RESEARCH ARTICLE

Spatial and temporal variability of the bacterial community in different chemotypes of the New Zealand marine sponge *Mycale hentscheli*

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Abstract

Molecular fingerprinting of 16S rRNA genes using terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) was used to characterize the temporal and spatial variability among sponge-associated bacteria from *Mycale hentscheli* having distinct bioactive chemotypes. Cluster analysis of T-RFLP and DGGE profiles from *M. hentscheli* chemotypes largely grouped sponge microbial diversity to their distinct chemotype pattern. Repeat sampling of individual *M. hentscheli* at one location over a 21-month period showed that the T-RFLP profiles from individual sponges had similarity indices ranging from 60% to 82% and calculated DGGE similarities between 23% and 95%. However, a portion (> 35% from DGGE and > 19% from T-RFLP) of the microbial community from *M. hentscheli* appeared to be spatially conserved through all *M. hentscheli* populations. Sequence analysis of DGGE band fragments showed a similarity among the bands originating from different individuals, different times, and different locations. The sponge-associated relationship of these bands was confirmed, with sequences having similarity to sponge-associated bacteria reported from global locations. This study highlights the spatial and temporal complexity in the distribution of bacterial communities associated with different chemotypes of the marine sponge *M. hentscheli*.

Introduction

Marine sponges are recognized as one of the best sources of novel bioactive compounds for biomedical applications with antiviral, antitumour, antimicrobial, antifungal, and cytotoxic activities (Blunt et al., 2009). The New Zealand marine sponge *Mycale (Carmia) hentscheli* Bergquist and Fromont (Bergquist & Fromont, 1988) (class Demospongiae: order Poecilosclerida: family Mycalidae) produces three classes of bioactive compounds; mycalamide (this includes mycalamides A, B, and D), pateamine, and peloruside A (Ferry et al., 1988; Northcote et al., 1991; West et al., 2000a, b). All three compounds derive their cytotoxic effects from the induction of apoptosis.

The finite supply of wild sponges and the extremely low concentrations of bioactive compounds are major limitations for a sustainable supply of bioactive compounds. Surveys of wild *M. hentscheli* have revealed a variation in the concentrations of mycalamide, pateamine, and peloruside A from different geographic regions and at different times of the year. Bioactive compound concentrations were site specific and showed spring–summer maxima (Page et al., 2005b), which further complicate the potential for utilizing wild sources for the supply of these compounds.

Chemical synthesis of marine bioactive compounds is frequently not viable because of the low yield and the expense of producing large complex molecules. One option is to use aquaculture for supply; however, this is dependent not only on explant survival and growth rate but also on maintaining the natural synthesis of the desired bioactive compounds. Trial aquaculture experiments have found that bioactive compound production in *M. hentscheli* varies with the location, season, and farming methods (Duckworth & Battershill, 2003; Page et al., 2005a, b), and there is currently no consistency in bioactive compound production from aquaculture (Page et al., 2005b). The specific cause of this
variation is unclear. There is evidence that the ecological function of sponge bioactive compound production is to control predators (Pawlik et al., 1995; Chanas et al., 1996), competitors, or fouling organisms (Engel & Pawlik, 2000; Kelly et al., 2003; Lee & Qian, 2004), and that compound production might be regulated in response to these ecological factors.

There is evidence that the numerous biologically active secondary metabolites, polyketides, and nonribosomal peptides, discovered in marine sponges, tunicates, and bryozoans during pharmaceutical screening, may be derived from microorganisms (Piel et al., 2005; Piel, 2009). Mycalamide A, from M. hentscheli, bears a close structural similarity to pederin, a compound originally isolated and characterized from the Paederus fuscipes beetle, and likely synthesized by an uncultured bacterial symbiont closely related to Pseudomonas aeruginosa (Piel, 2002). If the sponge-associated bacteria produce the metabolites, then it may be possible to provide a continuous supply of the biologically active compounds by laboratory culture of the bacteria.

Sponges are highly efficient filter feeders and there is active uptake and digestion of bacterial cells from seawater. Up to 60% of the total sponge biomass has been estimated to comprise bacteria (Imhoff & Stohr, 2003). Bacterial associations or specific symbioses have been described in sponges (Santavy et al., 1990; Schmidt et al., 2000; Hentschel et al., 2001; Webster & Hill, 2001) and other marine invertebrates (Haygood & Davidson, 1997). Relatively uniform sponge specific, but phylogenetically diverse microbial communities have been reported (Wilkinson et al., 1981; Hentschel et al., 2002; Hill et al., 2006). Surveys of sponges from the Mediterranean Sea, Red Sea, Sea of Japan, and the Pacific by Hentschel et al. (2002) reported 14 different monophyletic sponge-specific bacterial clusters belonging to seven bacterial divisions. In another study, bacterial communities of Antarctic sponge species were consistently detected within a particular species across McMurdo Sound regardless of the sample location (Webster et al., 2004). Bacterial community composition in the marine sponge Cymbastela concentrica from Australia was reported to be different between tropical and temperate environments, but very similar over a 500 km temperate range of the south-eastern Australian coast (Taylor et al., 2005). In reviewing the diversity of microorganisms from sponges, Taylor et al. (2007) reported that sequences representing 16 bacterial phyla have been recovered from sponges, and noted that the sponge-associated microbial community contains a mixture of generalist and specialist microorganisms and that these microbial communities are generally stable in both space and time.

Different chemotypes have been reported in M. hentscheli (Page et al., 2005b). Geographic and temporal differences in the production of bioactive compounds from M. hentscheli have resulted in this sponge having distinct chemotype differences (Duckworth & Battershill, 2003; Page et al., 2005b). The evidence that bacteria play a role in the production of some bioactive compounds (Piel et al., 2005) suggests that one approach to understanding any bacterial role is to compare the microbial community composition of sponges having these different chemotypes. In this study, we hypothesize that the chemotypes of M. hentscheli correlate to the associated bacterial community. To facilitate a rapid high-throughput survey of abundant members of the microbial community associated with M. hentscheli having different chemotype profiles, DNA fingerprinting tools terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) were used to investigate bacterial spatial and temporal variation.

Materials and methods

Sponge collection

Whole sponge or partial specimens of M. hentscheli having different chemotypes were collected from wild populations at seven sites from around New Zealand over a 5-year period (Fig. 1). Samples were collected by SCUBA diving to a depth of 30 m and were transferred underwater to sealable plastic bags containing seawater, brought to the surface, and stored on ice for transport to the laboratory (approximately 2 h). To sample microorganisms, small sections of sponge tissue were sampled from the pinacoderm through the mesohyl layer to approximately 2–3 cm depth. Tissue samples were stored in RNAlater™ (Ambion, Applied Biosystems Inc., Foster City, CA) to stabilize and protect cellular RNA and DNA. A subsample of this tissue was used for DNA extraction and was excised longitudinally from the original tissue.

Temporal study

Ten explants from wild M. hentscheli established on ropes as part of an aquaculture trial at Capsize Point in Pelorus Sound (Page et al., 2005a) were used to monitor the temporal changes in sponge-associated microbial communities. Subsamples of the captive explants were collected, as described above, at six time points over a 21-month period: 22 May 2003, 6 October 2003, 30 January 2004, 22 April 2004, 14 June 2004, and 16 February 2005. Captive sponges were monitored for health indicators at every sampling interval. Repeat sampling was not detrimental to sponge health, and aquaculture studies have demonstrated explant survival and rapid growth rates using these sponge culture methods (Page et al., 2005a). Fouling and reduction in
biomass enabled visual identification of dead or dying sponges, which were removed from the experiments.

**DNA extraction and purification**

Total DNA was extracted from approximately 0.2 g sponge tissue using the FastDNA® Kit as described by the manufacturer (BIO 101, QBioGene Inc., Carlsbad, CA). Purified DNA was quantified (DyNA Quant 200 Fluorometer, GE Healthcare) and resuspended to 10 ng μL⁻¹ in dH₂O and stored at 4 °C.

**PCR for T-RFLP analysis**

PCR products were amplified in a 50-μL reaction containing 1 × PCR buffer (15 mM Tris-Cl pH 8.0, 50 mM KCl, and 2.5 mM MgCl₂), 100 μM each of dATP, dCTP, dGTP, and dTTP (Roche, Mannheim, Germany), and 1 U Taq DNA polymerase (Eppendorf HotMaster™, Hamburg, Germany). Eubacterial-specific oligonucleotide primers (0.2 μM) FAM27F (5’-FAM-AGA GTT TGA TCM TGG CTC AG-3’) and VIC1087R (5’-VIC-CTC GTT GCG GGA CTT AAC CC-3’) (Marchesi et al., 1998; Osborn et al., 2000) were used to amplify a 16S rRNA gene fragment of about 1000 bp. Primers were 5’ end labelled with the phosphoramidite dyes 6-FAM and VIC, respectively (Applied Biosystems Inc.). Two microlitres (20 ng) of purified DNA was used as an amplification template. All amplifications were performed in a Thermocycler 2700 (Applied Biosystems Inc.) for 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min 30 s, followed by a final extension step of 72 °C for 7 min. Amplification was confirmed by agarose gel electrophoresis (1.0% in 1 × TBE) with ethidium bromide staining and viewed by UV transillumination. The PCR products were purified using the GENECLEAN® Turbo for PCR Kit (QBioGene Inc.) and the DNA was eluted in a final volume of 30 μL. The purified PCR products were quantified (DyNA Quant 200 Fluorometer) and resuspended to a final concentration of 10 ng μL⁻¹ in dH₂O.

**T-RFLP analysis**

The purified PCR product (250 ng) was digested with 20 U Alul (Invitrogen, Carlsbad, CA) in a 30-μL reaction volume at 37 °C for 3 h, and the enzyme was inactivated by heating at

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**Fig. 1.** Mycale hentscheli sampling sites, and major current and eddy systems around New Zealand: DUC, D’Urville Current; EAC, East Auckland Current; ECC, East Cape Current; SOC, Southland Current; TAC, Tasman Current; WAC, West Auckland Current; WEC, Westland Current; WCC, Wairarapa Coastal Current; ECC, East Cape Eddy; WAE, Wairarapa Eddy. Chemotypes for *M. hentscheli* (A) to (F).
65 °C for 20 min. Restriction digests were desalted to remove ions that might interfere with capillary electrophoresis by diluting products to a total volume of 480 μL in sterile water and concentrating on Microcon YM30 filter columns (Millipore, Bedford, MA). Fluorescently labelled terminal restriction fragments were separated and visualized by automated detection using an ABI 3730 Genetic analyzer (Applied Biosystems Inc.).

T-RFLP profiles were analysed using DAX acquisition and analysis software (version 7.3, van Mierlo Software Consultancy). A LIZ 500 internal size standard (Applied Biosystems Inc.) was added to each sample and was used to normalize all sample peak heights. T-RFLP’s (FAM and VIC traces) between 75 and 450 bp with peak areas > 100 fluorescence units were recorded. This peak information was tabulated as binary presence (1), absence (0) data for each sponge sample. Cluster analysis was performed on the binary data using the UPGMA procedure (unweighted pair-group with mathematical averages) (SAS release 9.1, SAS Institute Inc., Cary, NC). Sorenson’s similarity (Cs) index (Horswell et al., 2002) was used to calculate % similarities between samples based on the presence or absence of T-RFLP’s in all samples, where $Cs = (2N_{AB}/(N_A + N_B)) \times 100$, where $N_{AB}$ is the number of matching peaks, $N_A$ is the total number of peaks in Sponge A, and $N_B$ is the total number of peaks in Sponge B, where a Cs of 0% indicates that the samples are completely different, and a Cs of 100% indicates that the samples are identical.

PCR for DGGE analysis

PCR amplification for DGGE analysis was performed using a eubacterial-specific forward primer HDA1-GC (Walter et al., 2000) (5’-GGC TGG CGG GCG GCC GCC CCG GGC GGG GCG GCC GAC TCC TAG GGG AGG CAG CAG T-3’; boldface type indicates the GC clamp) and reverse primer R518 (Lane, 1991) (5’-CGT ATT ACC GCG GCT GCT GG-3’) to amplify a 179-bp fragment of the V2–V3 region of the 16S rRNA gene. The final primer selection was based on a series of primer trials targeting the 16S rRNA gene, where the DGGE patterns of different length PCR products were compared. The primer combination yielding the greatest frequency and the best distribution of bands was selected. The PCR reaction conditions were as described previously for T-RFLP, with the exception of an annealing temperature of 56 °C.

DGGE gel analysis

DGGE analysis of PCR amplicons was performed using a DCode Universal mutation detection system (Bio-Rad, Hercules, CA) with a 6% (w/v) polyacrylamide gel in 1 × TAE using a 30–55% denaturing gradient (100% denaturants consisting of 40% v/v formamide and 7 M urea). DGGE gels were run at 60 °C and 130 V for 4.5 h. Ethidium bromide-stained gels were visualized and captured using a MultiDoc-IT imaging system (UVP, Upland, CA). Statistical processing of bands from these gel images was performed using BIONUMERICS version 5.10 (Applied Maths, Kortrijk, Belgium), and analysed by clustering using the Dice binary coefficient and UPGMA as the clustering method. Unlike the T-RFLP data, where peak presence/absence was used to calculate relationships, DGGE percent similarity values calculated from the Dice coefficient analysis were used to compare the similarities between individual sponge samples.

T-RFLP and DGGE reproducibility

To evaluate the variation in comparing bacterial communities from *M. hentscheli*, the degree of variation between replicate samples from within the same sample (triplicate PCR amplifications from a single sponge DNA sample), and within sponge DNA samples (triplicate DNA extractions from the same sponge), was assessed using T-RFLP and DGGE.

Sequencing and identification of DGGE bands

Twelve bands were excised from DGGE gels using a sterile scalpel blade. DNA was eluted and purified from the bands as described by Tannock et al. (2004). PCR products were generated from the purified bands using the primer pair R158 and HDA1 without the GC clamp, and modified amplification conditions of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min 30 s (20 cycles), followed by a final extension step of 72 °C for 7 min. The resulting PCR products were purified using the GENECLEAN® Turbo for PCR Kit (QBioGene Inc.) and ligated into PCR®-4-TOPO® cloning vector (Invitrogen), and then transformed into chemically competent One Shot® TOP10 *Escherichia coli* cells using the TOPO TA Cloning Kit for Sequencing (Invitrogen) according to the manufacturer’s instructions.

DGGE PCR was used to directly screen the transformants. The migration of the amplified inserts was compared by DGGE alongside the original total community DGGE PCR to confirm that the cloned fragment matched the original band of interest. Only cloned fragments that migrated as a single band to the position identical to the originally excised band were selected for sequencing. An alkaline lysis procedure (Sambrook & Russell, 2001) was used to isolate the plasmid DNA of selected transformants from 2 mL of overnight culture. Recombinant clones containing the correct inserts were sequenced and analysed. For sequencing, the insert DNA was amplified using primers M13F and M13R (Invitrogen) and sequenced using internal primers T3 and T7 (Invitrogen). DNA sequencing was performed by the Allan Wilson Centre Genome Sequencing service at Massey.
University using the BigDye™ Terminator version 3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems Inc.) on an ABI3730 DNA sequencer (Applied Biosystems Inc.). Sequences were compared with those in the NCBI GenBank database using the BLAST algorithm (Altschul et al., 1997). Sequences were checked using CHIMERA CHECK (Maidak et al., 1997) on the RDP II database to ensure that no sequence was chimeric. The unique sequences obtained during this study are available at GenBank under accession numbers GQ280798–GQ280805.

**Chemical analysis**

Chemical analyses of selected *M. hentscheli* for levels of mycalamide A, pateamine, and peloruside A were performed following the methods described in Page et al. (2005b).

**Results**

**T-RFLP and DGGE sampling reproducibility**

Triplicate T-RFLP profiles from the same sponge DNA had a similarity index of 89–91% (mean = 90 ± 1%), and the reproducibility for DGGE fingerprint patterns from triplicate amplifications ranged from 89% to 100% (mean = 97.4 ± 4.5%). Triplicate DNA samples prepared from the same sponge showed a T-RFLP similarity index ranging from 90% to 96% (mean = 92 ± 3%), while DGGE profiles exhibited a greater range from 45.5% to 91.7% similarity (mean = 69.1 ± 23%). Visual examination of the replicate DGGE gels showed that the major band positions among replicate samples were identical (data not shown). The lower mean similarity of triplicate DNA samples prepared for DGGE was due to the variation in staining intensity resulting in bands not being readily detected by the BIONUMERICS software. Attempts were made to minimize this variation by standardizing band matching values to BIONUMERICS band analysis parameters (minimum profiling = 5%, ‘grey zone’ = 5%).

Sponge tissue was always sampled from the outer surface (pinacoderm) to approximately 2–3 cm depth inside the sponge mesohyl. To validate the sampling approach, T-RFLP profiles from samples taken from the mesohyl and pinacoderm layers of an *M. hentscheli* specimen were compared. This analysis showed a 91% similarity between these inner and outer layers (data not shown). This value was within the range detected between triplicate DNA samples prepared from the same sponge (90–96%).

**Spatial variation of bacterial communities associated with *M. hentscheli***

T-RFLP and DGGE analysis of bacterial communities from wild populations of *M. hentscheli* (Fig. 1) showed that geographically separated *M. hentscheli* had detectable differences in bacterial communities in addition to having distinct chemotype profiles. T-RFLP electropherograms (Table 1) of representative samples from each site indicated a greater diversity (represented by the number of peaks) in the sponge-associated microbial community from northern sample sites (Hohura Harbour and Spirits Bay) than in mid-latitude (Kapiti Island, Mahanga Bay, and Pelorus Sound) and southern New Zealand sites (Port Chalmers and Doubtful Sound).

Analysis of bacterial T-RFLP fingerprints (Fig. 2) showed geographical clustering of sponge specimens from within Port Chalmers (chemotype E: PC5, PC8, PC16), Doubtful Sound (chemotype F: FI02, TC33, TC17, FI07, TC18, FI21), and Kapiti Island (chemotype B: K150, K172, K256, K152, K265) areas. However, *M. hentscheli* samples from Pelorus Sound separated into three clusters, and there was no clear separation of the two chemotypes described from Pelorus Sound (chemotypes C and D). The two Northland samples (chemotype A: N7354 and N7362) did not cluster together.

DGGE cluster analysis (Fig. 3) of the same set of *M. hentscheli* samples showed a different distribution in the cluster associations. Bacterial communities from Port Chalmers and Doubtful Sound were split, with two Port Chalmers specimens (PC5, PC8) having a low (35%) similarity to all other specimens. Mid-latitude Pelorus Sound and Kapiti Island samples formed separate clusters, with one Pelorus Sound branch having 77% similarity to two samples from Mahanga Bay. Samples from Northland, which did not cluster using T-RFLP, clustered with DGGE, but were only 57% similar to the next closest cluster (from Port Chalmers and Doubtful Sound).

The clusters generated from each of the molecular fingerprinting methods generally grouped microbial communities to geographic location and by extension to the designated sponge chemotype. T-RFLP and DGGE analyses indicated differences in sponge microbial communities between extreme northern (Spirits Bay, Hohura Harbour) and southern (Doubtful Sound, Port Chalmers) sites. The T-RFLP patterns from Northland sites had more peaks than from any other sites (Table 1), and both samples were not tightly clustered to other sponge specimens (Figs 2 and 3). While Northland *M. hentscheli* were positive for mycalamide, the chemotype of these samples was not completely determined as no testing was conducted to determine the levels of pateamine or peloruside A. The chemotype of Doubtful Sound specimens was unique, having only pateamine with no mycalamide or peloruside A (chemotype F, Table 1). Samples from the other southern sample site at Port Chalmers contained mycalamide.

Specimens from central New Zealand (Pelorus Sound, Kapiti Island, and Mahanga Bay) had either a peloruside-negative or a peloruside-positive chemotype (designated...
Table 1. Bioactive compound occurrence and T-RFLP electropherograms from wild *Mycate hentschelli* from around New Zealand

<table>
<thead>
<tr>
<th>Chemotypes</th>
<th>Locations</th>
<th>Mycalamide</th>
<th>Peloruside A</th>
<th>Pateamine</th>
<th>T-RFLP electropherograms (FAM trace)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Northland – Hohura Harbour</td>
<td>Y</td>
<td>nt</td>
<td>nt</td>
<td><img src="image1.png" alt="Graph" /></td>
</tr>
<tr>
<td>A</td>
<td>Northland – Spirits Bay</td>
<td>Y</td>
<td>nt</td>
<td>nt</td>
<td><img src="image2.png" alt="Graph" /></td>
</tr>
<tr>
<td>B</td>
<td>Kapiti Island</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td><img src="image3.png" alt="Graph" /></td>
</tr>
<tr>
<td>C</td>
<td>Wellington Harbour – Mahanga Bay</td>
<td>Y</td>
<td>N</td>
<td>Var</td>
<td><img src="image4.png" alt="Graph" /></td>
</tr>
<tr>
<td>D</td>
<td>Pelorus Sound* – Capsize Point</td>
<td>Y</td>
<td>Y</td>
<td>Var</td>
<td><img src="image5.png" alt="Graph" /></td>
</tr>
<tr>
<td>C</td>
<td>Pelorus Sound* – Tennyson Inlet</td>
<td>Y</td>
<td>N</td>
<td>Var</td>
<td><img src="image6.png" alt="Graph" /></td>
</tr>
<tr>
<td>E</td>
<td>Port Chalmers</td>
<td>Y</td>
<td>Var</td>
<td>Y</td>
<td><img src="image7.png" alt="Graph" /></td>
</tr>
</tbody>
</table>
chemotypes C and D). Although specimens from this area were clustered geographically, with DGGE profiles having 60–95% similarity, there was no apparent site-specific and chemotype-related subclustering of the bacterial communities (Figs 2 and 3).

**Temporal variability of bacterial communities associated with *M. hentscheli***

To examine sponge microbial community changes within a population over time, individual captive *M. hentscheli* were resampled six times from Pelorus Sound over a 21-month period. Sorensen's similarity indices of the individual sponge microbial communities over time calculated from T-RFLP profiles (Fig. 4) showed that the similarity of the bacterial communities associated with these *M. hentscheli* were variable within and between sponges. For example, comparisons of consecutive samples from 3 October to 4 January were 83% and 87% similar from sponges P1 to P10, respectively, which indicates that a reasonably conserved and stable bacterial community persisted in these sponges over this period. Sponge samples having low similarity values were sponge P3 between 3 May and 3 October (55%) and sponge P8 between 4 January and 4 April (52%). Generally, for an individual sponge, bacterial community composition showed the highest similarity between consecutive sampling periods, with a few exceptions [P3: 3 May–3 October (55%) and P7: 4 June–5 February (59%)].
The low similarity levels between consecutive samples suggested a change in the sponge bacterial community composition over time, although there were instances where individual sponge bacterial communities appeared stable for 5 months or more [P7: 3 May–3 October (78%), 3 May–4 January (81%), and 3 May–4 April (80%)]. Changes in the bacterial composition were evident from three sponges (P6, P7, and P8) examined in 5 February. Low T-RFLP similarity values (≤ 60%) indicated that the T-RFLP pattern had changed from that recorded 8 months previously.

DGGE profiles from *Mycale hentscheli* revealed a maximum of 25–30 identifiable bands, suggesting a lower detectable species richness compared with T-RFLP. It is possible that the differences in similarity were due to differences in the resolution between the two molecular fingerprinting techniques, as a larger number of peaks were detected using

**Fig. 3.** Cluster analysis of microbial community DGGE fingerprints associated with *Mycale hentscheli* sampled from around New Zealand. Bars represent the SD at each branching level.
T-RFLP vs. the smaller number of bands from DGGE. DGGE fingerprint profiles of sponges showed that dominant bands were not only visible over time, but were common among many sponge samples (data not shown). Bacterial community variability from temporal sponge samples ranged from 22.9% to 95% (Dice similarity) using DGGE (Fig. 4), and 52% to 87% (Sorenson’s similarity) from T-RFLP (Fig. 4). DGGE analysis showed that sponge bacterial communities were relatively conserved over time (P7: 3 May–3 October (95%), P17: 3 May–3 October (93.8%)). Lower similarity from DGGE analysis was recorded between sponges sampled at longer time intervals (P8: 4 June–5 February (22.9%), P8: 3 October–5 February (26.7%), P10: 3 May–4 April (34.5%)). Comparisons of both T-RFLP and DGGE profiles over the 21-month sampling period showed that a large proportion of the detectable sponge-associated microbial community had changed, with < 50% similarity for DGGE and < 70% similarity for T-RFLP.

Sponges attached to fixed lines were observed to have a life span of approximately 2 years, after which time the sponge mass regressed and fouling of the sponge surface was observed. Of the seven original experimental sponges, only three could be sampled at 5 February as two of the original captive sponges had died (P17 and P10), and two others were visibly unhealthy and fouled by other marine organisms (P1 and P3).

Chemical analysis of sponges P17, P1, and P8 (Table 2) from a localized area in Pelorus Sound showed no seasonal correlation and low variation in peloruside A from each of the three sponges (P17: 138–195 μg g⁻¹, P1: 115–247 μg g⁻¹, P8: 56–103 μg g⁻¹). Mycalamide levels varied among individuals for each of the three sponges. Pateamine was only detected once from sponge P8.

Sequencing of DGGE bands

Twelve bands from M. hentscheli DGGE fingerprints sampled from four locations were sequenced (Fig. 5) and their similarities to other bacteria were determined (Table 3). Six bands aligned to uncultured bacterial sequences (1A, 6D, 1C, 4A, 6A, 3C) with sequence similarities ranging from 95% to 98%. Other bands were homologous to uncultured bacterial sequences reported from marine sponges (8B, 11A, 11A-2, 5D) with 96–100% similarity or from marine-derived sources (12B, 4C). Sequences were predominantly grouped in the Bacteroidetes, Verrucomicrobia, Alpha-, and
**Gammaproteobacteria.** The sequence of band 11A, a band isolated from a Mahanga Bay sponge, was identical to an uncultured sponge-associated bacterium reported from two sponges (*Axinella corrugata, Microciona prolifera*) and from a coral (*Muricea elongata*). Band 11A-2, excised from a similar position, was identical to the sequence of an uncultured bacterial clone characterized from the marine sponge *Tethya californiana*. Band 5D was identical to an uncultured bacterial clone, but closely related to coral and marine sponge-derived sequences. DGGE bands having the same migration position had identical sequences (6A and 3C; 12B and 4C; 1A, 1C, and 6D) (Fig. 5).

### Discussion

This study examined the relationship of different chemotypes of *M. hentscheli* with their associated bacterial communities. This is the first time that an attempt has been made to link distinctive sponge chemotypes to bacterial composition through spatial and temporal sampling of sponges.

DNA fingerprinting tools T-RFLP and DGGE were used to survey the sponge-associated microbial community. Analysis of T-RFLP profiles showed clustering of microbial communities from Doubtful Sound, Kapiti Island, and Port Chalmers (Fig. 2), but less correspondence between microbial community profiles from Pelorus Sound, where peloroside-positive and -negative chemotypes were recorded. A similar spatial clustering pattern was apparent from the DGGE analysis (Fig. 3), but not for Kapiti Island and Doubtful Sound. The clusters generated from each of the molecular fingerprinting methods generally grouped microbial communities to geographic location and by extension to the designated sponge chemotype. However, site-specific differences in the clustering associations between T-RFLP and DGGE raised the question of the ability of each method to accurately describe the relationships between the sponge microbial communities. An example of the divergence in the clustering relationships is the separation of the two Northland samples in the T-RFLP cluster, but not in DGGE. Determining the reliability of both methods for assessing community structure is difficult from these results. The nearest approximation of reliability was an analysis of the reproducibility of both techniques. From this, T-RFLP profiles showed better reproducibility (> 90% similarity from triplicate samples) than DGGE (> 45%).

While T-RFLP and DGGE rely on the analysis of PCR-amplified 16S rRNA gene fragments to characterize the predominant microbial community, there are inherent limitations associated with both methods. The key when applying these techniques is to minimize these limitations such that any change in the microorganisms detected by these methods represents a natural change and is not an artefact of the analysis. Both T-RFLP and DGGE have been well validated in the literature for microbial community analysis, and every attempt was made in this study to standardize sampling, DNA extraction, PCR conditions, and analysis methods to minimize errors. In addition to inherent PCR biases (such as amplification efficiency),

<table>
<thead>
<tr>
<th><em>M. hentscheli</em> samples</th>
<th>Sample date</th>
<th>Peloroside A ($\mu$g g$^{-1}$)</th>
<th>Mycalamide A ($\mu$g g$^{-1}$)</th>
<th>Pateamine ($\mu$g g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>May 2003</td>
<td>131.7</td>
<td>282.6</td>
<td>0</td>
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<tr>
<td></td>
<td>October 2003</td>
<td>114.8</td>
<td>262.2</td>
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</tr>
<tr>
<td></td>
<td>January 2004</td>
<td>138.0</td>
<td>262.9</td>
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<td>April 2004</td>
<td>191.5</td>
<td>239.0</td>
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**Table 2.** Bioactive compound levels from temporal *Mycale hentscheli* samples from Pelorus Sound

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**Fig. 5.** Composite image of DGGE profiles of 16S rRNA gene fragments from *Mycale hentscheli*. All labelled bands were excised and analysed by sequencing. *Mycale hentscheli* samples: A1P1, Jan1P1, Jun5P3, and MJP32.13 all sourced from Pelorus Sound; MAH2, Mahanga Bay; PCS, Port Chalmers; FI02 and TC17, Doubtful Sound.
DGGE relies on the visualization and assignment of bands, whereas T-RFLP is less limiting for analysis as this technique relies on GeneScan detection of fluorescent terminal fragments. For example, T-RFLP analysis of spatial sponge samples detected 339 unique peaks, of which 60% and 40% were contributed by the FAM- and VIC-labelled primers, respectively. By comparison, with 25–30 detectable bands, DGGE had noticeably lower species richness compared with T-RFLP and may be less sensitive for less abundant bacterial species.

Two different similarity indices were used to process and compare the sponge temporal data. T-RFLP similarity indices were calculated directly from the binary peak data by Sørenson’s similarity index, whereas similarities for DGGE banding patterns were generated using the Dice coefficient as part of the BIOPHORES analysis software. While valid comparisons can be made of the temporal and spatial data using each fingerprinting method, a more cautious interpretation should be made of any method–method similarity comparisons.

Unlike many other marine sponges studied, where significant differences have been reported in the microbial communities from different subregions of the sponge tissue (Thiel et al., 2007; Meyer & Kuever, 2008), *M. hentscheli* is described in the taxonomic literature as a ‘thick encrusting amorphous sponge’ (Bergquist & Fromont, 1988). The sampling approach used in this study for *M. hentscheli* was validated and standardized in an attempt to minimize any variation that might be a result of the sampling procedure.

The geographic variation and seasonal abundance of bacterial communities and bioactive compounds noted in this and other studies (Page et al., 2005b) may be influenced by environmental factors such as sea-surface temperatures. New Zealand’s landmass extends for 10° of latitude, from subtropical through to sub-Antarctic waters (Fig. 1). The Southland Current influences sea-surface temperatures at the southern-most sampling sites (Doubtful Sound and Port Chalmers), where annual sea-surface temperatures vary between 11–15 and 8–15 °C, respectively. The major current influencing the Northland sample sites is the East Auckland Current that originates from the tropics, and annual coastal sea-surface temperatures in Northland range from 14 to 20 °C. Other environmental factors such as nutrient availability, competition, and predation as well as sponge physiology and nutritional history have also been postulated as the causative agents of the observed seasonal variation (Thompson et al., 1987; Duckworth & Battershill, 2003; Page et al., 2005a). To add to this complexity, the abundance of cytotoxic metabolites in *M. hentscheli* differs not only over the geographic range of New Zealand (Table 1) (Perry et al., 1988; Northcote et al., 1991; West et al., 2000a, b) but also varies seasonally in the wild and in aquaculture experiments (Page et al., 2005b). This seasonal variability is in response to as yet unidentified stimuli. Temperature does appear to have a major influence on the growth rates of *M. hentscheli* (Page et al., 2005b). *Mycale hentscheli* grows the fastest during spring, summer, and autumn, and this period correlates not only with seasonal peaks in bioactive compound production but also the increased activity of competing surface fouling organisms (Page et al., 2005b). From the temporal study of *M. hentscheli*, fouled sponge would often be completely degraded within 4 weeks. Therefore, age, longevity, and condition of the sponge might also influence bacterial community associations. This suggests a possible ecological role for these bioactive chemicals as a defense mechanism against fouling, competition, and predation (Page et al., 2005b) and is supported by several reports that propose the role of host-associated bacteria in the production of cytotoxic compounds in marine invertebrates (Haygood et al., 1999; Davidson et al., 2001; Piel, 2009).

The structural similarities of mycalamide A to the bacterial-derived bioactive compound pederin suggest that bioactive compounds from *M. hentscheli* are bacterially derived (Piel, 2002). However, determining a specific association between the described bacterial community and the chemo-types of *M. hentscheli* is more problematic.

From this study, the spatial clustering and overall similarity of T-RFLP and DGGE fingerprints suggests that a detectable portion ( > 35% of the DGGE bands and > 19% of the T-RFLP peaks) of the microbial community was common to *M. hentscheli* across a range of geographic locations. It was also apparent that some microbial species were more conserved and resident in individual *M. hentscheli*. This was particularly evident from the DGGE banding patterns (Fig. 5), where bands were conserved and occurred across a number of spatial and temporal *M. hentscheli* (Fig. 5: bands 6A and 3C). Sequencing of these DGGE bands confirmed that the same bacterial species were present from *M. hentscheli* sampled at different times of the year and from different geographic locations (Table 3). One possible limitation of sequencing the DGGE bands was the short length of sequence (175–200 bp). However, the analysed region targeted a variable (V2–V3) region of the 16S rRNA gene, which has been shown to have considerable sequence diversity among different bacteria (Chakravorty et al., 2007). Analysis of the DGGE fragment sequences indicated that there was enough variation to obtain good species-level sequence identities. Sequencing confirmed the sponge-associated relationship of many of these DGGE bands with sequences having > 96% similarity to sponge or coral-associated bacteria.

The ubiquitous occurrence of sponge-specific bacterial communities was observed where DGGE band fragments matched sequences reported from sponges sampled from different regions for example, *M. prolifera* from North America (band 8B), *A. corrugata* from the Caribbean (band...
Table 3. 16S rRNA gene sequence comparison of DGGE bands of bacteria associated with Mycale hentscheli, with 16S rRNA gene database sequence entries

<table>
<thead>
<tr>
<th>DGGE band (GenBank accession no.)</th>
<th>Mycale samples</th>
<th>Length of sequence (bp)</th>
<th>Phylogenetic group*</th>
<th>Closest relative of sequence from described bacterial species (GenBank accession no.)</th>
<th>Sequence similarity (%)</th>
<th>Closest related database sequence (GenBank accession no.)</th>
<th>Sequence similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band 1A (GQ280798)</td>
<td>A1P1</td>
<td>195</td>
<td>Bacteroidetes; Flavobacteriaceae</td>
<td>Winogradskyella poriferorum strain UST030701-295 (AY848823)</td>
<td>95</td>
<td>Uncultured bacterium clone SS1_B_04_12 (EU050899)</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Jun5P3</td>
<td></td>
<td></td>
<td>Tenacibaculum sp. D38BY (EU021293)</td>
<td>96</td>
<td>Mucus bacteria 68 (AY654806)</td>
<td>96</td>
</tr>
<tr>
<td>Band 4A (GQ280799)</td>
<td>A1P1</td>
<td>175</td>
<td>Alphaproteobacteria Rhodobacteraceae</td>
<td>Salipiger mucosus strain LMG22090 (DQ915629)</td>
<td>97</td>
<td>Uncultured bacterium clone Sb105 (DQ861201)</td>
<td>98</td>
</tr>
<tr>
<td>Band 6B (GQ280800)</td>
<td>Jan1P1</td>
<td>201</td>
<td>Deltaproteobacteria; Desulfovibrionaceae</td>
<td>Desulfovibrio cavernae strain H1MT (AJ621885)</td>
<td>89</td>
<td>Uncultured bacterium clone P9X2b711 (EU491200)</td>
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<tr>
<td></td>
<td>Jun5P3</td>
<td></td>
<td></td>
<td>Uncultured bacterium clone SS1_B_02_91 (EU050885)</td>
<td>95</td>
<td>Uncultured bacterium clone SS1_B_02_91 (EU050885)</td>
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<tr>
<td>Band 11B (GQ280801)</td>
<td>MAH2</td>
<td>175</td>
<td>Alphaproteobacteria; Kordiimonadaceae; Kordiimonas</td>
<td>Kordiimonas gwangyangensis strain GW14-5 (AY682384)</td>
<td>97</td>
<td>Uncultured Kordiimonas sp. clone AC3_C1 16S rRNA gene, partial sequence (EF092170). Specific host – marine sponge Axinella corrigata</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Source – marine bacteria from marine sediments</td>
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<td>Uncultured alphaproteobacterium clone MPWIC_D06 16S rRNA gene, partial sequence (EF414194). Specific host – marine sponge, Microciona prolifera</td>
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<tr>
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<td>Source – coral, Muricea elongata</td>
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<td>Gammaproteobacterium MOLA 531 (AM990755). Specific host – marine sponge Petrosia ficiformis</td>
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<td>Uncultured gammaproteobacterium clone MPWIC_A09 (EF414223). Specific host – marine sponge, Microciona prolifera</td>
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<td>Specific host – marine sponge, Microciona prolifera</td>
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<td>Band 8B (GQ280802)</td>
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<td>Uncultured bacterium clone BT49MF48D11 (AF365561). Source – coral associated</td>
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<td></td>
<td>Source – coral associated</td>
<td>89</td>
<td>Uncultured Verrucomicrobia bacterium clone LD1-P81 (AY14329). Source – marine sediments</td>
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<tr>
<td>Band 12B (GQ280803)</td>
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<td>202</td>
<td>Bacteria; Verrucomicrobia</td>
<td>Source – isolated from toxic dinoflagellate Alexandrium minutum Roseaspora sp. J052 (DQ869302)</td>
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<td>TC17</td>
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<td>Source – isolated from toxic dinoflagellate Alexandrium minutum Roseaspora sp. J052 (DQ869302)</td>
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<td>Source – isolated from toxic dinoflagellate Alexandrium minutum Roseaspora sp. J052 (DQ869302)</td>
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<td>Band 11A-2 (GQ280804)</td>
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<td>Uncultured bacterium clone 101-31 (EF157247). Specific host – coral, Oculina patagonica</td>
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<td>Source – isolated from toxic dinoflagellate Alexandrium minutum Roseaspora sp. J052 (DQ869302)</td>
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<td>Band 5D (GQ280805)</td>
<td>MJP32.13</td>
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<td>Alphaproteobacteria Rhodospirillaceae</td>
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<td>Uncultured bacterium clone F12P4Mbd (FJ156460)</td>
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</table>

*Phylogenetic analysis: sequences were compared with those in the GenBank database using the BLAST algorithm (Altschul et al., 1997). Sequences were checked with CHIMERA CHECK (Maidak et al., 1997) on the RDP II database to ensure that no sequence was chimeric.
A temporal study observed a > 75% level of similarity between two C. concentrica sponge samples collected at an interval of 76 days (Taylor et al., 2005), and consistent seasonal bacterial structure was observed in the sponge Mycale adhaerens Bowerbank (Lee et al., 2006). Cultivation studies have also established that bacteria can maintain a highly specific and stable relationship with the sponge host (Santavy et al., 1990; Olson et al., 2000; Hentschel et al., 2001; Webster & Hill, 2001; Thakur et al., 2004). A culturable alphaproteobacterium (designated NW001) was always associated with the marine sponge Rhapaloeides odorabile irrespective of spatial and temporal variations (Webster & Hill, 2001). It may be that the abundance of conserved sponge-associated bacteria plays an important role in bioactive compound production, although this relationship has yet to be demonstrated.

While cluster associations between both fingerprinting methods did not specifically correlate, this research provides evidence to support the hypothesis that the chemotypes of M. hentscheli correlate to an associated bacterial community. The variation in DGGE or T-RFLP patterns observed in the microbial communities from spatially isolated M. hentscheli does suggest that there is an association between the microbial community and sponge chemotype. At the temporal scale, evidence for this association was less evident as chemotype patterns did not change significantly while a portion of the microbial community varied over time. A future approach to better define sponge–bacteria relationships to M. hentscheli chemotypes will be the construction of 16S rRNA gene clone libraries to identify unique OTUs to characterize the chemotype-specific fraction of the sponge bacterial community.

While evidence points to a microbial producer of bioactive compounds in M. hentscheli (Piel, 2002), other ecological factors may also play a significant role in regulating the production of these compounds. Just what these factor are (e.g. location, temperature, depth, sunlight, water quality, competition, and predation) requires further investigation. This study provides a basis for further investigations to identify and screen for key producer microorganisms from M. hentscheli or as a mechanism to target biosynthetic genes directly.

Acknowledgements

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References


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