and 5 g tryptone per l of BM. The pH was adjusted to 7.5 and 20 g Bacto Agar added for preparation of agar plates. Two commercially available agar media, Marine Agar (MA; Difco; non-selective marine bacteriological medium) and TCBS Agar (Oxoid; *Vibrio*-selective medium) were prepared according to the manufacturer's instructions. Marine Broth (MB) was prepared based on the formula provided for MA by the manufacturer, without the addition of agar. Sodium-free MB was prepared as above but with $Na^+$-containing chemical constituents of MB replaced with equivalent $K^+$ chemicals.

Bacterial isolation and purification

Tissue samples (1 - 2 $cm^3$) excised from the surface of samples of the parent sponges were washed in ASW, rinsed with 70 % ethanol and blot-dried on sterile absorbent paper. The surface layers of the excised samples were removed using a scalpel (to minimise contamination from surface-associated bacteria) and the remaining tissue (1 - 2 g) was weighed, cut finely and crushed in 10 ml final volume of ASW in a mortar and pestle. The resulting sponge suspensions (or seawater samples) were serially diluted in ASW and 100 µl of each dilution was plated onto LM, MA and TCBS (March sampling occasion) or MA only (June and August). MA alone was used following the initial sampling as this medium was found to support growth of all isolates obtained. Plates were incubated at room temperature (approx. 22° C). Colonies were counted after 7 d and concentrations calculated as colony forming units per g of tissue or per ml of seawater. Representative colony types were transferred to MA (all isolates were found to grow on this medium) and purified by sequential subculture.

Bacterial characterisation

Purified bacteria were partially characterised, compared and tentatively identified on the basis of reactions in a range of biochemical and other tests (REICHELT & BAUMANN, 1973; KREIG & HOLT, 1984) including colony morphology (colony growth on MA); cell

morphology (light microscopy of broth cultures in MB and from Gram-stained cells); motility (hanging drop microscopy of cells grown for 24 h in MB); ability to ferment glucose (growth and acid production was assessed 48 h after stab inoculation of duplicate tubes of BM [1 % agar] amended per l with 10 g glucose, 5.0 g yeast extract and 0.01 g phenol red. One tube was sealed with 2 % Bacto agar); requirement for $Na^+$ ions for growth or growth stimulation (assessed by comparative growth of isolates inoculated into MB and sodium-free MB. The $Na^+$-requiring marine bacterium *Vibrio harveyi* was included as a control); production of poly-β-hydroxybutyrate (PHB) from glucose (assessed microscopically by formation of cellular inclusions of PHB after 72 h in BM amended by the replacement of $NH_4Cl$ with 0.2 g/l $(NH_4)_2SO_4$ and the addition of 4.0 g/l glucose); production of oxidase (assessed by the formation of a purple colour when cells grown on MA were scraped off and exposed on filter paper to 1 % tetramethyl-para-phenylene-diamine dihydrochloride), and catalase formation (assessed by gas bubble formation when MA-grown cells were placed in a drop of hydrogen peroxide).

Cellular fatty acid composition of isolates was characterised and compared using a commercially available analysis and identification system (MIDI System, Newark, Delaware). The MIDI system database is derived from spectrographic profiles of fatty acid methyl esters (FAME) prepared from cells of known bacteria grown at 28° C for 24 h on trypticase soy broth agar (TSBA). The profiles of unidentified bacteria are compared to profiles in the database, and the resulting Similarity Index can provide a reliable indication of bacterial identity (PAISLEY, 1999). As most of the bacterial isolates failed to grow on TSBA an amended method was adopted. This involved growing isolates for 48 h at 22° C on MA, saponification of cells (approx. 40 mg), methylation of the liberated cellular fatty acids, partitioning the resulting fatty acid methyl esters into an organic phase, and finally separation by capillary gas chromatography (Hewlitt-Packard model 6890). Due to the altered bacterial growth conditions prior to FAME extraction, MIDI system matches for unknown isolates with known bacteria in the database were treated with suspicion and are not included in analysis. However, as a consistent method had been applied to all the unknown isolates in this study, direct FAME profile comparisons and grouping amongst the sponge isolates was possible. The resulting FAME groups represented bacteria which had a Euclidian distance of less than 10% in cluster analysis; a figure reported to equate to species groupings (PAISLEY, 1999).

Assays of antibiotic activity

For assays of antibiotic activity, overnight broth cultures [Oxoid Meuller-Hinton (MH) broth; 37° C] of the test organisms *Escherichia coli* (ATCC 10481), *Staphylococcus aureus* (NCTC 6571) and *Candida albicans* (ATCC 10231) were spread-inoculated onto MH agar plates, into which wells (5 mm diam.) were cut and inoculated with sponge or bacterial extracts. The test plates were incubated overnight at 37° C and inhibition zones noted and measured. Extracts of sponge tissue were prepared by repeated freeze-thawing of sponge samples previously stored at -20° C, and collection and centrifugation of the resulting liquid, 50 µl of which was added to wells in the test plates. For extracts of bacterial isolates, 1 ml of culture from overnight growth in MH broth was centrifuged to remove bacterial cells (*i.e.* detecting only extracellular products), and 50 µl of supernatant was added to wells in the test plates. The above assays were for water soluble componds. Assays for lipid soluble antimicrobial chemicals were not undertaken. The isolate constituting FAME group 14 produced an antibiotic compound, and the growth stage at which this occurred was assessed by assaying subsamples of bacterial culture fluid, as described above, taken at intervals during growth for 30 h in MH broth. The temperature sensitivity of the active component from this bacterium was assayed by heating culture fluid to 100° C for 10 min or 56° C for 20 min prior to antimicrobial testing. An indication of the compound's molecular size was obtained by assaying for activity following dialysis (MW 12,000 units) for 4 days in MH broth.

## RESULTS

A total of 133 bacterial isolates were cultured from 5 sponges and seawater collected from South Mole, Fremantle, on two occasions during 2001 (Tab. I). A further 3 isolates were obtained from a *C. australiensis* specimen held for 18 months in a flow-through seawater aquarium at the Department of Fisheries Marine Research Laboratories, Waterman, WA.

**Tab. I.** Culturable bacteria concentrations and isolates from temperate marine sponges and seawater.

| Sample | Sampling occasion# | Bacterial concentration (colony forming units $g^{-1}$ sponge tissue or $ml^{-1}$ seawater) | No. of Isolates |
|---|---|---|---|
| *Tethya ingalli* | 1 | $1.3 \times 10^5$ | 9 |
| | 3 | $9.5 \times 10^4$ | 11 |
| *Coelosphaera* sp. | 1 | $2.9 \times 10^4$ | 17 |
| | 3 | $2.1 \times 10^4$ | 5 |
| *Chondrilla australiensis* | 1 | $3.0 \times 10^{7*}$ | 12 |
| | 2 | ND | 3 |
| | 3 | $3.9 \times 10^4$ | 12 |
| *Echinodictyum clathrioides* | 1 | $5.4 \times 10^4$ | 16 |
| | 3 | $9.8 \times 10^4$ | 0^ |
| *Ircinia* sp. | 1 | $2.0 \times 10^6$ | 11 |
| | 3 | $1.7 \times 10^5$ | 8 |
| Seawater | 1 | $2.0 \times 10^{2*}$ | 28 |
| | 3 | $2.5 \times 10^2$ | 4 |

* Colony counts on LM medium but all others on MA; # 1=March 2002, 2= June 2002, 3= August 2002, (1 and 3 from South Mole, 2 from an aquarium-held sample); ^ All isolates died during purification subculturing. ND = Not Determined

Concentrations of culturable bacteria (Tab. I) in sponge tissues were in the order of $10^4$-$10^5$/g tissue for *T. ingalli*, *E. clathrioides* and *Coelosphaera* sp., and were similar on both sampling occasions. For *C. australiensis*, the bacterial counts were higher by 3 orders of magnitude on the first sampling occasion ($10^7$/g), while for *Ircinia* sp. they were higher by one order of magnitude ($10^6$/g). Bacterial concentrations in the water column were similar on both occasions and considerably lower in comparison to sponge tissue (~$10^2$/ml).

Based on cellular fatty acid composition, the 136 isolates could be assigned to 32 FAME groupings (Tab. II). Of these groups, 18 were only ever detected in sponge tissue, 6 were only found from the water column and 8 were detected in the tissue of at least one sponge and the surrounding water on at least one occasion. The greatest diversity of types was obtained from *Ircinia* sp. (13), while only 8 groups were recovered from *C. australiensis*.

Of the 18 groups found only in sponge tissue, 10 were only found in one sponge species: groups 8 and 19 in *T. ingalli*; groups 10 and 14 in *Coelosphaera* sp.; groups 13, 18 and 20 in *C. australiensis*; group 11 in *E. clathrioides*, and groups 7 and 22 in *Ircinia* sp. The remaining 8 groups (2, 4, 5, 12, 16, 21, 27, 30), were found from more than one sponge. Only one bacterial group (25) was found at some time during the sampling program in every sponge, and was also detected in the water column.

**Tab. II.** Association of cellular fatty acid bacterial groups with sponge tissue and the water column.

| Bacterial Group | Group present in | | | | Group associated with particular sources (sponges or seawater) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sponge only | Sponge & water | Water only | All sponges | *Tethya ingalli* | *Coelosphaera* sp. | *Chondrilla australiensis* | *Echinodyctium clathrioides* | *Ircinia* sp. | Seawater |
| 1 | | | X | | | | | | | X |
| 2 | X | | | | X | | | X | X | |
| 3 | | X | | | X | X | | | | X |
| 4 | X | | | | X | X | X | | X | |
| 5 | X | | | | X | | | | X | |
| 6 | | | X | | | | | | | X |
| 7 | X | | | | | | | | XR | |
| 8 | X | | | | XR | | | | | |
| 9 | | | X | | | | | | | X |
| 10 | X | | | | | XR | | | | |
| 11 | X | | | | | | | XR | | |
| 12 | X | | | | | | | X | X | |
| 13 | X | | | | | | XR | | | |
| 14 | X | | | | | XR | | | | |
| 15 | | X | | | X | | | | | X |
| 16 | X | | | | X | X | | | X | |
| 17 | | | X | | | | | | | X |
| 18 | X | | | | | | XR | | | |
| 19 | X | | | | XR | | | | | |
| 20 | X | | | | | | XR | | | |
| 21 | X | | | | X | X | | | | |

| Bacterial Group | Group present in | | | | Group associated with particular sources (sponges or seawater) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sponge only | Sponge & water | Water only | All sponges | *Tethya ingalli* | *Coelosphaera* sp. | *Chondrilla australiensis* | *Echinodyctium clathrioides* | *Ircinia* sp. | Seawater |
| 23 | | | X | | | | | | | X |
| 24 | | X | | | | X | | | | X |
| 25 | | X | | X | X | X | X | X | X | X |
| 26 | | | X | | | | | | | X |
| 27 | X | | | | | | X | | X | |
| 28 | | X | | | | X | X | X | X | X |
| 29 | | X | | | X | X | | X | X | X |
| 30 | X | | | | | X | | X | X | |
| 31 | | X | | | X | | X | X | | X |
| 32 | | X | | | | X | | | X | X |
| Total | 18 | 8 | 6 | 1 | 12 | 12 | 8 | 8 | 13 | 14 |

X = bacterial group present; XR = bacterial group present and only found from that source; = Bacterial group present on each sampling occasion;
= Bacterial group occurs in more than one sponge but not in the water column.

**Tab. III.** Biochemical and other characteristics of sponge bacteria grouped according to their cellular fatty acid profiles.

| Bacterial group based on FAME profiles | Biochemical and other characteristics | | | | | | |
|---|---|---|---|---|---|---|---|
| | Oxidase | Catalase | Relations to $O_2$ | Motility | $Na^+$ requirement* | PHB from glucose | Tentative identification |
| 3, 7, 10, 11, 15, 16, 17, 23, 26, 27 | + | + | OF | + | + | +/- | *Vibrio/Photobacterium* |
| 2, 6, 14, 24 | + | - | OF | + | + | - | *Vibrio* |
| 12, 28, 31 | + | +/- | OF | + | + | +/- | *Vibrio/Photobacterium* |
| 4, 5, 30 | +/- | + | OF | + | + | +/- | *Vibrio/Photobacterium* |
| 32 | +/- | +/- | OF | + | + | +/- | *Vibrio/Photobacterium* |
| 25 | +/- | +/- | OF | + | +/- | +/- | *Vibrionaceae* or *Enterobacteriaceae* |
| 29 | +/- | +/- | OF | +/- | + | +/- | *Vibrionaceae* |
| 9 | + | + | OF | - | + | - | ND |
| 19 | + | - | OF | - | + | - | ND |
| 1 | + | + | OF | + | - | NT | *Aeromonas/Plesiomonas* (*Vibrionaceae*) or *Enterobacteriaceae* |
| 8 | + | - | OF | - | - | NT | *Enterobacteriaceae* |
| 21 | + | - | O/OF | +/- | + | - | *Vibrionaceae* or *Pseudomonadaceae* |
| 13, 18, 20, 22 | NT | | | | | | |

+ = positive result for indicated test; - = negative result for indicated test; +/- = variable result for indicated test; O = oxidative metabolism; OF = fermentative or oxidative metabolism; * = $Na^+$ required or stimulatory for growth; NT - Not Tested; ND - Not Determined

A consistent association was found on each sampling occasion between bacteria and sponge tissue for 5 of the bacterial groups (4, 8, 10, 25 and 28). Group 4 bacteria were found on both sampling occasions in *T. ingalli*, *Coelosphaera* sp. and *C. australiensis* from South Mole, and from the aquarium-held *C. australiensis*. This bacterium was also found in *Ircinia* sp. on the second collection from South Mole, but never found in the water column. Based on colony characteristics, group 4 bacteria were the dominant type in *C. australiensis* on all sampling occasions and one of the dominant bacteria in the tissues of the other three sponges on at least one of the sampling occasions. *T. ingalli* and *Coelosphaera* sp. were also found to contain group 8 and 10 bacteria, respectively, on both sampling occasions, but neither group was detected in other sponge species or the water column. Group 25 bacteria were found on both sampling occasions in *T. ingalli* and *Ircinia* sp., but were also found on at least one occasion in all other sponges and the water column. Group 28 bacteria were also found on both occasions in *Ircinia* sp. but on at least one occasion in all other sponge species (with the exception of *T. ingalli*) and the water column. Bacteria in groups 26 and 32 were found in seawater on both occasions from South Mole: the former was never found associated with sponge tissues but the latter was recovered from tissues of one of the sponges on at least one sampling occasion.

Bacteria belonging to the 32 FAME groupings were further characterised in a range of biochemical and other tests, to enable tentative identifications (Tab. III). Bacteria in all groups except 1 and 8, and some in group 25, had a requirement for the presence of $Na^+$ in the growth medium, or grew better in its presence. All were Gram -ve straight or curved rods, and most could be assigned, on the basis of the tests undertaken, to the family *Vibrionaceae* or to particular genera within that family. Group 8 was assigned to the family *Enterobacteriaceae*, while groups 1 and 25 could belong to either the non sodium-requiring genera of the *Vibrionaceae* (*Aeromonas* and *Plesiomonas*) or the *Enterobacteriaceae*. It was not possible to assign groups 9 and 19 to any family. Bacteria from group 21 gave conflicting results with some isolates being strict aerobes while others were facultatively anaerobic. The aerobes in group 21 had characteristics consistent with the family *Pseudomonadaceae*.

Undiluted tissue extracts of three of the five sponge species, *Ircinia* sp., *E. clathrioides* and *T. ingalli* were active against the Gram positive bacterium *Staphylococcus aureus*, the extract of *Ircinia* sp. producing the largest growth inhibition zones (9 mm diam.). No inhibition of *Escherichia coli* (Gram negative bacterium) or *Candida albicans* (yeast) was detected. Only one bacterial isolate, the bacterium comprising FAME group 14 and obtained from *Coelosphaera* sp., produced antimicrobial activity and this was only noted against *S. aureus*. The isolate was tentatively identified as *Vibrio* sp. (G -ve, facultatively anaerobic, curved rod morphology, oxidase and catalase positive, requirement for $Na^+$ for growth). Growth curve assays (Fig. 1) indicated that the antimicrobial activity was produced during stationary growth phase and in association with the formation of a black, extracellular, water soluble pigment in the culture medium. The antimicrobial activity was non-dialysable (12000 MW). Heating to 56° C for 20 min caused no reduction in activity relative to controls (15 mm diameter inhibition zones), but heating to 100° C for 10 min reduced inhibition zones in assays to 13 % of controls.

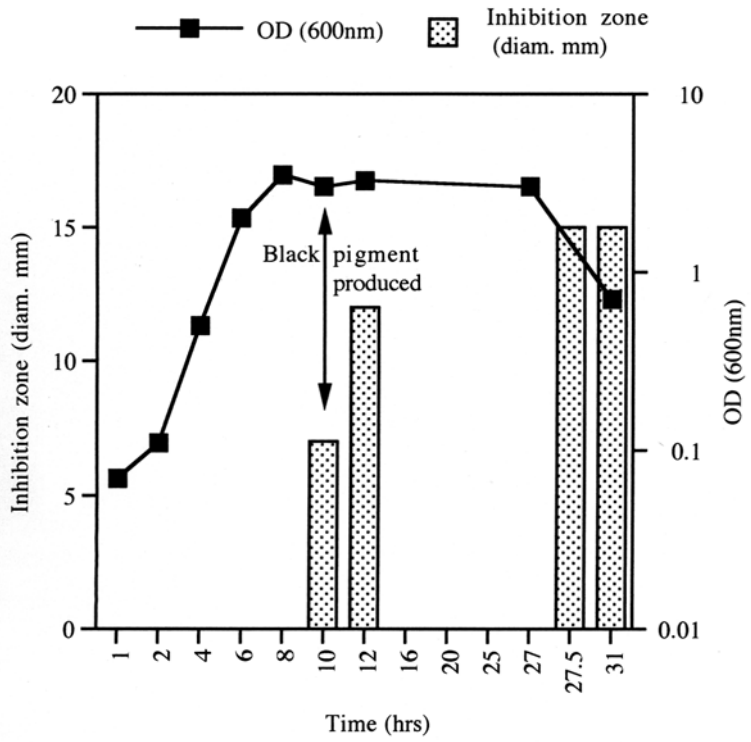


**Fig. 1.** Antibiotic activity and pigment production in relation to growth of FAME group 14 bacterium

## DISCUSSION AND CONCLUSIONS

In this study we found evidence for the association of particular marine bacteria with common temperate Western Australian marine sponges. For some bacteria there was evidence of a permanent relationship with their sponge host, suggestive of mutualistic symbiotic associations. We also found antimicrobial activity in extracts of some of the sponges we investigated and in one sponge bacterial isolate.

A diverse range of bacteria and other microbes have been found in association with sponges (SANTAVY *et al.,* 1990; WEBSTER *et al.,* 2001; reviewed by LEE *et al.,* 2001) and other marine organisms (LARKUM *et al.,* 1987; DUGLAS, 1994; ROHWER *et al.,* 2001). Microbes may be present in low concentrations in sponge tissues, but can comprise 60 % of the sponge biomass (WILKINSON, 1978a) and it is common for their concentrations to exceed those in the surrounding water column by orders of magnitude (*e.g.* SANTAVY & COLWELL, 1990; FRIEDRICH *et al.,* 1999; HENTSCHEL *et al.,* 2001). Many of the previously described bacteria in marine sponges and other organisms are members of the γ-subdivision of the *Proteobacteria* (DISTEL *et al.,* 1988, 1991; FRIEDRICH *et al.,* 1999; LOPEZ *et al.,* 1999; WEBSTER *et al.,* 2001). In some

sponges γ proteobacteria are major components of the bacterial populations present and SANTAVY *et al.* (1990) found that members of the genera *Vibrio* and *Aeromonas* (γ-*Proteobacteria*; *Vibrionaceae*) dominated the culturable bacteria from the sponge *Ceratoporella nicholsoni*. Based on limited phenotypic data, we also found evidence in this present study that the majority of culturable bacteria from *C. australiensis, T. ingalli*, *E. clathrioides*, *Coelosphaera* sp. and *Ircinia* sp belonged to the *Vibrionaceae*, although the presence of *Enterobacteriaceae* was also indicated. Amongst these bacteria we found 32 groups, based on cellular fatty acid profiles, with quite variable distribution and occurrence in the habitats we examined. These FAME groupings may represent individual bacterial species (PAISLEY, 1999), although the anomalous result for group 21 indicates the need for caution in interpretation as it is apparent that some bacteria can have similar FAME profiles despite belonging to markedly different physiological groupings of bacteria. Our results indicated that most bacterial groups (18/32) were associated only with sponge tissues and were not present in the surrounding water column, paralleling similar findings (WILKINSON, 1978b; WILKINSON *et al.,* 1981; SANTAVY & COLWELL, 1990; BURJA *et al.,* 1999). An important implication of these studies is that a significant proportion of marine bacterial diversity is found only in association with marine biota. These results emphasise the need for future research to more fully document microbial biodiversity and examine the relationships between symbiotic bacteria and marine vertebrates, invertebrates and plants. Such knowledge is essential in management processes aimed at ensuring that biodiversity protection and conservation is achieved in marine habitats (COLWELL, 1997).

Despite the abundant evidence for significant populations of microbes associated with sponges, there is only a limited number of studies which provide evidence of permanent associations between sponges and microbial symbionts. The microbes involved include archaea (PRESTON *et al.,* 1996; SCHLEPER *et al.,* 1997, 1998; MARGOT *et al.,* 2002), α-*Proteobacteria* (WEBSTER & HILL, 2001a) and cyanobacteria (USHER *et al.,* in press). While the majority of bacterial groups detected in the present study were only found in sponge tissues, most of these were not present on all sampling occasions. Although these bacteria were never found in the water column, it suggests they may be transient in the sponge or occurring at densities too low to be detected in our study. We did however find evidence that some bacterial groups are permanently associated with particular sponges. For example, group 4 bacteria dominated the culturable community from *C. australiensis* on both sampling occasions (autumn and late winter), and was also the dominant bacteria in the specimen of this sponge which had been held under aquarium conditions for 18 months. The same bacterial group was also consistently found associated with two other sponges *T. ingalli* and *Coelosphaera* sp., and was at times the dominant culturable bacterium in their tissues. This bacterium appears to belong to either the *Vibrio* or *Photobacterium* genera. The occurrence of these consistent associations provides strong circumstantial evidence that this bacterium is a permanent resident in the tissues of these sponges, reinforced by its apparent absence from the surrounding water.

Bacteria-sponge associations have been suggested to be based on a variety of potential benefits to the symbiotic partners. These include direct contributions to host nutrition (REISWIG, 1975, 1981; WILKINSON & FAY, 1979; WILKINSON &

GARRONE, 1980; VACELET, 1982; WILKINSON *et al.,* 1999); degradation and recycling of toxins or animal wastes (VACELET, 1975; WILKINSON, 1978b), and host protection through amensalism, or production of feeding deterrent or antifouling chemicals (BAKUS *et al.,* 1986; PAUL, 1992; UNSON *et al.,* 1994). The ecological role of such associations has yet to be clearly established. However, the occurrence of sponge species with permanent microbial symbionts able to be cultured and amenable to laboratory manipulation, such as found here in *C. australiensis*, *T. ingalli*, and *Coelosphaera* sp., provides the opportunity to investigate in detail the basis of the symbiotic association involved.

Antimicrobial activity against *S. aureus* was detected in crude extracts of *Ircinia* sp., *E. clathrioides* and *T. ingalli.* Only water soluble antimicrobial chemicals would have been readily detected but lipid soluble ones may also have been present but not extracted. Sponges are considered a rich source of novel metabolites (THOMPSON, 1985; FAULKNER, 2000), many of which are bioactive (MUNRO *et al.,* 1999; LEE *et al.,* 2001). There is increasing evidence that many of these compounds are produced by bacteria living symbiotically with the sponges (STIERLE *et al.,* 1988; IMAMURA *et al.,* 1993; BEWLEY *et al.,* 1996; JENSEN & FENICAL, 1996). However no bacteria cultured from *Ircinia* sp., *E. clathrioides* or *T. ingalli* produced antimicrobial activity in culture. This gives rise to a number of possible explanations: the activity may be of sponge origin, may be derived from a specific sponge-bacterium interaction, or is produced by symbiotic bacteria not able to be cultured in this study. The latter is highly likely considering that typically <1 % of bacteria are able to be cultured from natural habitats (HUGENHOLTZ *et al.,* 1998; WEBSTER *et al.,* 2001).

Only one of the cultured bacteria, an isolate from the sponge *Coelosphaera* sp., exhibited antimicrobial activity. Extracts of this bacterium inhibited growth of *S. aureus* (Gram +ve bacterium), but not *E. coli* (Gram -ve) or *C. albicans* (yeast). The bacterium was only recovered from *Coelosphaera* sp. and only on one sampling occasion, suggesting it may not be a permanent symbiont in that sponge. The bacterium, which had a FAME profile (group 14) distinguishing it from all other bacteria isolated in this study, had phenotypic characteristics typical of species of the genus *Vibrio*, members of which have previously been shown to be associated with marine sponges and to produce antibacterial compounds (ELYAKOV *et al.,* 1991; OCLARIT *et al.,* 1994; HENTSCHEL *et al.,* 2001).

The antimicrobial compound produced by the isolate cultured from *Coelosphaera* sp. was apparently a secondary metabolite, produced during the stationary growth phase. While the chemical nature of the active component remains to be investigated in detail, preliminary results indicate that it is a relatively large compound (non-dialysable), stable to moderate temperatures (56° C) but largely denatured by heating to 100° C. Its detection in the culture medium coincided with the production of a black, water-soluble pigment, and while the pigment may be responsible for the observed antimicrobial activity, a causal relationship was not examined.

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