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Humpback whales harbour a combination of specific and variable skin bacteria

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Summary

Investigations of marine mammal skin-associated microbiota are limited to cultivation-based studies of lesioned individuals, resulting in a lack of understanding about the composition of 'normal' skin-associated microbial communities, their variation among individuals, and whether or not the microbial communities change with host health or environmental exposures. In this study, bacterial communities associated with the skin of 19 North Pacific humpback whales (*Megaptera novaeangliae*), including skin from three health-compromised individuals, were examined using small subunit ribosomal RNA gene-based culture-independent techniques. These analyses revealed that the skin-associated bacteria were significantly different from free-living bacterial communities in the surrounding seawater. Two novel groups within the *Flavobacteriaceae* family of the *Bacteroidetes* phylum were found to be associated with multiple whales, including a species within the *Tenacibaculum* genus that associated with 95% of the individuals. Statistical analyses revealed that a group of eight 'healthy' whales harboured similar microbial communities, while the health-compromised and other 'healthy' animals harboured communities that were unique to the specific animal. These results describe two components of the whale skin bacterial community: a specific and potentially co-evolved fraction, and a more variable microbial community fraction that may offer a diagnostic-type tool for investigating the health and life-related events of these endangered animals.

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Introduction

Microorganisms are abundantly associated with mammals, and recent studies have demonstrated that the composition of these communities can be related to the health and ecology of the host (Ley *et al.*, 2008; Costello *et al.*, 2009; Grice *et al.*, 2009; Hartman *et al.*, 2009; Larsen *et al.*, 2010). Marine mammals are uniquely exposed to a diverse and dynamic suite of free-living marine bacteria, and their epithelial skin layer, their largest organ, is in constant contact with these microorganisms. Observations of skin lesions are frequently reported for marine mammals, and their occurrence has been linked to a variety of microorganisms including *Achromobacter* sp., *Actinobacteria* sp., *Escherichia coli*, *Pseudomonas* sp., *Staphlococcus* sp., *Streptococcus* sp. and *Vibrio* sp. (Varaldo *et al.*, 1988; Buck *et al.*, 1991; Moore *et al.*, 2003; Hamilton and Marx, 2005; Bearzi *et al.*, 2009). However, these studies all utilized cultivation-dependent techniques, which typically exclude the dominant members of many communities (Amann *et al.*, 1995; Rappé and Giovannoni, 2003), and are without comparison to non-lesioned skin. Examining the role of microorganisms in marine mammal skin lesions requires a broader understanding of the epithelial microbial community from individuals that appear healthy. Additionally, utilization of molecular microbial ecology techniques that reduce the biases inherent to cultivation-based methodology will heighten our understanding of marine mammal skin-associated bacterial communities.

It is not known if microorganisms are transient colonizers of the skin surface of marine mammals, or if they are adapted to the specific characteristics of this environment, such as frequent skin sloughing, host defences, exposure to UV radiation, rapid pressure changes and frequent contact with the aerobic environment. The skin microbiota of migratory marine mammals is of particular interest because it is exposed to a variety of oceanic habitats that can harbour different bacterioplankton communities (Rusch *et al.*, 2007). Humpback whales (*Megaptera*

novaeanglia) undergo the longest documented migration of any mammal, travelling across ocean biomes from high-latitude feeding grounds to lower latitudes for breeding and calving (Stone *et al*., 1990; Clapham, 1996). The population structure of humpbacks has been investigated (Baker *et al*., 1990; 1993), but no known studies have examined their skin microbiology.

This study focuses on bacterial communities associated with the skin of North Pacific humpback whales (*Megaptera novaeangliae*) present near the Hawaiian Islands, their major breeding grounds (Calambokidis *et al*., 2001). The animals sampled in this study underwent migration routes to Hawaiian waters, but varied in sex, age and exposure to stressors. This study utilized cultivation-independent, small-subunit ribosomal RNA (SSU rRNA) gene-based molecular techniques in order to describe and compare the bacterial communities associated with normal (i.e. 'healthy') and health-compromised humpbacks, as well as communities inhabiting the surrounding seawater.

Results and discussion

Description of samples

Skin (1.7–68.8 mg) was sampled from humpback whales in the vicinity of the Hawaiian Islands using biopsy darts (Palsbøll *et al*., 1991) targeting the dorsal region of individuals, or from sloughed skin recovered from acoustic tags or skimmed from the water following breaching (Table 1). Skin was also recovered from three health-compromised animals, and lice from one of the individuals. The health-compromised animals included a female calf, estimated to be several weeks old, that stranded ashore at Puamana, Maui on 25 February 2008 and died soon after reaching shore. Within an hour of the death, biopsy samples were taken from below the head, dorsal portion of the head and peduncle area, although the body location was not noted for the individual samples. An adult male entangled in over 45 m of nylon line (entangled whale #1_ENTI) and heavily colonized by cyamid amphipods (whale lice) was found off the island of Maui on 2

Table 1. Description of humpback whale skin, lice and seawater samples and method of analysis.

Sample name	Sample type	Whale health	Age	Sex[a]	Latitude	Longitude	Analysis[b]
WH44	Skin biopsy	Normal	Adult	M	21°33.245	158°16.404	T-RFLP
WH45 (a,b)[c]	Skin biopsy	Normal	Adult	U	21°22.450	158°09.366	T-RFLP
OSW1[d]	Seawater				21°25.640	158°12.740	T-RFLP
WH46	Skin biopsy	Normal	Adult	U	21°35.763	158°17.699	T-RFLP
WH47	Skin biopsy	Normal	Adult	M	21°35.791	158°17.629	T-RFLP
WH48	Skin biopsy	Normal	Adult	F	21°35.791	158°17.629	T-RFLP
WH49 (a,b)	Skin biopsy	Normal	Adult	M	21°35.630	158°18.121	T-RFLP
WH50	Skin biopsy	Normal	Adult	M	21°35.636	158°18.003	T-RFLP
WH51	Skin biopsy	Normal	Adult	M	21°35.636	158°15.847	T-RFLP, clone library
OSW2[d]	Seawater				21°35.970	158°16.660	T-RFLP
CALF	Skin biopsy	Normal	Calf	U	21°34.792	158°19.200	T-RFLP, clone library
WH53	Skin biopsy	Normal	Adult	F	21°33.306	158°17.422	T-RFLP
OSW3[d]	Seawater				21°33.000	158°16.140	T-RFLP
OSW4[d]	Seawater				21°34.172	158°17.350	T-RFLP
WH54	Skin biopsy	Normal	Adult	M	21°35.710	158°15.754	T-RFLP
OSW5[d]	Seawater				21°34.180	158°17.870	T-RFLP
WH55	Skin biopsy	Normal	Adult	F	21°36.200	158°08.800	T-RFLP
OSW6[d]	Seawater				21°36.200	158°08.800	T-RFLP
WH56	Skin biopsy	Normal	Adult	M	21°37.946	158°12.565	T-RFLP
WH44M	Skin from tag	Normal	Adult	M	20°49.027	156°41.990	T-RFLP
WH57	Skin from tag	Normal	Adult	M	20°46.719	156°47.997	T-RFLP
WH57BR (a,b)	Skin from breach	Normal	Adult	M	20°46.719	156°47.997	T-RFLP
MSW1[d]	Seawater				20°46.719	156°47.997	T-RFLP
WH61 (a,b)	Skin from tag	Normal	Adult	M	20°45.409	156°39.037	T-RFLP
MSW2[d]	Seawater				20°44.769	156°39.767	T-RFLP
ENT1 (a,b)	Skin from line	Compromised	Adult	M	20°49.249	156°44.505	T-RFLP, clone library
LICE (a,b)	Lice from ENT1	Compromised			20°49.249	156°44.505	T-RFLP
MSW3[d]	Seawater				20°45.409	156°39.030	T-RFLP
ENT2	Skin biopsy	Compromised	Adult	U	20°45.300	156°27.600	T-RFLP
H-C CALF (a,b,c)	Skin biopsy	Compromised	Calf	F	20°85.588	156°66.474	T-RFLP

a. M, male; F, female; U, unknown.

b. Prior to nucleic acid extraction, 300 μl of DNA lysis buffer was added to the thawed humpback whale skin and lice samples. DNA was extracted from all samples using a modified version of the DNeasy Tissue Kit (Qiagen, Valencia, CA) (Becker *et al*., 2007) and quantified using the PicoGreen fluorescent assay (Invitrogen Corp., Carlsbad, CA) on a SpectraMax M2 plate reader (Molecular Devices Corp., Sunnyvale, CA).

c. Letters indicate replicate samples.

d. Seawater collected following acquisition of previously listed skin sample. The seawater samples were filtered onto 0.2 μm polyethersulfone membranes (Supor-200; Pall Corp., East Hills, NY) using a 60 ml hand-held syringe, stored in 300 μl of DNA lysis buffer [20 mM Tris HCl (pH 8.0), 2 mM EDTA (pH 8.0), 1.2% (v/v) Triton X-100 and 20 μg ml^{-1} lysozyme], and kept on ice for no more than 24 h before freezing to –80°C.

March 2007. The animal was completely freed of all gear, and skin and lice samples were obtained from the disentangled gear. The recovered gear was recognized as king crab potting gear last identified in the Bering Sea during 15–29 January 2007, and the animal was estimated to have migrated 4300 km to Hawaiian waters trailing the gear. Entangled whale #2 (ENT2), another adult whale, was reported off the island of Maui on 10 February 2008. Efforts to remove the heavy gauge line were unsuccessful, and a biopsy sample was obtained. All skin and lice samples were kept on ice for no more than 24 h before freezing to −80°C.

Small volume (220–240 ml) surface seawater (< 0.5 m) samples were collected following capture of selected whale skin samples and after the boat was moved from the immediate area in which the animal or pod had surfaced (Table 1).

Whales harbour distinct bacterial communities compared with seawater

Bacterial SSU rRNA genes associated with whale skin, whale lice and seawater were examined using terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu *et al*., 1997). The average number of T-RFs associated with healthy adult whale skin was 38 (± 21 standard deviation; $n = 19$). This value was lower than the surrounding seawater samples, which harboured 82 T-RFs (± 10 standard deviation; $n = 9$). A non-metric multi-dimensional scaling comparison of the T-RFLP profiles demonstrated that bacterial communities associated with seawater clustered together, and separately from those associated with whale skin (Fig. 1). A multi-response permutation procedure test comparing the seawater and whale skin T-RFLP profiles found the communities to be significantly distinct ($P < 0.001$, $A = 0.28$), regardless of whether the whale sample originated from sloughed skin or was acquired by biopsy. Thus, despite the immediate contact with seawater, humpback whales appear to harbour specialized bacterial communities that are both less diverse and phylogenetically distinct compared with seawater. Other marine animals including fish, squid and corals have similarly been shown to harbour specialized bacterial communities that differ from those in the surrounding water (Cahill, 1990; Barbieri *et al*., 2001; Rohwer *et al*., 2001). The humpback skin-associated bacteria are most likely specialized to the substrates on and within the epithelium.

Whales harbour both specific and variable bacterial community members

T-RFLP comparisons revealed similarity between epithelium-associated bacterial communities within a core group of eight apparently healthy animals ('healthy humpback core group', Fig. 1), despite differences in sex and the immediate geographic location from where the individuals were sampled. The remaining skin samples from apparently healthy individuals were more variable in bacterial community composition and did not cluster with this group; however, seven T-RFs from the 'healthy humpback core group' were present in > 50% of the T-RFLP profiles from the other whales. In particular, the T-RF at 406 bp was the most widespread among samples: it was present in all but one of the whale skin samples, and was the dominant T-RF associated with the healthy calf. Additionally, this T-RF was associated with all three health-compromised whales, indicating that some bacterial community members remain associated with humpback skin in spite of exposure to stressors. The only other cultivation-independent survey of marine mammal-associated bacteria that we are aware of examined the upper respiratory tract of bottlenose dolphins (Johnson *et al*., 2009), and also revealed the presence of host-specific bacterial communities.

The skin samples clustering outside of the 'healthy humpback core group' harboured more variable bacterial communities (Fig. 1). The body location where samples originate from is a plausible source of variability so, to investigate this further, bacterial communities from replicate skin samples obtained from the same animal were compared using the Sorenson (Bray-Curtis) distance coefficient (Table 2). Replicate samples obtained from breachings, acoustic tags and biopsies from the stranded calf (obtained from unidentified locations on the animal) were the most variable, while biopsy samples obtained from near the dorsal fin (WH45 and WH49) or sloughed skin from the entangled animal exhibited lower variability in bacterial communities (Table 2).

While skin sample location may be one source of variability in the T-RFLP results, a number of samples obtained from dorsal biopsies clustered outside of the 'healthy humpback core group'. Bacterial communities

Table 2. Comparison of replicate whale skin specimens using Sorenson (Bray-Curtis) distance coefficient.

Sample name	Source of sample	Sorenson distance coefficient[a]
WH45 (a vs. b)	Dorsal biopsy	0.410
WH49 (a vs. b)	Dorsal biopsy	0.442
WH61 (a vs. b)	Acoustic tag	0.708
WH57BR (a vs. b)	Skimmed from water after breaching	0.639
ENT1 (a vs. b)	Fishing line	0.224
H-C CALF (a vs. b)	Biopsy	0.667
H-C CALF (a vs. c)	Biopsy	0.500
H-C CALF (b vs. c)	Biopsy	0.367

a. Distance measure was calculated from T-RFLP profiles using PC-ORD software.

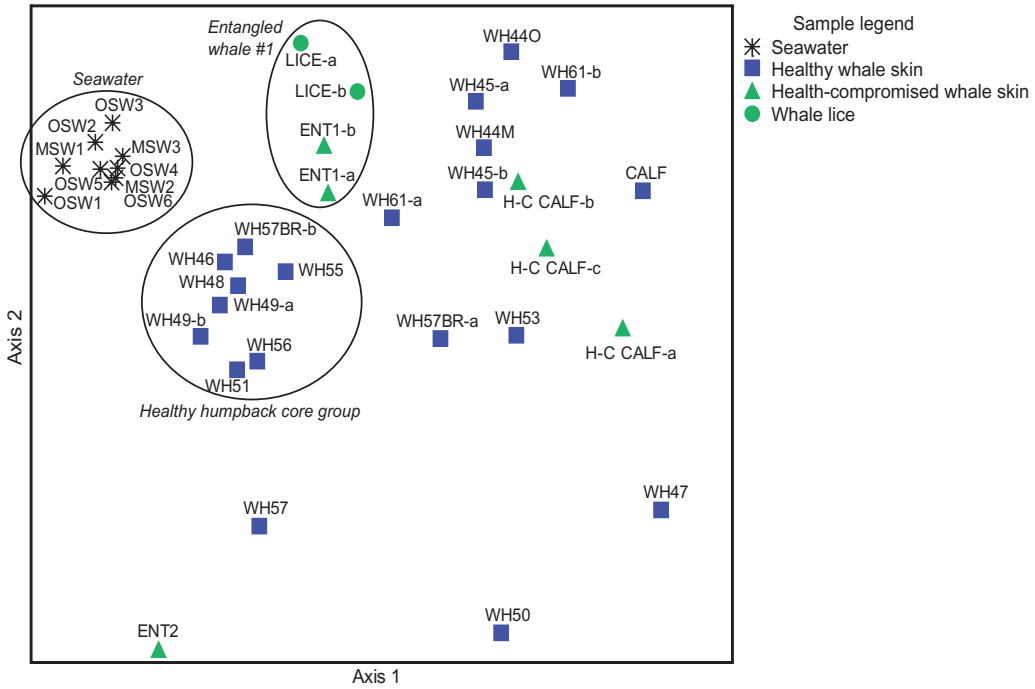

Fig. 1. Comparison of bacterial community T-RFLP profiles associated with the different humpback whale skin, lice and seawater samples via non-metric multidimensional scaling. The stress of the three-dimensional solution is 0.176. The abbreviations correspond to names in Table 1. Replicate samples are denoted with lower case letters a, b or c, and the circled groups were designated post-analysis to highlight the clustering of these samples. For T-RFLP analysis, bacterial SSU rRNA genes were first amplified via PCR using oligonucleotide primers 27F-B-FAM (5′-AGRGTTYGATYMTGGCTCAG-3′) and 519R (5′-GWATTACCGCGGCKGCTG-3′), with 'FAM' indicating 5′ end-labelling with the FAM fluorochrome. Each 50 μl PCR reaction consisted of 2 U of Sahara enzyme (Bioline USA, Taunton, MA), 1× Sahara reaction buffer, 2 mM Sahara $MgCl_2$, 200 μM of each dNTPs, 200 nM of each primer and 10 ng of genomic template (100 ng of template was used for several samples which did not amplify at a lower concentration). The reactions were preformed in a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA) with an initial denaturation step at 95°C for 3 min, followed by 30 cycles of a touchdown protocol consisting of 95°C denaturation for 30 s, 65–50°C annealing for 1 min (decreasing by 0.5°C each cycle), and 72°C extension for 2 min, with a final extension step at 72°C for 20 min. Samples not amplifying under these conditions were subjected to two to five cycles at an annealing temperature of 50°C. Amplification products were purified using the microcentrifuge protocol of the QIAquick PCR Purification Kit (Qiagen, Valencia, CA), and subsequently restricted in a 10 μl reaction containing 100 ng of purified PCR product, 2 μg of bovine serum albumin (BSA), 1× enzymatic reaction buffer, and 5 units of HaeIII restriction endonuclease (Promega, Madison, WI) for 7 h at 37°C. Restriction digests were purified using the microcentrifuge protocol of the QIAquick Nucleotide Removal Kit (Qiagen) and 30 ng μl^{-1} of each product was subsequently electrophoresed on an ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, CA). Operational taxonomic units (OTUs) were identified as peaks for fragment positions 34 through 550. To account for small differences in the amount of DNA loaded on the ABI 3100, the data set was normalized to include peaks representing greater than 0.05% of the total profile area. Each position was then scored for presence or absence of an OTU, producing a binary sequence for each sample. The matrix was analysed using non-metric multidimensional scaling (NMS) analysis with the Sorensen (Bray-Curtis) distance measure using slow and thorough autopilot mode using PC-ORD software (MjM Software Design, Gleneden Beach, OR).

associated with the palms of humans were recently shown to contain dominant and shared bacterial phylotypes, but also a great deal of interpersonal variation which could be attributed to individual habits (Fierer *et al.*, 2008). Whales falling outside of the 'healthy humpback core group' may similarly be characterized by differing habits, exposure to stressors, reproductive history or other life-related events that have affected their skin bacterial community. The health-compromised whales are an interesting example of the potential impact of stress-related events on skin microbiota. The health-compromised whales all experienced different life-related events (one adult migrated 4300 km entangled in gear, another adult was entangled for an unknown duration, and the calf was stranded on the beach), and each animal harboured a unique bacterial T-RFLP profile. In particular, the T-RFLP profiles from the two entangled whales (ENT1 and ENT2) did not overlap closely with profiles from the other whales, but the stranded calf samples (HC-CALF) clustered more closely to the 'healthy' whales (Fig. 1). The ENT1 and ENT2 whales were entangled in line for a long enough duration to be characterized as severely health-compromised, and an altered skin bacterial community may have developed during this time. However, the HC-CALF may have died due to drowning or starvation or other common birth or newborn-related factors. These factors typically occur over a short time frame, and an altered bacterial community may not be detectable during

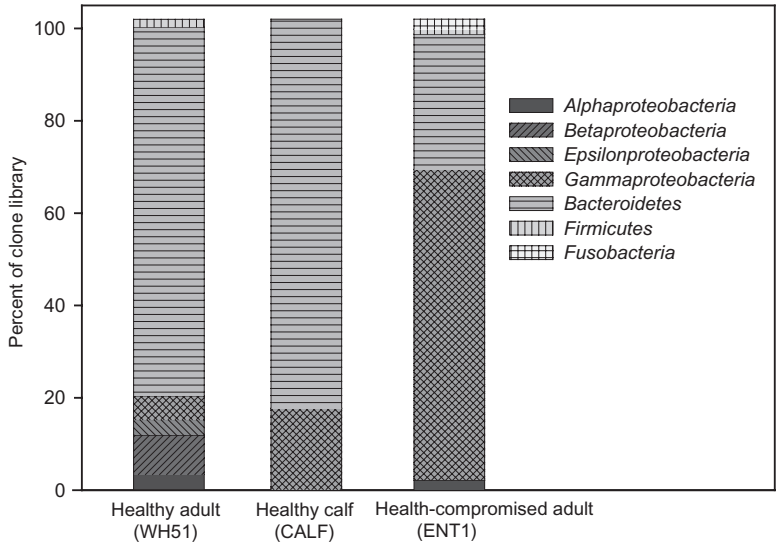

Fig. 2. Distribution of groups of skin bacteria identified in SSU rRNA gene libraries from a healthy adult (WH51, 60 clones), healthy calf (CALF, 23 clones) and health-compromised, entangled adult humpback whale (ENT1, 96 clones). The SSU rRNA gene was amplified from the genomic DNA samples using the 27F-B and 1492R (Lane, 1991) primers in a 20 μl reaction of the same reagent concentrations and reaction conditions as detailed for T-RFLP. To reduce the formation of heteroduplexes, a reconditioning PCR step was performed (Thompson *et al*., 2002). For reconditioning PCR, duplicate 50 μl reactions were prepared containing 100 ng of amplified DNA as template and the same concentration of reagents as for T-RFLP, and cycled with an initial denaturation step at 95°C for 5 min followed by two rounds of 95°C denaturation for 30 s, 55°C annealing for 1 min, and 72°C extension for 2 min, and concluding with a cycle containing an extension step of 20 min. The duplicate reactions were combined and purified as described for the T-RFLP PCR amplification products, and cloned using the pGem-T Easy system (Promega, Madison, WI). Clones were PCR amplified using primers targeting the M13 forward and reverse priming sites with conditions previously described (Vergin *et al*., 2001) and sequenced with the forward primer on an ABI 3730XL capillary-based DNA sequencer (Applied Biosystems). The resulting SSU rDNA clone sequences were checked for the presence of chimeras using the Chimera Detection program of the Ribosomal Database Project II (Cole *et al*., 2005), and by manual inspection of aligned sequences. Sequence alignments were constructed using the SILVA INcremental Aligner (SINA) (Pruesse *et al*., 2007) and then imported into the 'All-Species Living Tree' project SSU rRNA gene release 95 database (Yarza *et al*., 2008) using the ARB version 09.07.14prv software (Ludwig *et al*., 2004). Alignments of the clone sequences were refined manually by including nearest neighbours and taking into account the secondary structure information of the rRNA gene. Clone sequences were identified to the family level and closest recognized species or lineage, and classifications were verified using the Ribosomal Database Project classifier (Wang *et al*., 2007).

this period. Thus, in addition to location on the animal, time frame and health condition may also play a role in shaping the composition of bacterial communities associated with humpback whale skin.

Epibionts including lice and barnacles frequently reside on humpback whales, and entangled whale #1 was characterized by a heavy lice infestation. The lice were found to possess a bacterial community similar to that residing on the whale skin (Fig. 1). Although this is the first microbial examination of whale lice, the similarity between the two communities is not necessarily surprising because lice feed on skin (Roundtree, 1983; 1996). Lice are observed in high densities primarily on health-compromised humpbacks (Osmond and Kaufman, 1998), and may pose a threat as vector for the transmission of bacteria, including pathogens, within humpback whale populations.

In order to identify members of the bacterial communities associated with representative whale skin samples and correlate phylogenetic identities with particular T-RFs arising from the T-RFLP data set, SSU rRNA genes were polymerase chain reaction (PCR) amplified, cloned, and sequenced from a healthy adult (WH51; 60 clones), healthy calf (CALF; 23 clones), and a health-compromised adult (ENT1, replicate a; 94 clones). A health-compromised calf sample was not included in this analysis because the anatomical origin of the biopsy samples was not recorded. All sequences reported in this study have been deposited in GenBank under Accession Nos GU201952–GU202013. Phylogenetic analyses of the whale skin-associated bacteria revealed that the healthy adult and calf SSU rRNA gene clone libraries were predominantly (78–82% of library) composed of the bacterial phylum *Bacteroidetes* (Fig. 2, Tables S1 and S2). Members of the *Bacteroidetes* are also consistently associated with the human skin, the most characterized type of mammalian skin. Previous studies have shown that *Bacteroidetes* generally comprise a small (< 5%) percentage of the total bacterial community found on human skin (Fierer *et al*., 2008; Gao *et al*., 2008), which do not appear to be closely related to those found on humpbacks (< 80% SSU rRNA gene sequence identity). *Bacteroidetes* are also one of the dominant phyla of bacteria associated with whale falls (carcasses of large

whales on the seafloor) (Tringe *et al*., 2005), although are also distantly related to those found on living humpbacks (< 91% SSU rRNA gene sequence identity).

In contrast to the healthy animals, the skin from one of the health-compromised adults (ENT1) was comprised of ~28% of members of the *Bacteroidetes* phylum (Fig. 2). The skin from this whale contained a high percentage of diverse *Gammaproteobacteria* (64%), including *Pasteruella skyensis*, a bacterium previously found associated with diseased salmon (Birkbeck *et al*., 2002) (Fig. 2, Table S3). Compared with its healthy counterparts, this health-compromised whale appeared to harbour a phylogenetically unique skin bacterial composition. It is possible that behavioural alterations during the 4300 km migration of this entangled whale may have altered the skin-associated bacteria from a *Bacteroidetes*- to *Gammaproteobacteria*-dominated community. Examining skin bacteria prior to and following life-altering events such as entanglements or during the development of skin lesions or other disease symptoms is necessary to better understand the influence of life events or stressors on skin bacterial communities. Additionally, deeper sequencing of bacterial SSU rRNA genes associated with an increased number of skin samples will provide further insight into the quantity of rare and potentially unique bacteria.

No overlap was observed between the bacteria found on humpback whale skin and those previously cultivated from skin lesions from diverse marine mammals (Varaldo *et al*., 1988; Buck *et al*., 1991; Moore *et al*., 2003; Hamilton and Marx, 2005; Bearzi *et al*., 2009). This finding is probably a reflection of the differences between lesioned and non-lesioned skin, differences between marine mammal species, and the circumvention of cultivation biases afforded by the characterization of bacterial communities via cultivation-independent techniques.

Humpback-specific Flavobacteriaceae

Phylogenetic analyses of the *Flavobacteriaceae* family of the *Bacteroidetes* phylum revealed a novel *Flavobacteria* lineage ('humpback whale-associated group 1') consisting of clone sequences from both the healthy adult and calf samples (Fig. 3). The healthy adult clone library possessed the highest proportion of this lineage (65% of clones). Unfortunately, this novel lineage does not produce a T-RF fragment length that is detectable by the T-RFLP protocol employed in this study because it does not possess a HaeIII restriction site within the first 550 base pairs on the 5′ end of the molecule. Therefore, the presence of the humpback whale-associated group 1 could not be further assessed within the sample pool without additional analysis. Future studies of humpback whale associated bacteria should be designed to include this group.

The SSU rRNA gene clone libraries from the healthy adult, healthy calf and health-compromised adult all contained clones belonging to a second previously undescribed lineage, 'humpback whale-associated group 2', which was most closely related to the *Tenacibaculum* genus of the *Flavobacteriaceae* family of *Bacteroidetes* (Fig. 3). Representative clones from this group were subjected to T-RFLP analysis (as described in Fig. 1) in order to precisely determine their presence and abundance in the environmental sample T-RFLP profiles. This lineage was found to correspond to the T-RF at 406 bp that was present in all but one of the whale skin samples (95% of the samples). Despite the limited number of studies on marine mammal-associated bacteria, bacteria of the genus *Tenacibaculum* were previously found associated with the upper respiratory tract of bottlenose dolphins (Johnson *et al*., 2009) (Fig. 3). The dolphin-associated clone sequences were < 95% similar to those originating from humpback skin, and therefore appear to represent distinct microorganisms. Bacteria within the genus *Tenacibaculum* have been previously isolated from marine habitats (Frette *et al*., 2004; Yoon *et al*., 2005; Choi *et al*., 2006; Jung *et al*., 2006; Banning *et al*., 2010), and a variety of marine animals (Heindl *et al*., 2008; Wang *et al*., 2008) including diseased fish (Pineiro-Vidal *et al*., 2008a,b). Several of the *Tenacibaculum* spp. isolates are predators of other bacteria (Banning *et al*., 2010), and thus it is possible that *Tenacibaculum* spp. on humpbacks play a role in limiting bacterial growth on the skin. However, it can be difficult to assess the potential role of undescribed bacteria based on phylogenetic similarities (e.g. Rocap *et al*., 2003). Thus, additional research is necessary to assess the impact of this unique group of marine bacteria on the health and ecology of humpback whales and other marine mammals.

The prominent association between bacteria belonging to the *Tenacibaculum* lineage and the humpback calf indicates that specific humpback skin microbial communities may be established early during development. Since these bacteria have not been previously detected in seawater despite considerable efforts to characterize marine bacterioplankton communities (Giovannoni and Rappé, 2000; Treusch *et al*., 2009), it is most probable that the bacteria are acquired from other humpback whales. These communities could be transmitted in uterus during development of the foetus, or from post-birth activities such as skin contact during nursing or close contact swimming.

Conclusions

This study describes two components to the humpback whale skin bacterial community. The first consists of the

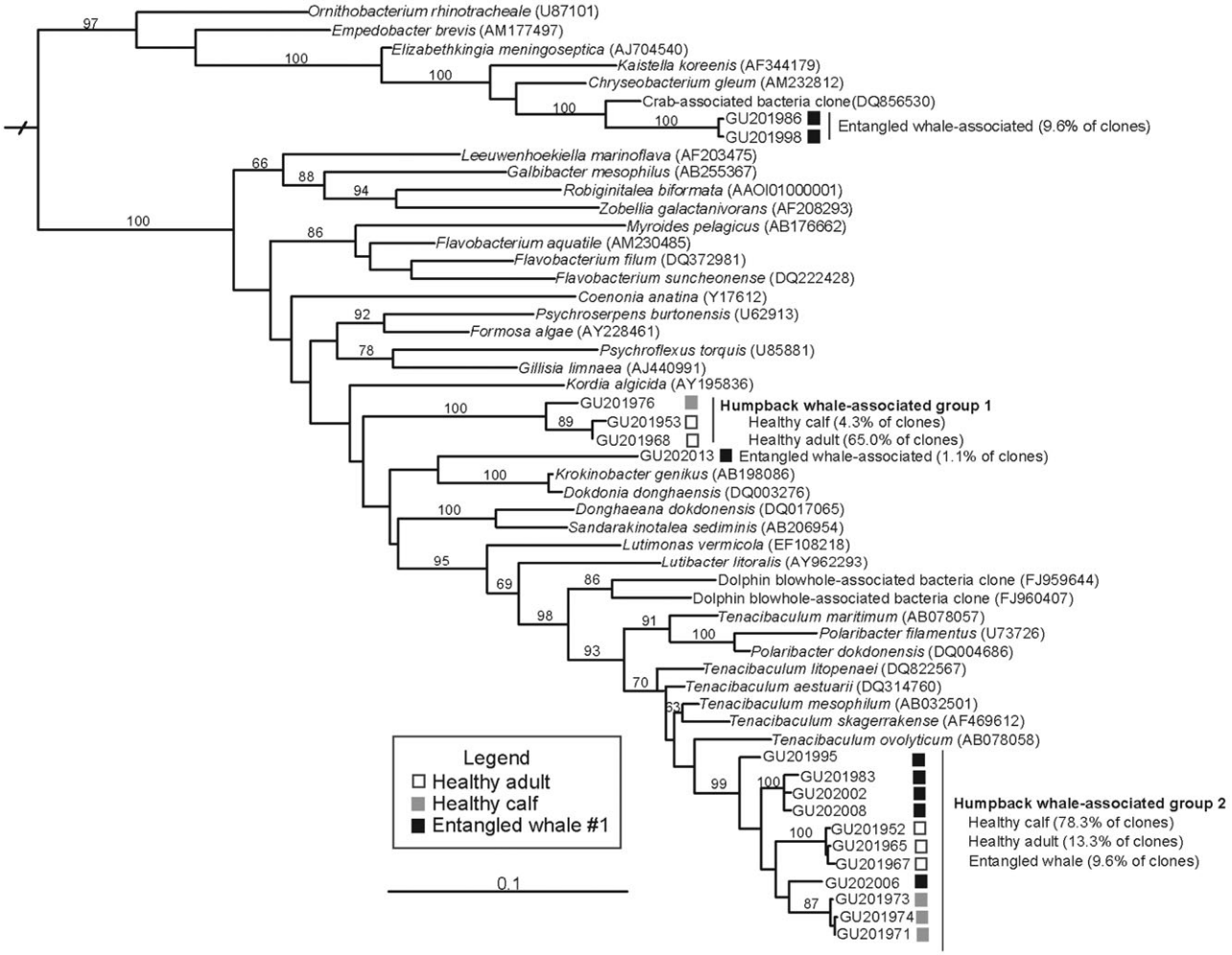

Fig. 3. Phylogenetic relationships between bacterial SSU rRNA gene clones obtained from skin from a healthy adult humpback (WH51), a healthy adult calf (CALF) and a health-compromised, entangled adult (ENT1) and reference sequences of recognized species from the *Flavobacteriaceae* family identified from the 'All-Species Living Tree' project SSU rRNA gene database, and additional reference sequences obtained from environmental gene clones from the SILVA SSURef 95 database and GenBank (Altschul *et al*., 1997). The scale bar corresponds to 0.10 substitutions per nucleotide position. Only bootstrap values greater than 60% are listed. To construct the phylogeny, a filter was applied to the sequences using the 'filter by base frequency' tool in ARB, and the filtered alignment used to construct a phylogenetic tree using the RAxML-HPC BlackBox version 7.2.6 maximum likelihood method with 1000 bootstrappings (Stamatakis, 2006) in the Cipres Portal version 2.1 (http://www.phylo.org). The tree was rooted and displayed using the Interactive Tree of Life software (Letunic and Bork, 2006). The outgroup includes the *Bacteroidetes* species *Algoriphagus ratkowskyi* (AJ608641), *Flexibacter roseolus* (AB078062), *Flexithrix dorotheae* (AB078077), *Sphingobacterium spiritivorum* (EF090267) and *Chitinophaga pinensis* (AF078775).

novel *Tenacibaculum* group ('humpback-associated group 2') that appears to be prevalent in the North Pacific population of humpback whales. Further analysis will reveal if the 'humpback-associated group 1' *Flavobacteriaceae* are also widespread among this population. Additionally, it is possible that these bacterial groups are broadly associated with the *M. novaeangliae* species as a whole. Analysis of bacterial communities associated with skin from geographically distinct populations of humpbacks, including those inhabiting the South Pacific, North Atlantic, South Atlantic and Indian Ocean, will provide insight into whether these novel bacterial groups may have co-evolved with the species.

The second component to the humpback whale skin bacterial community is the variable fraction. Unique T-RFs were frequently present in the skin-associated bacterial profiles, and the presence of these bacterial community members may be related to specific exposures or health-related factors. For example, the palms of humans were recently shown to harbour distinct bacterial communities that were proposed as a forensic identification tool to match objects to the handler (Fierer *et al*., 2010). It is possible that humpback whale skin bacterial communities may similarly be a useful diagnostic-type tool to learn about their habits, exposures and possibly even health state. For example, comparisons between the data pre-

sented here and skin bacterial communities associated with North Pacific humpback whales residing in their Bering Sea feeding grounds would be valuable for investigating the effects of oceanic environment on skin microbiota. Additionally, increased research on health-compromised whales, especially those possessing skin lesions, will help in evaluating whether skin microbiota can serve as a useful diagnostic tool to learn about the health of this endangered species.

The T-RFLP and SSU rRNA gene cloning and sequencing methodology employed in this study was designed to provide a preliminary assessment of bacteria associated with the skin of humpback whales, and indentify candidates for the most common community members. We expect that future studies of humpback whale associated bacteria will incorporate this information in the design of future investigations.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Phylogenetic identification of SSU rRNA gene clones from the skin of a healthy adult humpback whale (WH51) ($n = 60$ clones).

Table S2. Phylogenetic identification of SSU rRNA gene clones from the skin of a healthy calf humpback whale (CALF) ($n = 23$ clones).

Table S3. Phylogenetic identification of SSU rRNA gene clones from the skin of a health-compromised humpback whale (ENT1) ($n = 94$ clones).

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