RESEARCH ARTICLE

Bacterial communities associated with healthy and *Acropora* white syndrome-affected corals from American Samoa

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Abstract

Acropora white syndrome (AWS) is characterized by rapid tissue loss revealing the white underlying skeleton and affects corals worldwide; however, reports of causal agents are conflicting. Samples were collected from healthy and diseased corals and seawater around American Samoa and bacteria associated with AWS characterized using both culture-dependent and culture-independent methods, from coral mucus and tissue slurries, respectively. Bacterial 16S rRNA gene clone libraries derived from coral tissue were dominated by the *Gammaproteobacteria*, and Jaccard's distances calculated between the clone libraries showed that those from diseased corals were more similar to each other than to those from healthy corals. 16S rRNA genes from 78 culturable coral mucus isolates also revealed a distinct partitioning of bacterial genera into healthy and diseased corals. Isolates identified as Vibrionaceae were further characterized by multilocus sequence typing, revealing that whilst several *Vibrio* spp. were found to be associated with AWS lesions, a recently described species, *Vibrio owensii*, was prevalent amongst cultured *Vibrio* isolates. Unaffected tissues from corals with AWS had a different microbiota than normal *Acropora* as found by others. Determining whether a microbial shift occurs prior to disease outbreaks will be a useful avenue of pursuit and could be helpful in detecting prodromal signs of coral disease prior to manifestation of lesions.

Introduction

Coral reefs occupy < 1% of the earth's ocean surface, though harbour one of the most diverse ecosystems on the planet and are of tremendous economic value providing substantial revenue to coastal communities through fishing, tourism and coastal protection (Moberg & Folke, 1999; Rosenberg *et al.*, 2007; Bourne *et al.*, 2009). Reef-building corals are comprised of a complex interaction between the coral host, endosymbiotic microalga (*Symbiodinium* spp.) and close associations with a broad spectrum of unicellular and multicellular organisms (Ainsworth *et al.*, 2010), including bacteria (Rohwer *et al.*, 2001, 2002; Bourne & Munn, 2005), archaea (Kellogg, 2004; Wegley *et al.*, 2004), viruses and fungi (Knowlton & Rohwer, 2003; Patten *et al.*, 2008; van Oppen *et al.*, 2009) and hydrozoans (Pantos & Bythell, 2010). This multi-organismal association is referred to as the coral holobiont (Rohwer *et al.*, 2002), and the relationship between the coral's constituent partners is thought to be dynamic, with its homoeostasis integral to the health of the coral (Rohwer *et al.*, 2002). Within individual corals, there also exist a number of microhabitats (including the coral surface mucus layer, coral tissues and coral skeleton), and these each may contain distinct and diverse microbial communities (Bourne & Munn, 2005; Rosenberg *et al.*, 2007; Ainsworth *et al.*, 2010).

Recent decades have seen a rise in reports of coral mortality in global reef ecosystems (Hughes *et al.*, 2003; Lesser, 2004; Bourne *et al.*, 2009), and despite a number of biotic and abiotic factors having been identified as major contributors to the general decline (Rosenberg *et al.*, 2007), causal relationships have yet to be clearly defined (Work *et al.*, 2008). Climatic and anthropogenic stressors may compromise coral host immunity, thereby predisposing them to opportunistic infection (Harvell

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et al., 1999, 2001; Bally & Garrabou, 2007; Lesser *et al.*, 2007). Diseases of corals are emerging as a very real threat worldwide (Rosenberg *et al.*, 2007), and a significant proportion of current research efforts are focused on identifying the microbial agent(s) responsible, although other causes certainly exist. The complexity of the coral holobiont, limited understanding of host physiology and lack of systematic deductive approaches to disease investigations has led to uncertainty.

A number of studies have examined differences in microbial communities between healthy and diseased corals in an effort to understand disease pathogenesis (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2004; Sunagawa *et al.*, 2009). There exists great intra- and intercolony variation in microbiota (Pantos *et al.*, 2003; Barneah *et al.*, 2007), and it is possible that coral-associated microbiota may undergo dramatic changes before manifestation of lesions on the coral host (Pantos *et al.*, 2003).

'White syndromes' (WS) are one of the most prevalent tissue loss diseases of reef-building corals globally (Willis *et al.*, 2004) and are characterized by an acute or subacute loss of tissue leading to exposure of the underlying coral skeleton. In the field, WS can affect multiple (Dalton *et al.*, 2010) or single (Aeby *et al.*, 2011) genera of corals. Whilst it is often assumed that WS are infectious, one hypothesis is that they are the result of programmed cell death (PCD) within the coral tissues, although a microbial component was not ruled out as bacteria can induce a characteristic PCD response (Ainsworth *et al.*, 2007a, b). In contrast, other studies of WS (Sussman *et al.*, 2008; Luna *et al.*, 2010) have provided evidence for the involvement of *Vibrio* spp. associated with lesions, illustrating that WS probably have multiple different causes.

One of the most common and damaging coral diseases on the reefs within American Samoa is *Acropora* white syndrome (AWS) (Fig. 1a) (Aeby *et al.*, 2009). As the name suggests, AWS appears to exclusively affect scleractinian corals of the family Acroporidae, resulting in a clear zone of demarcation between healthy tissue and white skeleton. AWS results in a progressive tissue loss leading to partial to total colony mortality depending on the coral species affected and has been found to be transmissible through direct contact in controlled studies (Aeby *et al.*, 2011); histological analyses of tissues from American Samoan corals with AWS revealed mostly tissue necrosis or wound repair processes to be evident (Work & Aeby, 2011). Our objective was to characterize the diversity of bacteria in samples from normal and lesion tissues of corals affected by AWS and compare this to unaffected corals from American Samoa.

Materials and methods

Sample collection

Samples from *Acropora hyacinthus* were collected from reefs (< 10 m in depth) in Vatia Bay, on the north side of the island of Tutuila (140°14′S, 1700°40′W), American Samoa, over a 48-h period during June 2008 (Fig. 1b). Six healthy and seven colonies manifesting AWS were sampled. We collected lesioned fragments (DD) from diseased colonies and apparently healthy tissue from diseased (HD) and healthy (HH) colonies into plastic bags. Separately, triplicate water samples (15 mL) were taken from the water column (30 cm) above each area sampled. Samples were stored at 4 °C until laboratory processing.

Bacterial isolation and selection media

Coral mucus (1 mL) was obtained by syringe from each coral fragment surface and serially diluted 10-fold (10^{-1} to 10^{-7}) in 0.22-μm-filter-sterilized artificial sea water (ASW) (Instant Ocean; Spectrum Brands, Madison, WI). ASW (5 mL) was then added to the coral fragments, and tissues removed by airbrushing and aliquoted into 1.5-mL microfuge tubes. Mucus and tissues from diseased (DD) corals were sampled from tissues immediately adjacent to lesions bordering exposed coral skeleton. Water column samples were serially diluted 10-fold (10^{-1} to 10^{-5}) in (ASW). Seawater and coral mucus were streaked onto marine agar (MA) (BD, NJ) for the isolation of general marine bacteria and *Vibrio* species-selective thiosulphate citrate bile sucrose (TCBS) agar and incubated at 28 °C for 48 h. Morphologically distinct colonies were isolated and restreaked on MA and TCBS prior to cryopreservation in 15% (v/v) glycerol at −80 °C and subsequent DNA extraction. Samples from the coral mucus and water were processed 2–3 h after collection for culture-based analysis, whilst tissue samples were stored at −20 °C for subsequent DNA extraction and clone library analyses.

DNA extraction and PCR amplification

Total genomic DNA was extracted from bacterial isolates using the Wizard Genomic DNA Kit (Promega, Madison, WI) and from coral tissues using a MoBio PowerPlant DNA Isolation kit (MoBio, Carlsbad, CA). Bacterial DNA was amplified using the 63F (Marchesi *et al.*, 1998) and 1389R (Osborn *et al.*, 2000) primer pair targeting the bacterial 16S rRNA gene. Isolates putatively identified as members of the Family *Vibrionaceae* (based on growth on TCBS and 16S rDNA gene sequence) were further characterized by multilocus sequence typing (MLST) using primer pairs recA-01-F and recA-02-R, rpoA-01-F and

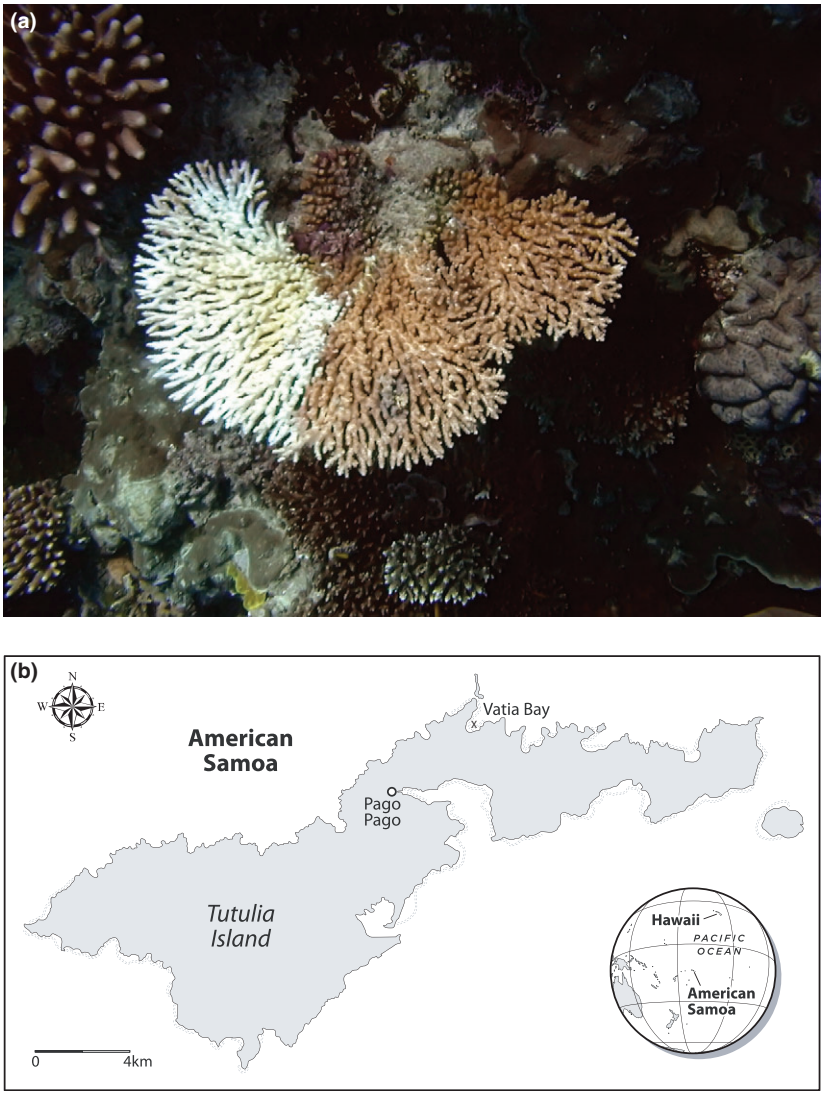


Fig. 1. (a) AWS on *Acropora* coral. (b) American Samoa. The location of the sampling site (in Vatia Bay) is denoted by an 'X'.

rpoA-03-R, and pyrH-04-F and pyrH-02-R (Thompson *et al.*, 2005). Reaction mixtures comprised 1.25 mM $MgCl_2$, 200 μM dNTP, 2 U of iTaq DNA polymerase (Biorad, Hercules, CA), 200 nM of each primer, 400 ng μL^{-1} nonacetylated BSA (Kreader, 1996), 1 μL (2–10 ng) of sample genomic DNA and nuclease-free water to bring the total volume to 50 μL. PCR conditions followed those exactly as described for the primers 63F-1389R (Osborn *et al.*, 2000) and recA-01-F, recA-02-R, rpoA-01-F, rpoA-03-R, pyrH-04-F and pyrH-02-R (Thompson *et al.*, 2005).

Clone library construction and sequencing

The PCR products were purified from agarose gels using the Nucleospin Extract II kit (Machery-Nagel, Düren, Germany), cloned into the pCR2.1-TOPO cloning vector and transformed into OneShot TOP10 competent cells as specified by the manufacturer (Invitrogen, Carlsbad, CA). Clones were checked for inserts by PCR amplification using M13 forward (−20) (5′-GTAAAACGACGGCCAG-3′) and M13 reverse (5′-CAGGAAACAGCTATGAC-3′) primers (Invitrogen) and sent to Australian Genome Research Facility Ltd (AGRF, Brisbane, Australia) for sequencing, using the 63F forward primer.

Sequence analysis

Clonal sequences were trimmed using the Lasergene SeqMan II software package (DNAStar, Madison, WI) and partial 16S rRNA genes (300–700 bp in length) aligned and classified using the Greengenes Database and Tools

(http://greengenes.lbl.gov/) (DeSantis *et al.*, 2006). Sequence data for 16S rRNA genes from culturable isolates were compared with those in the NCBI database using the Nucleotide Basic Local Alignment Search Tool (BLASTN) algorithm (Altschul *et al.*, 1990) and submitted under GenBank Accession Nos. GU903192–GU903269. For the MLST analyses, sequence data for 16S, *recA*, *rpoA* and *pyrH* genes for representative *Vibrio* type strains were retrieved from The Taxonomy of the *Vibrios* website (http://www.taxvibrio.lncc.br/). The four genes for each *Vibrio* type strain (as well as for each putative *Vibrio* isolate) were concatenated and aligned (Sawabe *et al.*, 2007) using the Lasergene EditSeq and MegAlign software packages (DNAStar). The neighbour-joining method (with the CLUSTALW package in MEGALIGN) was used to construct a rooted phylogenetic tree; *Campylobacter jejuni* was included as an outgroup. Sequence data for the recA (400–700 bp), rpoA (300–700 bp) and pyrH (300–500 bp) genes for putative *Vibrio* isolates were submitted under GenBank Accession Nos. JN715078–JN715125; sequence data for 16S rRNA genes from clone libraries were submitted under GenBank Accession Nos. JN215787–JN216827.

Statistical analysis

All statistical analyses were performed using R (R Development Core Team, 2010). A Shannon–Wiener diversity index (Shannon, 1948) was used to characterize species diversity and evenness in pooled libraries of the healthy and diseased coral samples. The Jaccard's similarity coefficients (Jaccard, 1901) between the coral sample sets were also calculated.

Results

Culture-independent bacterial diversity associated with healthy and diseased *A. hyacinthus* tissues

In June 2008, visual coral surveys were conducted around the main island of Tutuila, American Samoa to identify sites displaying signs of AWS (Fig. 1b). The highest disease level was found in Vatia Bay, with an AWS prevalence of 2.9% (Aeby *et al.*, 2011), and therefore, samples were taken from both healthy and AWS-affected specimens at this site. Fifteen 16S rRNA gene clone libraries (comprising 1255 clonal sequences) were derived from replicate healthy tissue (HH, $n = 3$), healthy tissue from diseased colonies (HD, $n = 5$), lesion tissue (DD, $n = 5$) and water column (W, $n = 2$). The diversity of the microbial communities associated with the corals and water column comprised members of commonly occurring marine and coral bacterial phyla including the *Proteobacteria*, *Cyanobacteria*, *Firmicutes* and *Actinobacteria* (Fig. 2). The *Proteobacteria* dominated and contained representatives from the *Alpha-*, *Beta-*, *Epsilon-* and *Gamma*-classes. Amongst *Proteobacteria*, the *Alphaproteobacteria* dominated water column clone libraries (32%), whilst comprising only 2% of each of the coral (HH, HD and DD) clone libraries. *Epsilonproteobacteria* were found at consistently low levels (2–4%) in the coral samples but were absent from water column samples. *Beta-* and *Gammaproteobacteria* were ubiquitous amongst the coral samples but rare in the water column. Clones related to the *Alphaproteobacteria*, *Sphingomonas* spp. and *Novosphingobium* spp. were found in both HD and DD coral tissues but not in tissues sampled from healthy colonies of corals, whilst clones affiliated with the *Betaproteobacteria Polaromonas* spp. and *Herbaspirillum* spp. and *Gammaproteobacteria Actinobacillus* spp., *Haemophilus* spp. and *Pasteurella* spp. were found consistently in both healthy and diseased coral tissues.

Cyanobacteria were the next dominant group comprising mainly *Gloeobacter* spp. and *Prochlorothrix* spp. *Flavobacteria* were obtained at much higher levels (14%) in the water column samples when compared with the coral samples (< 2%). In contrast, members of the *Bacilli* were recovered from all healthy and diseased corals but not water column samples (Fig. 2).

The diversity and evenness of microbial communities in HH coral tissue samples was greater than that in tissue from HD and DD corals (Table 1). Whilst the species richness index was lowest in HH samples, the species evenness value was highest when compared with those of the other samples – correspondingly, the similar values for both species richness and evenness in the HD and DD samples suggest that despite the higher overall number of clones, some clones were more abundant than others. The Jaccard's similarity coefficients demonstrated that the microbial communities in the HD and DD corals ($J = 0.52$) are more similar to each other than to the HH corals ($J > 0.62$).

Culturable bacterial communities associated with healthy and diseased *A. hyacinthus* mucus

Of 316 isolates initially screened from coral mucus samples, 78 morphologically distinct isolates were obtained, comprising 26, 20, 20 and 12 isolates from DD, Water, HH and HD samples, respectively (Table 2). Sequence analysis of the 16S rRNA gene identified the majority as *Gammaproteobacteria* (44%) and *Bacilli* (34%), the remainder comprising *Actinobacteria* (18%) and *Alphaproteobacteria* (3%). At the genus level, most *Gammaproteobacteria* belonged to the genera *Vibrio* (21%) and *Pseudoalteromonas* (14%), whilst the *Bacilli* were dominated by the genus *Bacillus* (17%).

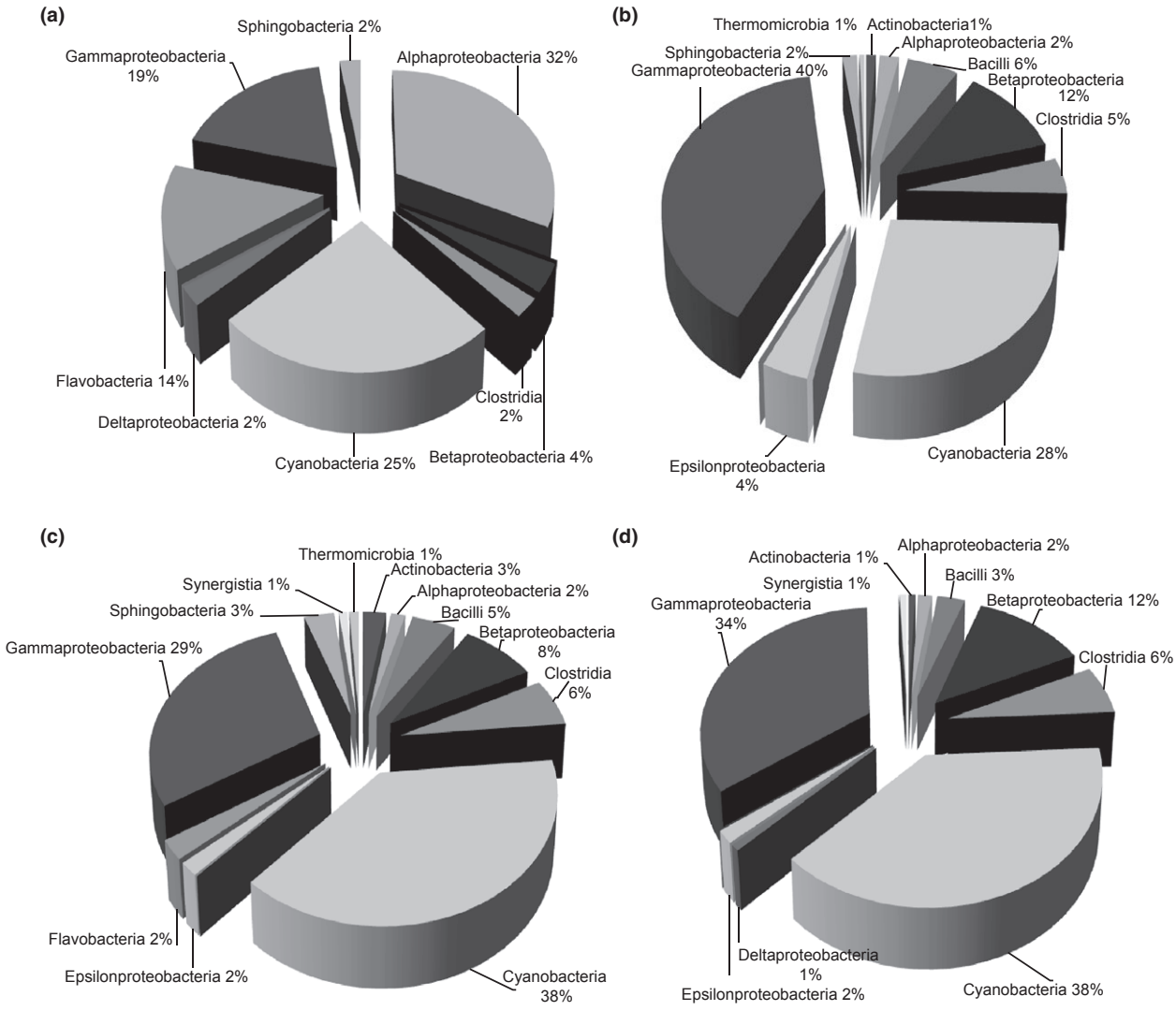


Fig. 2. Bacterial diversity (by class) from pooled clone libraries of 16S rDNA gene sequences sampled from (a) the water column immediately above corals (W) [n = 92]; (b) coral fragments from colonies that showed no signs of disease (HH) [n = 207]; (c) coral fragments from an area of apparently healthy tissue on the diseased colony (HD) [n = 409]; and (d) coral fragments from an area of diseased tissue on the diseased colony (DD) [n = 547].

Distinct partitioning of the culturable isolates was seen amongst sample types and the water column with the majority of the isolates only retrieved from a single-sample type. For example, *Microbacterium* spp., *Micrococcus* spp., *Pseudomonas* spp. and *Salinicoccus* spp. were only found in HH samples, whilst *Brachybacterium* spp., *Exiguobacterium* spp., *Brevibacterium* spp., *Psychrobacter* spp. and *Nesterenkonia* spp. were associated within lesions (DD samples) only. Isolates related to *Pseudoalteromonas* spp. and *Staphylococcus* spp., however, were found in mucus from all coral tissue samples, regardless of disease state, whilst bacteria similar to *Vibrio* spp. were ubiquitously distributed in all sample types, including the water column above the corals.

MLST identified two isolates (AmSamHH29 and AmSamHD46) as probable *Vibrio harveyi* and *Vibrio alginolyticus*, respectively, based on > 99% MLST sequence similarity. One-third (four) of the *Vibrio* isolates were 97–99% MLST identical to a newly identified species, *Vibrio owensii* (Cano-Gómez *et al.*, 2010), and of those, three (AmSamDD45, AmSamDD48 and AmSamDD85) were in mucus from AWS disease lesions. The remaining eight putative *Vibrio* isolates could not be readily identified with MLST and were labelled as Family *Vibrionaceae*,

Table 1. Shannon–Wiener diversity indices for tissue microbial communities retrieved from coral fragments taken from colonies that showed no signs of disease (HH), coral fragments taken from an area of apparently healthy tissue on the diseased colony (HD) and coral fragments taken from an area of diseased tissue on the diseased colony (DD)

Coral tissue sample	Species richness*	Species diversity†	Species evenness‡
HH	30	3.02	0.89
HD	38	2.97	0.82
DD	36	2.89	0.81

*Species richness = total number of OTUs.
†Species diversity = Shannon diversity index.
‡Species evenness = Shannon's equitability index [Shannon diversity index/log (species richness)].

whilst a single isolate (AmSamHH17), which was identified as a *Vibrio* when its individual housekeeping gene sequences were compared with published sequences, could not ultimately be resolved using MLST analyses and may possibly be a novel *Vibrio* species.

Discussion

The bacterial communities associated with the mucus and tissues from healthy corals are distinct from those of AWS-diseased corals. Interestingly, the communities in the tissues of HD and DD corals are more similar to each other than to the HH corals, and findings that agree with others (Pantos *et al.*, 2003) that showed that microbial communities of diseased corals exhibit a 'whole community' response, such that communities isolated from healthy and diseased tissues on the same coral will be very similar, regardless of the disease state of local tissues. These results suggest that microbial ecology analyses of apparently healthy corals may prove to be indicative of disease prior to the coral host exhibiting any pathological symptoms; however, this can only be confirmed by monitoring colony microbiota prior to and during manifestation of disease. Both diversity and evenness of microbial communities in HH coral tissue samples were greater than from HD and DD corals, and species richness was lower in apparently healthy coral colonies than either the HD or DD samples, findings concordant with previous studies (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002; Pantos *et al.*, 2003; Sekar *et al.*, 2006). Whilst the abundance of operational taxonomic units (OTU) within the HH tissue microbial communities was more evenly distributed amongst these species, microbial communities in HD and DD samples comprised OTUs that were noticeably more abundant than others – which is to be expected in diseased corals where one or more potential pathogens or secondary microbial colonizers may dominate the microbial community.

Clone libraries associated with *Acropora* corals comprised members of the *Proteobacteria*, *Cyanobacteria*, *Firmicutes* and *Actinobacteria*, with the following members of the *Proteobacteria* dominating: *Alpha-*, *Beta-*, *Epsilon-* and *Gamma*-classes. The *Alphaproteobacteria* are some of the most abundant organisms in the sea (comprising the ubiquitous SAR11 cluster of organisms (Giovannoni *et al.*, 1990)) and are found associated with corals and sponges (Lampert *et al.*, 2006), and one of these, *Sphingomonas*, is associated with White Plague disease of *Montastrea annularis* corals (Pantos *et al.*, 2003). In our study, *Sphingomonas* spp. and *Novosphingobium* spp. were found in both HD and DD but not HH coral tissues. In contrast to the coral tissue samples in this study, the *Alphaproteobacteria* dominated the water column clone libraries (Fig. 2) as expected (Kooperman *et al.*, 2007). The *Gammaproteobacteria* dominated microbial communities from *Acropora* spp. in American Samoan corals in accordance with other studies of this genus in the Pacific (Bourne, 2005; Littman *et al.*, 2009) and Atlantic (Frias-Lopez *et al.*, 2002), but dominant groups of bacteria vary with coral species and habitat (Kooperman *et al.*, 2007; Littman *et al.*, 2009).

In both healthy and diseased coral mucus samples, the dominant culturable bacteria were the *Gammaproteobacteria* (comprising 44% of the isolates) as this group is readily enriched in culture media (Eilers *et al.*, 2000; Fuchs *et al.*, 2000; Allers *et al.*, 2008). However, in this study, they also predominated in clone libraries from tissue samples and are thus likely representative of the *Acropora* coral microbial community. *Vibrio* spp., *Staphylococcus* spp. and *Pseudoalteromonas* spp. were associated with mucus sampled from all healthy and diseased coral tissues. *Pseudoalteromonas* have caused mortality in marine crustacea (Costa-Ramos & Rowley, 2004) and algae (Lovejoy *et al.*, 1998) but like *Staphylococcus* spp., which may be found associated with skin and mucus membranes in warm-blooded organisms, there are no reports of either of these causing disease in corals. Members of the genus *Staphylococcus* are more commonly considered as terrestrial organisms, and so their presence in marine environments is unusual; however, the fringing reefs surrounding American Samoa are located very close to shore, and it is likely that runoff from the island would transport surface soils and associated microorganisms onto the reefs. A closer examination of the distribution of the different bacterial genera also indicates that some overlap exists between the coral microbiota and the surrounding seawater. Whilst this phenomenon has been previously documented (Kooperman *et al.*, 2007), the variations in proportional abundance of different niches

Table 2. Cultured bacterial isolate 16S rRNA gene sequences from mucus samples of healthy and diseased *Acropora* corals

Isolate accession no.	ID	Nearest phylogenetic relative [accession number]	% Similarity (> 500 bp)
GU903197	AmSamW5	*Bacillus oshimensis* [EU977653]	98.8
GU903198	AmSamW6	*Pseudoalteromonas piscicida* [FJ457196]	99.8
GU903204	AmSamW11ii	*Brevundimonas* sp. [EU841506]	100.0
GU903211	AmSamW21	*Pseudoalteromonas piscicida* [FJ457196]	99.8
GU903214	AmSamW23	*Bacillus horikoshii* [AB043865]	100.0
GU903223	AmSamW35	*Bacillus horikoshii* [AB043865]	100.0
GU903225	AmSamW37	*Oceanobacillus picturae* [GQ903468]	99.5
GU903227	AmSamW39	*Bacillus oshimensis* [EU977653]	99.2
GU903232	AmSamW43i	*Alteromonas macleodii* [FJ161362]	99.8
GU903231	AmSamW43ii	*Vibrio harveyi* [FJ937878]	100.0
GU903241	AmSamW53	*Pseudoalteromonas mariniglutinosa* [AB257337]	100.0
GU903243	AmSamW55	*Pseudoalteromonas piscicida* [FJ457196]	99.8
GU903244	AmSamW56	*Vibrio* sp. [FJ457416]	99.5
GU903247	AmSamW60	*Vibrio aestuarianus* [FJ161294]	100.0
GU903249	AmSamW62	*Bacillus horikoshii* [AB043865]	100.0
GU903250	AmSamW63	*Bacillus* sp. [AB112008]	98.7
GU903258	AmSamW72	*Vibrio parahaemolyticus* [EU155529]	100.0
GU903259	AmSamW73	*Vibrio* sp. [EU697086]	98.7
GU903263	AmSamW78	*Kytococcus sedentarius* [CP001686]	99.8
GU903269	AmSamW86	*Bacillus horikoshii* [AB043865]	99.8
GU903192	AmSamHH1	*Staphylococcus saprolyticus* [DQ644501]	99.8
GU903193	AmSamHH2	*Micrococcus luteus* [FJ440954]	100.0
GU903194	AmSamHH3i	*Pseudomonas fluorescens* [AB266613]	100.0
GU903195	AmSamHH3ii	*Microbacterium paraoxydans* [FJ357595]	100.0
GU903196	AmSamHH4	*Pseudoalteromonas mariniglutinosa* [AB257337]	100.0
GU903201	AmSamHH9	*Staphylococcus saprolyticus* [GU197531]	100.0
GU903202	AmSamHH10i	*Pseudomonas fluorescens* [AB266613]	100.0
GU903203	AmSamHH10ii	*Microbacterium paraoxydans* [GU223117]	100.0
GU903206	AmSamHH14	*Staphylococcus saprolyticus* [GU097199]	100.0
GU903207	AmSamHH15	*Pseudoalteromonas* sp. [FJ457155]	100.0
GU903208	AmSamHH17	*Vibrio harveyi* [FJ937878]	100.0
GU903218	AmSamHH27	*Pseudoalteromonas* sp. [EU440054]	100.0
GU903219	AmSamHH29	*Vibrio harveyi* [AY332404]	99.9
GU903230	AmSamHH42	*Vibrio* sp. [EU372937]	99.5
GU903238	AmSamHH51i	*Salinicoccus hispanicus* [NR_025645]	98.6
GU903242	AmSamHH54	*Kytococcus sedentarius* [EU443746]	100.0
GU903251	AmSamHH65	*Photobacterium* sp. [FJ457450]	100.0
GU903252	AmSamHH66	*Micrococcus luteus* [CP001628]	98.6
GU903260	AmSamHH74	*Kytococcus sedentarius* [CP001686]	99.7
GU903266	AmSamHH83	*Pseudoalteromonas* sp. [FJ457155]	100.0
GU903199	AmSamHD7	*Bacillus oshimensis* [EU977653]	98.8
GU903200	AmSamHD8	*Pseudoalteromonas piscicida* [FJ457196]	99.8
GU903217	AmSamHD26	*Kocuria rhizophila* [EU554435]	99.8
GU903228	AmSamHD40	*Xanthomonas* sp. [DQ213024]	99.9
GU903234	AmSamHD46	*Vibrio* sp. [EU581714]	99.5
GU903239	AmSamHD52i	*Planococcus* sp. [AY538695]	96.6
GU903240	AmSamHD52ii	*Jeotgalicoccus halotolerans* [NR_025643]	100.0
GU903246	AmSamHD59	*Bacillus* sp. [AB112008]	98.7
GU903254	AmSamHD68	*Stenotrophomonas* sp. [AY259519]	100.0
GU903256	AmSamHD70	*Photobacterium damselae* [EF643517]	100.0
GU903265	AmSamHD81	*Bacillus* sp. [AB112008]	98.4
GU903267	AmSamHD84	*Vibrio* sp. [EU697086]	98.8
GU903205	AmSamDD12	*Psychrobacter submarinus* [NR_025457]	100.0
GU903209	AmSamDD19	*Brachybacterium paraconglomeratum* [EU660359]	99.8
GU903210	AmSamDD20	*Staphylococcus* sp. [FJ752530]	99.8
GU903213	AmSamDD22i	*Oceanobacillus picturae* [AB491184]	99.9

Table 2. Continued

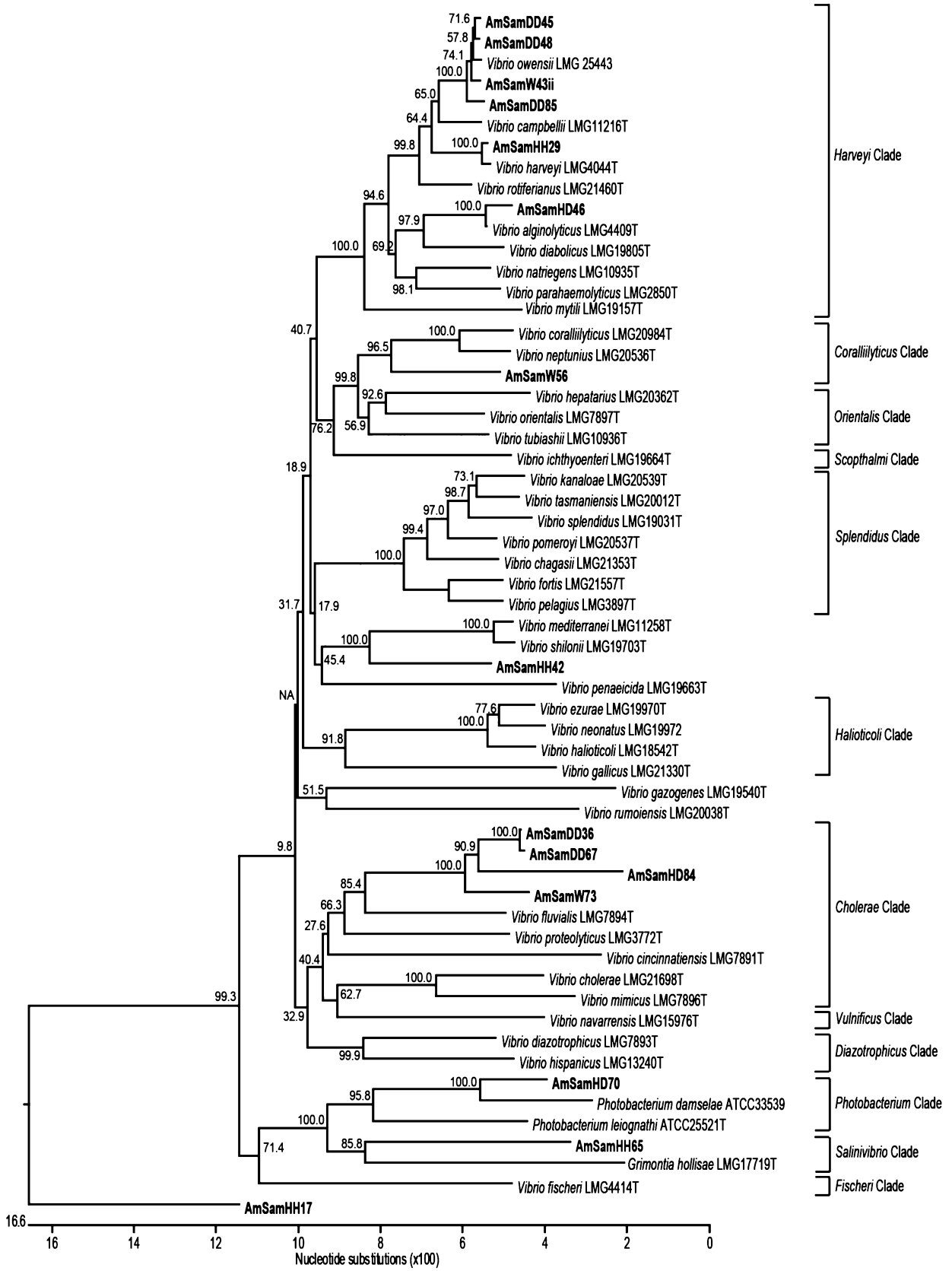
Isolate accession no.	ID	Nearest phylogenetic relative [accession number]	% Similarity (> 500 bp)
GU903212	AmSamDD22ii	*Nesterenkonia lacusekhoensis* [NR_028928]	99.1
GU903215	AmSamDD24	*Pseudoalteromonas* sp. [FJ457155]	100.0
GU903216	AmSamDD25	*Pseudoalteromonas piscicida* [FJ457196]	99.7
GU903220	AmSamDD31	*Exiguobacterium* sp. [GU339294]	99.8
GU903221	AmSamDD32	*Brevibacterium* sp. [EU873272]	99.1
GU903222	AmSamDD33	*Bacillus horikoshii* [AB043865]	100.0
GU903224	AmSamDD36	*Vibrio* sp. [EU697086]	100.0
GU903226	AmSamDD38	*Vibrio harveyi* [FJ161347]	97.9
GU903229	AmSamDD41	*Oceanobacillus picturae* [AB539828]	100.0
GU903233	AmSamDD45	*Vibrio campbellii* [FM204856]	100.0
GU903235	AmSamDD47	*Nesterenkonia* sp. [GQ280020]	99.8
GU903236	AmSamDD48	*Vibrio campbellii* [FM204856]	100.0
GU903237	AmSamDD49	*Brevibacterium* sp. [EU873272]	99.3
GU903245	AmSamDD57	*Thalassobius* sp. [FJ403051]	99.8
GU903248	AmSamDD61	*Nesterenkonia sandarakina* [GU112980]	99.6
GU903253	AmSamDD67	*Vibrio* sp. [EU022568]	99.3
GU903255	AmSamDD69	*Brachybacterium* sp. [GU064364]	96.8
GU903257	AmSamDD71	*Bacillus* sp. [FJ763975]	99.7
GU903261	AmSamDD75	*Staphylococcus warneri* [GU397393]	99.9
GU903262	AmSamDD76	*Nesterenkonia sandarakina* [GU112980]	100.0
GU903264	AmSamDD80	*Bacillus* sp. [AB112008]	98.5
GU903268	AmSamDD85	*Vibrio harveyi* [GQ487488]	99.8

The sequences in the table have been grouped by sample type, the water column immediately above corals (ID AmSamWX), coral tissue from healthy colonies with no disease lesion (ID AmSamHHX), coral tissue from healthy fragment on diseased colonies (AmSamHDX) and coral tissue from lesion area of diseased colonies (AmSamDDX).

by different groups and genera confirm previous findings (Ritchie, 2006) that the coral host demonstrates a strong selection pressure on its associated microbes.

The pathogenicity of *Vibrio* spp. in marine environments is well documented (Thompson *et al.*, 2004; Austin *et al.*, 2005), but more specifically, they have previously been implicated in coral disease (Banin *et al.*, 2000; Ben-Haim *et al.*, 2003; Cervino *et al.*, 2008; Sussman *et al.*, 2008; Luna *et al.*, 2010). Accordingly, TCBS selective medium was used in the hope of isolating a possible causative agent of AWS. The *Vibrionaceae* are difficult to classify to species level using 16S rRNA gene sequences alone (Thompson *et al.*, 2005), so 15 isolates affiliated to *Vibrio* spp. using 16S rDNA gene data (Table 2) were further characterized by sequence analysis of several housekeeping genes (*recA*, *rpoA* and *pyrH*). The combination of several loci used in the MLST approach led to a more robust identification of *Vibrio* spp.; four isolates (AmSamW43ii, AmSamDD45, AmSamDD48 and AmSamDD85) that were classified as either *Vibrio rotiferianus*, *Vibrio campbellii* or *V. harveyi* (using individual gene markers) were conclusively grouped (97–99% sequence identity) as members of a novel *Vibrio* species, *V. owensii* (Cano-Gómez *et al.*, 2010) using the MLST approach (Fig. 3). *Vibrio owensii* was initially isolated from cultured lobsters (Cano-Gómez *et al.*, 2010), and so this is the first record of this organism being associated with lesions in corals. Luna *et al.* (2010) found *V. harveyi* associated with WS in *Pocillopora damicornis* from the Indian Ocean; in our study, an isolate closely related to *V. harveyi* was recovered only from the water column. The coral pathogen *Vibrio coralliilyticus* was not recovered from any of the coral samples, despite being previously identified as a causative agent of WS in *Acropora* corals in the Indo-Pacific (Sussman *et al.*, 2008). However, a single isolate, AmSamW56, did cluster with members of the *Coralliilyticus* clade (Fig. 3), but a diagnostic test for the *V. coralliilyticus* zinc-metalloprotease gene *VcpA* (B. Wilson *et al.*, unpublished data) was negative (data not shown). *Vibrio* spp. (AmSamHH17, AmSamHH29 and AmSamHH42) were also associated with

Fig. 3. Phylogenetic tree (using the neighbour-joining method) of culturable *Vibrionaceae* isolated from the water column immediately above corals (W); coral fragments taken from colonies that showed no signs of disease (HH); coral fragments taken from an area of apparently healthy tissue on the diseased colony (HD); and coral fragments taken from an area of diseased tissue on the diseased colony (DD). *Camplylobacter jejuni* was included as an outgroup. Figures at nodes indicate bootstrap values for 1000 bootstrap repetitions.



healthy corals, so the role of *Vibrionaceae* in coral disease remains unclear. The *Vibrio* species are ubiquitous marine organisms, some of which are pathogenic with infection manifested typically as necrosis secondary to production of extracellular proteases in a wide range of organisms (Austin *et al.*, 2005).

In conclusion, whilst there was no clear candidate(s) responsible for causing AWS apparent from either the culturable isolates or clone library data, there were distinct differences between the microbial communities of healthy and diseased corals. The bacteria associated with healthy tissues on diseased colonies were more similar to diseased tissues on the same colony than to tissues from healthy colonies. Shifts in microbial diversity are indicative of an unhealthy coral holobiont, and further studies that map the disease progression from initial onset are required. Future investigative efforts must therefore take into account the multifactorial nature of coral aetiology studies and combine a number of molecular, physiological and histopathological tools when assessing coral health, especially regarding those corals that may have already progressed into a diseased state prior to the appearance of any visible signs.

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