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RESEARCH NOTE

First characterization of gastrointestinal culturable bacteria of Patagonian toothfish *Dissostichus eleginoides* (Nototheniidae)

Primera caracterización de bacterias gastrointestinales cultivables en el bacalao de profundidad *Dissostichus eleginoides* (Nototheniidae)

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Abstract.- The Patagonian toothfish *Dissostichus eleginoides* is one of the most important fisheries from the Southern Ocean. The biology of this species is relatively well studied and some nutritionals issues have also been reported; however there is no information about the composition of the bacterial community of the gastrointestinal tract, which is essential to characterize the microbiota of this fish. The bacterial flora of *D. eleginoides* is here described for the first time using culturable methods. By applying traditional culture-based techniques and 16S rDNA sequencing methods it was possible to characterize the families Vibronaceae and Moraxellaceae, which were mainly represented by *Vibrio* and *Psychrobacter*, respectively. This Patagonian fish shows a microbiota very similar to other cold waters fishes.

Key words: Notothenioid, cold-water fish, subantarctic, Bacteria, 16S rRNA

INTRODUCTION

Commercial fishing of Patagonian toothfish *Dissostichus eleginoides* (Smitt, 1898) represents a global challenge over the sustainability in the exploitation of marine resources. This species is particularly vulnerable to over-exploitation because of their slow growth rate, late maturity and low fecundity (Horn 2002, Collins *et al*. 2010). It is in this scenario that Chile has been developing aquaculture research projects, which allowed to obtain information about the natural diet (Soto 2015), growth and survival rates in captivity (Gallardo 2016). In this process, it is essential to improve our knowledge about the bacterial flora of this fish species, because the gastrointestinal microbiota play a critical role in the physiology, nutrition and health of the host. Microbiome research in other teleost species, especially those important in an aquaculture perspective, has been scarce (Llewellyn *et al*. 2014). Substantial information about the gastrointestinal microbiota has been produced during the last years, but the focus was put on model and farmed fish species, such as zebra fish, turbot, salmon or trout (Nayak 2010, Ganguly & Prasad 2012, Llewellyn *et al*. 2014). Few microbiological studies have been conducted on cold-water fishes that have great ecological and economic importance, as halibut or Atlantic cod (Jensen *et al*. 2002, Ringø *et al*. 2006). To date there are no reports available of disease outbreaks and mortalities provoked by pathogenic bacteria in the Patagonian toothfish kept under captivity conditions.

The aim of this study was to evaluate the culturable microbiota retrieved from 3 sections of the digestive tract (stomach, middle- and distal intestine) of adult Patagonian toothfish, in order to obtain further information about culturable aerobic heterotrophic bacteria present in the gastrointestinal tract.

MATERIALS AND METHODS

Specimens of wild Patagonian toothfish were collected near Diego Ramirez Archipelago (56°29'S, 68°44'W), on board of a factory vessel, at 1500 to 1700 m of depth using long-line fishery. This was performed according the methodology of aquaculture broodstock selection (Gallardo 2016). Caught fishes were then transferred to Centro de Cultivos Marinos Bahía Laredo, located in Laredo Bay (52°58'S, 70°49'W) Punta Arenas, Chile. The fishes were kept for 6 months in 50 m^3 fiberglass circular tanks, with flow through system of seawater. The water temperature was 6.4 ± 1.4°C and the fishes were

fed 3 times a week, with a sausage that contained a fishmeal mixture rich in highly unsaturated fatty acids, in addition to fish oil, vitamins and immune stimulants. The feeding rate was 0.6 ± 0.25% of body weight per day and the growth rate was 0.06 ± 0.08% per day. Sixty fish were captured, 20% survived and acclimatized to the aquaculture confinement. Of these fishes, one was euthanized due to erratic behavior, hence providing an opportunity to assess the gastrointestinal microbiota of a specimen of *D. eleginoides*, that was been maintained under controlled conditions.

Isolation and identification of cultivable bacteria

The peritoneal cavity of the fish was aseptically opened with a sterile scalpel and then the gastro intestinal tract (GI) was aseptically excised and sectioned in: stomach, middle and posterior intestine. These sections were rinsed with sterile PBS (Phosphate Buffer Saline) to remove food debris. Tissue samples of intestinal wall used to cultivate bacteria were washed with sterile PBS. The side of the intestinal epithelium was rubbed on plates with solid nutrient media (Rogosa, R2A, Marine Agar and TCBS agar) to recover lactobacilli, heterotrophic bacteria, heterotrophic marine bacteria, and *Vibrio* spp., respectively. The plates were incubated in duplicate at 10°C for several days until bacterial growth was observed. Colony selection for sequencing processes was made according to morphology and analysis of 16S restriction fragments, as described by Urtubia *et al*. (2014). PCR amplifications were performed using the same condition as described above for 27F and 1422R primers. 70 isolates (20 from stomach, 22 from middle intestine and 27 from distal intestine) were identified using sequences analyses of 16S rRNA gene.

Data analysis

16S RNA genes sequenced from isolated strains were edited with DNA BASER Sequence Assembler v.4. The phylogenetic assignation was performed based on the sequence similarity using BLAST (Basic Local Alignment Search Tool) program (National Center for Biotechnology Information). The phylogenetic tree was created by means of Bosque software (Ramírez-Flandes & Ulloa 2008). 16S Ribosomal sequence were aligned with 3.6 MUSCLE software (Edgar 2004). The phylogenetic tree was inferred by maximum likelihood method based on the HKY85 model (Hasegawa *et al*. 1985). The phylogenetic inference corresponded to PhyML (Guindon & Gascuel 2003). Statistical evaluation of tree topologies analysis was performed with 1000 bootstrap repetitions. Sequences of isolated strains were submitted to GenBank database under accession numbers KP259730 – KP259799**.**

Results and discussion

The characterization and comparison of bacterial flora associated to the digestive tract achieved by cultivable methods is an important step to understand the functional roles that bacteria play and their relationship with the host, as well as the potential use as probiotics in aquaculture (Nayak 2010, Perez-Sanchez *et al*. 2014). This study describes for the first time, the culturable bacteria associated to the digestive tract of *D. eleginoides*. Because the Patagonian toothfish used in this study was part of the acclimatization process belonging to a reproduction experiment, no other specimens could be sacrifice to perform further bacterial studies. In the context of the opportunistic sampling, these preliminary results are important as provide a first view of the qualitative bacterial composition.

A total of 113 cultivable isolates were recovered from the 3 sections of the GI tract using different solid media. 53 colonies were obtained from Marine agar, 30 from TCBS and 30 using R2A medium. In contrast, no colonies were obtained in the Rogosa medium. Restriction fragments analysis of these strains allowed to differentiate 70 strains (21 from stomach, 22 from middle intestine and 27 from final intestine). These 70 isolates were purified and identified based on their 16S rRNA gene sequences. The predominant bacteria class was α-Proteobacteria (n= 63), whereas the least abundant classes were Actinobacteria (n= 7) and Firmicutes (n= 1), represented by genera *Nesterenkonia* (n= 1), *Rhodoglobus* (n= 4), *Frigoribacterium* (n= 1), *Acinetobacter* (n= 1) and *Sporosarcina* (n= 1), respectively (Fig. 1).

Several isolated cultivable bacteria from *D. eleginoides* belonged to genera described for cold environments were found in this study, such as *Psychrobacter*, *Frigoribacterium* and *Rhodoglobus*. This is consistent with the Southern Ocean environment. In this regard, water temperature may be a key factor affecting not only distribution of *D. eleginoides* but also bacterial composition along the digestive tract.

Results demonstrated that bacterial strains belonging to the family Vibrionaceae (44.3%) and Moraxellaceae (35.7%) were present in all 3 sections of the GI tract, showing a clear predominance in respect to other bacterial families. Moreover, Micrococcaceae (1.4%), Pseudomonadaceae (1.4%), Microbacteriaceae (8.6%), Sphingomonadaceae (5.7%), Xanthomonadaceae (1.4%) and Planococcaceae (1.4%) registered a low predominance in the intestinal tract sections. The most frequently recovered bacterial genera were *Vibrio* (n= 26) and *Psychrobacter* (n= 23), both from Proteobacteria (Fig. 2). The presence of *Vibrio* species in the microbiota of *D. eleginoides* is consistent with previous studies performed on several fish species (Nayak 2010). Vibronaceae was mainly

represented by *Vibrio* and *Psychrobacter,* in all 3 sections of the GI tract. It is evident that cold environments constitute an ecological niche for *Psychrobacter* bacteria in organisms that inhabit cold waters (Bozal *et al*. 2003). The genera *Vibrio* and *Psychrobacter* were highly represented in stomach (52.3 and 33.3%, respectively) and middle intestine (45.4 and 50.0%, respectively). In contrast, these genera only represented 18.5% of the bacterial isolates from distal intestine. *Aliivibrio* was detected only in this portion with equal percentage as *Vibrio*. Of this group, the most predominant isolates corresponded to *Vibrio tasmaniensis*, *Vibrio splendidus* and *Aliivibrio logei*. Consistent with these results, MacCormack & Fraile (1990) obtained mostly *Vibrio* spp. from the fish *Notothenia neglecta,* using cultivation-dependent approaches. The authors concluded that *Vibrio* species constitute the predominant indigenous intestinal microbiota found on this Antarctic notothenioidei fish. Furthermore, intestinal microbiota studies of *Notothenia coriiceps* and *Chaenocephalus aceratus*, using non-cultivable methods, showed a relatively low bacterial sequence diversity. The most dominating order in terms of abundance were gamma-Proteobacteria, *Photobacterium* sp., *Allivibrio* sp. and *Vibrio* sp. (Ward *et al*. 2009). Recently, Sedláèek *et al*. (2016) described the dominance of *Enterobacter* isolated from gut contents of several fish species inhabiting Weddell Sea in Antarctica (*Notothenia coriiceps*, *Trematomus bernacchii*, *Trematomus hansoni*, and *Trematomus newnesi*). Nevertheless, they used a specific media for *Enterobacter*, evidencing that different results can be obtained depending on the methodological approaches (this includes cultivable and non-cultivable methods). They even suggest that the results may vary depending on the type of sampled tissue (*e.g.*, intestinal contents or GI tract wall). Although, some *Vibrio* species could be considered as pathogenic bacteria, its presence in Patagonian toothfish intestinal tract seems to be normal. *Vibrio* has been frequently detected in the intestinal flora of different fish species, for example in halibut, tilapia, Atlantic cod and Atlantic salmon (Nayak 2010, Llewellyn *et al*. 2014). These results suggest that *Vibrio splendidus* and *Vibrio tasmaniensis* are native bacterial flora in *D. eleginoides*.

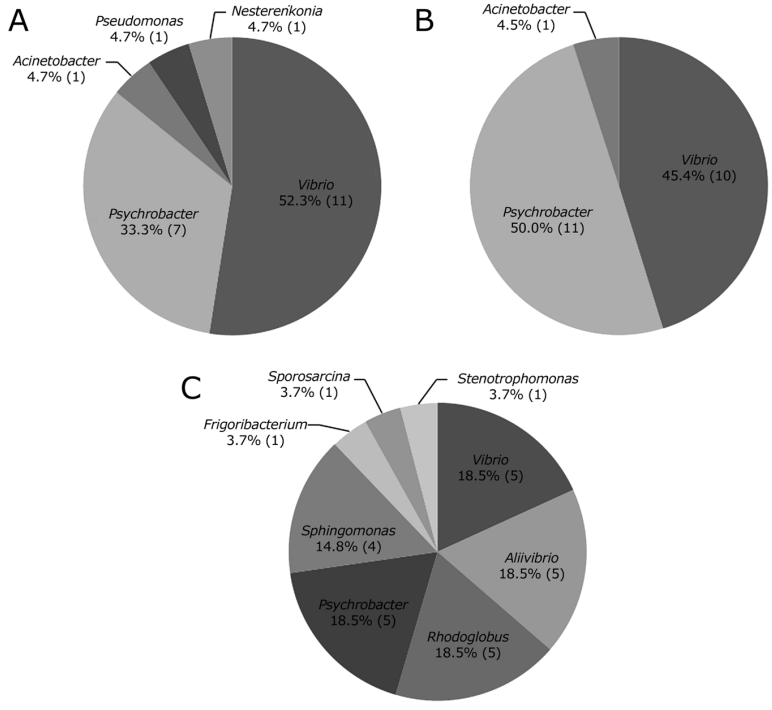

Figure 1. Composition of the culturable bacteria associated with the gastrointestinal tract of the Patagonian toothfish *D. eleginoides*. The number of strains for each bacterial genus is shown in parentheses. A) Stomach; B) Middle intestine; C) Distal intestine / Composición de las bacterias cultivables asociadas al tracto gastrointestinal del bacalao de profundidad *D. eleginoides*. Se muestra entre paréntesis el número de cepas pertenecientes a cada género bacteriano. A) Estómago; B) Intestino medio; C) Intestino distal

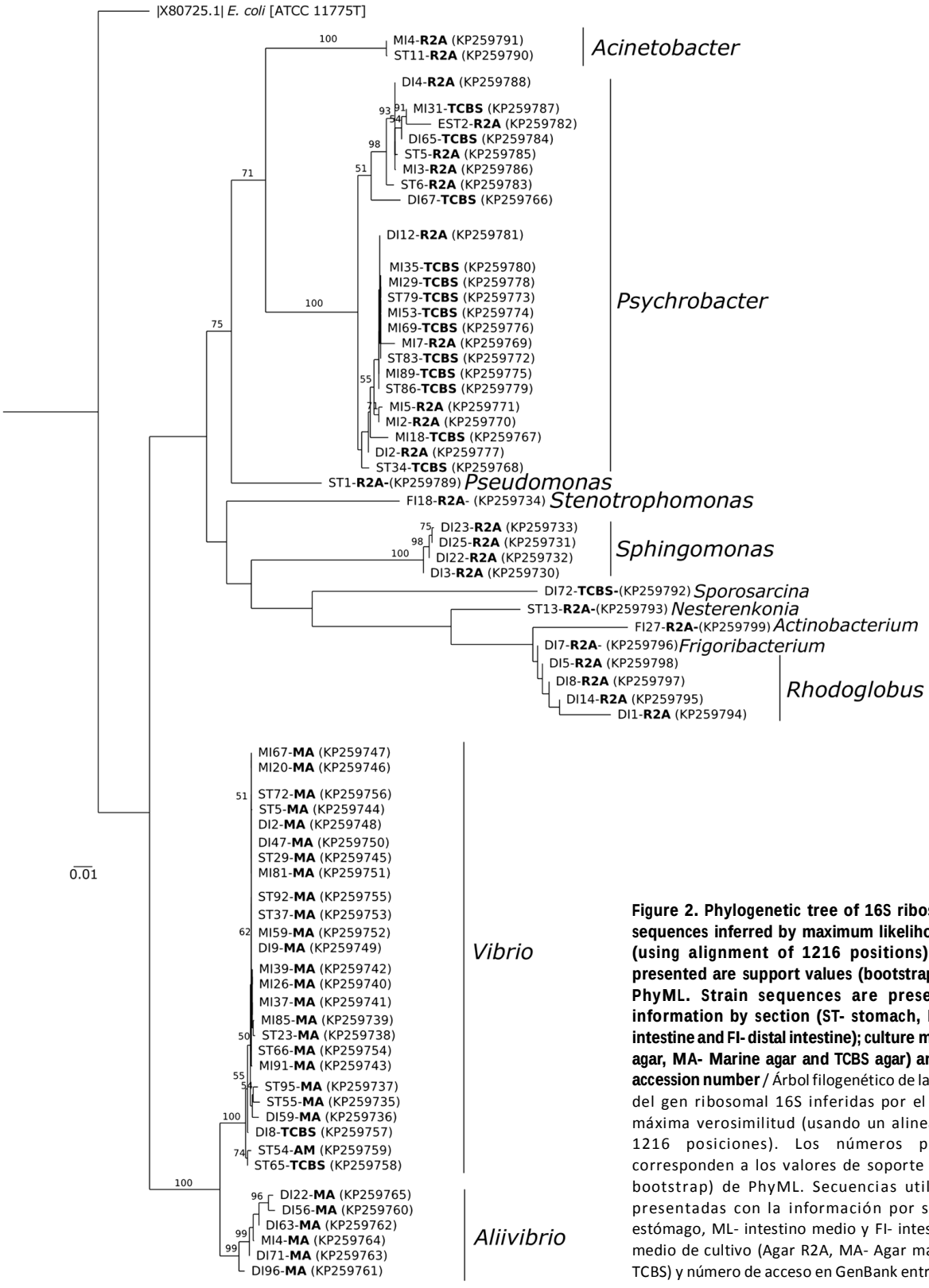

Figure 2. Phylogenetic tree of 16S ribosomal gene sequences inferred by maximum likelihood method (using alignment of 1216 positions). Numbers presented are support values (bootstrap values) of PhyML. Strain sequences are presented with information by section (ST- stomach, MI- middle intestine and FI- distal intestine); culture medium (R2A agar, MA- Marine agar and TCBS agar) and GenBank accession number / Árbol filogenético de las secuencias del gen ribosomal 16S inferidas por el método de máxima verosimilitud (usando un alineamiento de 1216 posiciones). Los números presentados corresponden a los valores de soporte (valores de bootstrap) de PhyML. Secuencias utilizadas son presentadas con la información por sección (ST- estómago, ML- intestino medio y FI- intestino distal), medio de cultivo (Agar R2A, MA- Agar marino y Agar TCBS) y número de acceso en GenBank entre paréntesis

The presence in the fish microbiome of several bacterial groups, such as Actinobacteria, may imply that these bacteria could be involved on bacterial control against fish pathogens, like *Vibrio* spp. (Sanchez *et al*. 2012). The phylum Actinobacteria was represented by 3 genera: *Nesterenkonia* sp. (one isolate from stomach), family Micrococcaceae; *Frigoribacterium* sp. (one isolate from distal intestine) and *Rhodoglobus* sp. (4 isolates from distal intestine with 99% of 16S rRNA sequence identity), which belong to the Microbacteriaceae family (Fig. 2). Species of the genus *Nesterenkonia* have been previously reported as halotolerant, being isolated from different saline ecosystems such an arid saline system (El Hidri *et al*. 2013) or a hypersaline Antarctic lake (Collins *et al*. 2002). Microbacteriaceae family represented by the genus *Rhodoglobus* had similar representation to the Vibrionaceae family in the distal intestine, but was only present in this segment. Four isolates showed 99% similarity with a *Rhodoglobus* sp. isolated from cold environment (Sheridan *et al*. 2003). This phylum has been reported in carnivorous fishes from marine and freshwater environments (Sullam *et al*. 2012). Further studies in *D. eleginoides* using cultivation independent approaches such as denaturing gradient gel electrophoresis (DGGE) or next generation sequencing (NGS) technologies will be necessary to assess with a higher resolution the complexity of bacterial community associated to GI tract. This type of studies will also help to understand how different nutritional and environmental conditions may influence digestive tract bacterial community structure in this species. A better knowledge of the intestinal bacterial community will play a key role in the comprehension of potential fish captivity consequences regarding this emerging species in aquaculture system.

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