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Characterization of the intestinal microbiota of two Antarctic notothenioid fish species

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Abstract The fish fauna of the Southern Ocean is dominated by species of the perciform suborder Notothenioidei, which constitute 46% of fish species and 90% of biomass. Notothenioids have undergone rapid morphological and ecological diversification and developed physiological adaptations to a cold, highly oxygenated environment. Microbes inhabiting animal intestines include those that perform essential nutritional functions, but notothenioid gut microbial communities have not been investigated using cultivation-independent approaches. We analyzed bacterial 16S rRNA gene sequences obtained from the intestinal tract of *Notothenia coriiceps* and *Chaenocephalus aceratus*, which differ in their pelagic distribution and feeding strategies. Both samples showed dominance of Gammaproteobacteria (mostly *Vibrionaceae*), as has been reported for temperate teleost species. Both samples showed low diversity relative to that reported for other fish microbiota studies, with *C. aceratus* containing fewer OTUs than *N. coriiceps*. Despite the small sample size of this preliminary study, our findings suggest that Antarctic notothenioids carry a gut microbiota similar in composition to that of temperate fish, but exhibiting lower species-level diversity. The omnivorous *N. coriiceps* individual exhibited greater diversity than the exclusively carnivorous *C. aceratus* individual, which may indicate that increasing herbivory in fish leads to gut microbe diversification, as found in mammals. Lastly, we detected members of taxa containing known microbial pathogens, which have not been previously reported in Antarctic notothenioid fish.

Keywords Antarctic microbiology · Notothenioid · Intestine · Bacteria · Diversity · 16S rRNA

Abbreviations

16S rRNA	16S ribosomal RNA
NCI	Notothenia coriiceps sequence library
CAI	Chaenocephalus aceratus sequence library
OTU	Operational taxonomic unit

Introduction

The modern fish fauna of the Southern Ocean is taxonomically restricted, highly endemic, and singularly dominated by species of the suborder Notothenioidei (Eastman 2005). The preponderance of the notothenioid fishes results from the unique paleoclimatic and paleogeographic history of Antarctica (Eastman 1993). With the opening of the Drake Passage (~34–30 Myr ago) and the establishment of the Antarctic Circumpolar Current, the Southern Ocean became thermally isolated, began to cool, and attained its present frigid temperatures (–2 to +2°C) by the mid-Miocene (14–10 Myr ago). During this interval, the shallow-water, cosmopolitan, and temperate fish fauna characteristic of the

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late Eocene (38 Myr ago) became largely extinct due to destruction of inshore habitat and changes in trophic structure caused by repeated ice sheet scouring of the continental margin (Eastman 2005). Thus, fish diversity was reduced and new ecological niches became available to other taxa, such as the notothenioid fishes, that were diversifying in situ. Today species of the notothenioid suborder constitute 46% of all the fish species of the Southern Ocean. At the high latitudes of the Antarctic continental shelf, notothenioid species dominance is particularly clear, encompassing 77% of species diversity and 90% of biomass.

The notothenioids have undergone rapid morphological and ecological diversification as they adapted to a cold, highly oxygenated environment over the past 10–14 Myr (Cheng and Detrich 2007). The acquisition of novel antifreeze proteins (Chen et al. 1997; Cheng and Chen 1999), loss of oxygen-binding hemoproteins (Cocca et al. 1995; Zhao et al. 1998; Sidell and O'Brien 2006; Near et al. 2006), and loss of an inducible heat shock response (Hofmann et al. 2000; Buckley and Hofmann 2004) by notothenioids are examples of novel traits that evolved in a perennially frigid environment. Musculoskeletal diversification away from the ancestral benthic condition toward pelagic or partially pelagic zooplanktivory enabled the taxon to exploit unfilled niches in the water column (Eastman 1993; Eastman 2005). Although notothenioids lack swim bladders, many species reduced body density to near neutral buoyancy, and some attained it (Eastman 1997; Eastman 1999; Near et al. 2007), through a combination of decreased skeletal mineralization and increased lipid deposition. Thus, in the dominant family Nototheniidae, approximately 50% of the Antarctic species are semipelagic, epibenthic, cryopelagic, and pelagic (Eastman 2005).

Given the ecological, morphological, and physiological diversification of the notothenioids, one may anticipate that their gut microbial communities have also evolved to accommodate the various trophic lifestyles of the hosts. However, notothenioid gut microbial communities have received relatively little attention. MacCormack and Fraile (1990, 1991) reported that the stomach microbiota of *Notothenia neglecta* (once distinguished from *Notothenia coriiceps* but now considered conspecific) is highly variable and probably related to microbiota that is ingested with food. The authors obtained mostly *Vibrio* spp. using cultivation-dependent approaches, and concluded that *Vibrio* spp. constitute the predominant indigenous intestinal microbiota of *N. neglecta*, in agreement with contemporaneous observations of other marine teleosts inhabiting a variety of environmental conditions. No cultivation-independent approaches were applied in these studies.

We have analyzed bacterial 16S ribosomal RNA sequences obtained from the intestinal tracts of *N. coriiceps* and *Chaenocephalus aceratus* species, which differ in their pelagic distribution and feeding strategies. *N. coriiceps* (yellowbelly rockcod or bullhead notothen) is a benthic omnivore that consumes small fish, krill, invertebrates, and kelp, whereas *C. aceratus* (blackfin or Scotia Arc icefish) is a benthopelagic ambush predator of fish that also raids in the water column to feed on krill (Eastman 1993). These preliminary findings provide a foundation for comprehensive characterization of the composition and function of notothenioid intestinal microbiota.

Materials and methods

Fish were collected by bottom trawling (100–200 m) from the *ARSV Laurence M. Gould* in Dallmann Bay (west of Brabant Island) in the Palmer Archipelago. Fish were transported alive to Palmer Station, Antarctica, and maintained at −1.5–0.0°C in aquaria supplied with running seawater. Specimens were euthanized, and the entire intestinal tract removed by dissection. A small section (approximately 1 cm^2) was excised from the foregut wall, rinsed with sterile saline to remove adherent luminal contents, and subjected to DNA extraction.

For this preliminary characterization, bulk genomic DNA was extracted and purified from foregut wall samples of one *Notothenia coriiceps* (henceforth referred to as NCI) and one *Chaenocephalus aceratus* (CAI), using the Ultra Clean soil DNA kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions. 16S rRNA genes were amplified by PCR using 27F (5′-GAGTT TGATCCTGGCTCAG-3′) and 1525R (5′-AGAAAGGA GGTGATCCAGCC-3′) primers, specific to the domain Bacteria, and Platinum Taq PCR supermix (Invitrogen, Inc., Carlsbad, CA). Amplifications were performed using a DNA Engine Tetrad PTC-225 thermal cycler (MJ Research, Waltham, MA) with an initial denaturation of 2 min at 94°C, followed by 29 cycles of 30 s at 94°C, 30 s at 55°C and 2 min at 72°C, with a final extension of 5 min at 72°C. A PCR negative control reaction in which the genomic DNA was replaced by an equivalent volume of sterile distilled water was also included. The resulting PCR products were cloned to generate bacterial 16S rRNA clone libraries from each fish using the TOPO TA cloning kit (Invitrogen, Inc.) according to the manufacturer's instructions. The nearly complete 16S rRNA gene sequences obtained from the two libraries using the cloning vector primers M13F and M13R corresponded to positions 27–1,545 of the *E. coli* sequence.

Sequence alignments were performed in the ClustalW package of MacVector 10.0 (MacVector, Inc., Cary, NC), and the libraries were screened for potential PCR-derived anomalies and chimeras using the MALLARD and

PINTAIL software as described by Ashelford et al. (2006). All sequences identified as anomalous were discarded from further analysis. Distance matrices were calculated using dnadist (PHYLIP package version 3.68), and used as input for DOTUR and LIBSHUFF. Number of operational taxonomic units (OTUs), Shannon's diversity index (H′), rarefaction curves and Chao1 estimates were determined using the DOTUR software package (Schloss and Handelsman 2005), with implementation of the complete linkage clustering algorithm. Comparisons of similarity between the two libraries were performed using LIBSHUFF (Singleton et al. 2001) and Jaccard values obtained from SONS (Schloss and Handelsman 2006) using the provided documentation. Phylogenetic affiliations of 16S rRNA gene sequences were assigned using the CLASSIFIER software provided by the Ribosomal Database Project Release 10 (Cole et al. 2007), and the nearest relative of 16S rRNA gene sequences was determined using BLAST to query the GenBank database (Altschul et al. 1990).

The phylogenetic tree of clone sequences related to *Neorickettsia/Ehrlichia* was constructed using the relevant sequences from each clone library, sequences identified by the analysis pipeline as being most closely related to the query sequence, and selected additional reference sequences. Alignments were generated using ClustalW as described above and included 1,360 nucleotide positions. Boot-strapped neighbor-joining trees were generated using SEQBOOT and CONSENSE (PHYLIP package version 3.68).

Sequences obtained in this study have been deposited in GenBank under the accession numbers FJ456353 to FJ456655 (for the CAI library) and FJ456656 to FJ456849 (for the NCI library).

Results and discussion

The foregut microbiota of the two notothenioid specimens were characterized by relatively limited sequence diversity, and the diversity of the *C. aceratus* (CAI) library was considerably lower than the diversity of the *N. coriiceps* (NCI) library (Table 1). Using DOTUR, the *N. coriiceps* sample (NCI) contained 17 OTUs (grouped at 99% sequence similarity), while the *C. aceratus* sample contained 6 OTUs. The ChaoI species richness estimator was also used to predict the number of expected OTUs in each library (if the community was completely sampled), which were 22 and 9 in the NCI and CAI libraries, respectively (Table 1). The rarefaction curve for the CAI library (at 99% similarity) approaches a plateau at 7 OTUs, whereas the curve for the NCI library (at 99% similarity) indicates that the number of OTUs should increase with additional sampling (data not shown). In addition, Shannon's diversity index (H′), which takes into account both species richness and evenness, was much lower in the CAI library (Table 1), most likely due to the dominance of a single OTU in the library (99% of clone sequences, Table 2). These results suggest that notothenioid fish gut microbiota may possess the lowest diversity of any fish gastrointestinal tract yet examined using cultivation-independent approaches (see Table 1), although these studies are admittedly small in number and several were conducted using density gradient gel electrophoresis (DGGE), from which the

Table 1 Comparisons between the NCI and CAI clone libraries and culture-independent diversity of gut microflora from other fish species

Fish sample	Number of sequences	OTUs[a]	H′[a]	Chao1[a]	Coverage[a]
Notothenia coriiceps	194	17	2.0	22	96%
Chaenocephalus aceratus	303	6	0.3	9	99%
Atlantic salmon (*Salmo salar* L.)[d]	–[b]	26[c]	–	–	–
Long-jawed mudsucker (*Gillichthys mirabilis*)[e]	88	13	–	–	–
Bluegill (*Lepomis macrochirus*)[f]	–	4–12[c]	–	–	–
Rainbow trout (*Oncorhynchus mykiss*)[g]	104	32	–	–	–
Takifugu niphobles[h]	250	24	–	–	–

[a] Calculated using OTU frequencies at 99% sequence similarity

[b] Data is not available or was not calculated

[c] Estimated from number of bands observed in denaturing gradient gel electrophoresis

[d] Liu et al. (2008)

[e] Bano et al. (2007)

[f] Uchii et al. (2006)

[g] Kim et al. (2007)

[h] Shiina et al. (2006)

Table 2 OTUs identified in the CAI and NCI libraries

OTU groupings (99% similarity)	OTU frequency	Best BLAST match[a]	% Identity[a]
Clone library CAI			
OTU1	**284**	***Photobacterium phosphoreum* (X74687.1)**	**98%**
OTU2	**4**	***Photobacterium phosphoreum* (EU881910.1)**	**99%**
OTU3	1	*Sphingomonas* sp. oral clone AV069 (AF385529.1)	99%
OTU4	12	*Photobacterium iliopiscarium* (AY849429.1)	99%
OTU5	**1**	***Ehrlichia* sp. 'trout isolate' (AF206298.1)**	**93%**
OTU6	1	*Moritella marina* (AJ297540.1)	99%
Clone library NCI			
OTU1	1	Uncultured bacterium clone (EU463379.1)	93%
OTU2	38	*Vibrio logei* strain T2110 (DQ318955.1)	99%
OTU3	34	*Vibrio logei* strain SR181 (AY292934.1)	99%
OTU4	**26**	***Photobacterium phosphoreum* (X74687.1)**	**99%**
OTU5	**4**	***Ehrlichia* sp. 'trout isolate' (AF206298.1)**	**93%**
OTU6	11	*Fusobacterium* sp. CSL-7530 (EU597748.1)	92%
OTU7	2	phototrophic eukaryote clone DYF38 (AY702173.1)	99%
OTU8	46	*Aliivibrio salmonicida* LFI1238 (FM178379.1)	99%
OTU9	**22**	***Photobacterium phosphoreum* (EU881910.1)**	**99%**
OTU10	1	*Ehrlichia* sp. 'trout isolate' (AF206298.1)	93%
OTU11	2	*Desulfovibrio desulfuricans* (AF192154.1)	85%
OTU12	2	*Mycoplasma moatsii* (AF412984.1)	93%
OTU13	1	Uncultured bacterium ARCTIC24_B_12 (EU795097.1)	99%
OTU14	1	*Desulfovibrio desulfuricans* (DQ092636.1)	86%
OTU15	1	*Shewanella sediminis* HAW-EB3 (CP000821.1)	97%
OTU16	1	*Moritella* sp. 56A1 (AB183749.1)	99%
OTU17	1	Uncultured bacterium clone C13 (DQ340200.1)	90%

[a] Determined from a representative sequence from each OTU

OTUs indicated in *bold* were found to be common to both libraries

number of OTUs can only be inferred, not determined. A further limitation of these studies, and of our own, is the use of only one PCR primer set. Frank et al. (2008) have demonstrated that use of a re-formulated 27F primer containing mixed sequences more accurately recovered microbial community structure than the single primer sequence used in our studies and those to which we compared our results. Use of these mixed primers is likely to retrieve greater diversity and is to be recommended for most studies, but in our case would have precluded direct comparison of our data with those from the microbiota of other fish. The relatively low diversity reported for fish gut microbiota in general (as compared to human, mammalian, and bird gut microbiota) may be related to the smaller population size—10^3–10^8 cfu/g in fish (Trust and Sparrow 1974; Trust et al. 1979; Huber et al. 2004; Kim et al. 2007) compared to 10^{11} cfu/g in mammals and birds (Moore and Holdeman 1974; Mead 1997). Diversity may also be reduced due to the paucity of anaerobes (dominant in the gut microbiota of other animals) in fish intestinal tracts (Kim et al. 2007). We infer that the notothenioid intestinal tract is substantially aerobic because these fish drink seawater rich in oxygen and support intestinal populations of helminth parasites that depend on oxygen for respiration (Dr. Joseph Eastman, pers. comm.).

Pairwise LIBSHUFF (Singleton et al. 2001) analysis of the two libraries revealed little similarity between the microbial communities in the two fish intestinal microbiota ($P = 0.001$). Using SONS (Schloss and Handelsman 2006), three OTUs were found to be common to both libraries (Table 2). The overwhelmingly dominant OTU1 (93.7% of clones) of the CAI library, related to *Photobacterium phosphoreum* (X74687.1), was also present in the NCI library (OTU4), where it constituted 13.4% of clones. Conversely, a second OTU, related to *P. phosphoreum* (EU881910.1), which was a minor component (OTU2, 1.3%) of the CAI library, constituted a substantial fraction (OTU9, 11.3%) of the NCI library. The third common OTU, which contained sequences most similar to a bacterial trout isolate 16S rRNA gene, was related to the genera *Ehrlichia* and *Neorickettsia*, members of which are associated with pathology of fish. This *Ehrlichia* sp.

(AF206298.1), the only minority OTU shared by the two libraries, was only distantly related to previously characterized *Ehrlichia/Neorickettsia* 16S rRNA sequences (93% sequence similarity). Figure 1 shows that our *Ehrlichia/Neorickettsia* related clones formed a monophyletic cluster within the dendrogram of other cultivated and uncultivated *Ehrlichia/Neorickettsia* spp., which suggests strongly that they may represent organisms unique to Antarctic notothenioid fish.

Of the 23 OTUs detected, 14 showed greater than 97% similarity with database sequences (Table 2) and therefore likely represent strains of previously identified species. Seven OTUs shared 90–93% similarity, consistent with potentially novel species-level diversity within previously classified genera, including the *Ehrlichia/Neorickettsia* related clones (Figure 1). One OTU (NCI OTU11) shared only 85% similarity with its closest database match, a strain of *Desulfovibrio desulfuricans*; this may indicate a novel family within the deltaproteobacteria.

Both samples showed dominance of Gammaproteobacteria (Table 2), as has been reported for many temperate marine species (Colwell 1962; Ohwada et al. 1980; MacFarlane et al. 1986; Spanggaard et al. 2000; Kim et al. 2007). However, the vast majority of *C. aceratus* gammaproteobacterial clones (99%) belonged to the genus *Photobacterium*, whereas the *N. coriiceps* sequences were more evenly distributed between *Photobacterium* and *Vibrio*. We detected no aeromonads or pseudomonads, which are frequently reported members of fish gut microbiota. *Pseudomonas* spp. were reported to be present in the cultivation-based study of *N. neglecta* (*coriiceps*) by MacCormack and Fraile (1990, 1991). Despite these differences, the fact that both our cultivation-independent study and the culturing methodology of MacCormack and Fraile (1991) recovered a majority of *Vibrio/Photobacterium* spp. argues that cultivation from fish intestinal samples may reflect actual microbial diversity better than that found with other environmental samples. This conclusion is consistent with the results of Spanggaard et al. (2000), in which 50% of the cultivation-independent microbial community of rainbow trout intestine was recovered by conventional culturing. However, this high level of culturability may be taxon-dependent, as Romero and Navarrete (2006) report that only 1% of juvenile coho salmon gut bacteria (dominated by *Shewanella*, *Aeromonas*, and *Pseudomonas*) could be recovered as cultivable bacteria.

N. coriiceps is omnivorous, and the specimen examined possessed greater microbial diversity than the exclusively carnivorous *C. aceratus* individual. Comprehensive analyses of vertebrate gut microbiota (albeit mostly mammalian) indicate that bacterial diversity increases from carnivory to omnivory to herbivory (Ley et al. 2008). This may indicate that increasing herbivory in notothenioid fish leads to gut microbe diversification, as found in mammals. Despite the relatively small sample size of this preliminary study, a substantial coverage of the two clone libraries was achieved (Table 1). An increased sampling effort would not be expected to appreciably increase the diversity of recovered 16S rRNA gene sequences as the dominance of a small number of phylotypes would likely result in a larger collection of *Vibrio* related sequences with few recovered rare sequences. However, our cultivation-independent approach has detected minority taxa not reported in the only previous culture-dependent analysis of notothenioid gut microbiota.

The presence of several sequences related to *Mycoplasma* (in NCI), *Ehrlichia/Neorickettsia* (in NCI and CAI), and 46 putative members of *Aliivibrio salmonicida* (in NCI) may indicate previously uncharacterized pathogenic burdens, although we cannot exclude the possibility that these organisms originated from the notothenioid diet and were therefore intestinal transients. It is also possible that the *Mycoplasma*-related OTU represents a member of the commensal microbiota, as has been inferred for the long-jawed mudsucker, in which mycoplasmas dominate

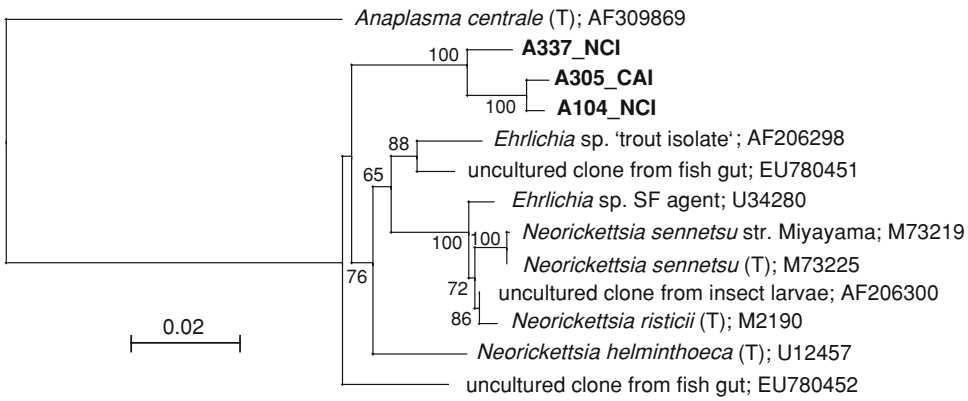

Fig. 1 Neighbor-joining phylogenetic tree of clones related to the genus *Neorickettsia*. Clone sequences from this study are indicated in *bold*. The scale bar represents the expected number of changes per nucleotide position. Bootstrap values ≥ 50% (10,000 iterations) are indicated at the nodes. (A305-CAI and A104_NCI were identified as the same OTU at 99% sequence similarity by SONS)

(Bano et al. 2007). Nearly all of these sequences match most closely to known or suspected pathogens of fish, but NCI OTU1 (single sequence) is most closely related to a sequence from mammalian fecal material (Ley et al. 2008). Other minority components included those related to *Sphingomonas*, *Fusobacterium*, *Desulfovibrio*, *Shewanella*, and algal plastids (the latter presumably derived from the kelp intake of *N. coriiceps*). NCI OTU13 is 99% similar to a metagenomic clone from the Arctic (Broad Institute Marine Metagenomics Project). This finding is not sufficient to infer bipolar distribution of a single species (Staley and Gosink 1999), but is an interesting preliminary finding. The lack of strictly anaerobic taxa detected by our study agrees with the cultivation studies of MacCormack and Fraile (1990, 1991), who also ascribed this to the high levels of dissolved oxygen in Antarctic waters. The approaches used in our study do not allow us to determine whether the microbial taxa detected constitute a true microbial microbiota or merely reflect microbial populations associated with the food source. However, *Vibrio* spp. are acquired early in fish larval development and are regarded as members of an indigenous microbiota, and *Aliivibrio salmonicida* (detected in this study) is able to colonize the fish intestinal tract (Hansen and Olafsen 1999). This makes it unlikely that we have assessed a community that is solely of dietary origin.

In summary, our results suggest that Antarctic notothenioids carry a gut microbiota similar to that of temperate fish in overall composition but exhibiting lower diversity at the species level. Extremely low temperatures may be driving the lower diversity of the notothenioid gut microbiota compared to that of more temperate fish. However, recent studies have shown that the bacterioplankton communities in the polar and temperate oceans are similar (Murray and Grzymski 2007), which suggests that opportunities for colonization may be comparable but host-specific factors affect colonization dynamics. A goal of future research will be to determine the specific environmental factors that limit the diversity of bacteria associated with the Antarctic notothenioids**.** We also detected the presence of potentially pathogenic microbes in apparently healthy Antarctic notothenioid fish, which raises the possibility that they may play a role in population dynamics of their host species.

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