

Composition of cultivable enteric bacteria from the intestine of Antarctic fish (family Nototheniidae)

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ABSTRACT: In this study, the intestinal bacteria of wild Antarctic fish (family Nototheniidae) were examined using traditional culture-based techniques. Bacterial flora of the gut contents of four marine fish species (*Notothenia coriiceps*, *Trematomus bernacchii*, *Trematomus hansonii*, and *Trematomus newnesi*) was investigated to describe the allochthonous bacteria inhabiting these Nototheniidae fish. A set of 43 fresh and healthy fish was analyzed and intestinal bacteria were retrieved using the dilution plate technique on selective media Endo agar and XLDA agar. A total of 133 different bacterial isolates were obtained and initially characterized by key phenotypical tests. Notothenoid fish gut microbiota showed low species diversity of isolates and intestines were inhabited by 3 different isolates per fish on average. The bacterial colonization of the intestine content of *Trematomus newnesi* was poor. Curiously, the Gram-negative non-fermenters, including *Pseudomonas* sp., *Vibrio* sp., and *Alcaligenes*-like isolates represented the majority (59%) of intestine isolates grown on the used selective media for enteric bacteria. Based on preliminary identification, only 54 isolates (41%) were tentatively determined as enteric bacteria. The identification of 54 isolates of Gram-negative fermenting rods to the species level was achieved using biochemical characterization by commercial kits ENTEROtest 24 and Biolog GN2 MicroPlate. Results showed that *Enterobacter cloacae* phenon and *Aeromonas hydrophila* were predominant bacterial species in the free-living fish intestine from the group of fermenting Gram-negative rods.

Keywords: gut microflora; selective media; identification; *Enterobacter*, *Aeromonas*

INTRODUCTION

The microbiota in the gastrointestinal tract can be divided into two major groups – autochthonous or allochthonous bacteria. The term autochthonous flora is often used synonymously with the term normal flora, while the allochthonous flora refers to the microorganisms which are incidental visitors of the gastrointestinal tract and which are rejected after some time (Ringo et al. 1995). Marine organisms share ecosystem with microorganisms of marine environments and seawater may function as a medium for both transport and growth of microorganisms (Hansen and Olafsen 1999). It

is generally accepted that the composition of the allochthonous (transient) intestinal tract microbiota is highly variable and is affected by many environmental conditions as salinity, temperature, etc. (Ringo et al. 1995; Pond et al. 2006; Liu et al. 2008), but stable in fish kept in defined conditions (Pond et al. 2006). Especially food accessibility, composition, and food changes may affect the bacterial diversity in fish intestine (Ringo and Strom 1994; Ringo et al. 2006). Moreover, the microbiotas of the fish intestine have been shown highly dependent on the bacterial colonization during early development (Ringo et al. 1995; Ringo and Birkbeck 1999). It is generally accepted that

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fish possess a specific intestinal microbiota that is affected by food starvation. The gastrointestinal tract of seawater fishes is a complex ecosystem containing a large number of resident (autochthonous) microorganisms, which may play a role in fish nutrition and health (Hansen and Olafsen 1999). The role of gut microbiota in fish probably varies with the fish species and the presence of normal microflora in the gut may prevent its colonization with potentially harmful bacteria (Ringo et al. 2006; Ward et al. 2009).

Microbiology of the intestinal tract of relatively few marine fish species, mostly commercially important wild-caught or farmed fish, has been investigated (Cahill 1990; MacCormack and Fraile 1990; Ringo et al. 1995, 2001, 2006; Hansen and Olafsen 1999; Kim et al. 2007, Liu et al. 2008). The gastrointestinal microflora of fish appears to be simpler than that of endotherms and predominant bacterial genera/species isolated from most fish guts have been aerobes or facultative anaerobes (Ringo et al. 1995, 2001). However, a few recent studies have reported a wider diversity of the gut microflora than believed previously (Ringo et al. 2006; Hovda et al. 2007; Ward et al. 2009), especially in the intestinal contents of freshwater fish (Gonzalez et al. 1999; Spanggaard et al. 2000; Wu et al. 2010; Cantas et al. 2012). In contrast to what is reported for seawater fish, the intestinal microflora of freshwater fish species tends to be dominated by members of the genera *Aeromonas*, *Plesiomonas*, representatives of the family Enterobacteriaceae, and obligate anaerobic bacteria (Hansen and Olafsen 1999). Wu et al. (2010) published that aeromonads are the most common organisms in the gut of freshwater fish and are commonly isolated from normal healthy fish. There is evidence that dense microbial populations occur within the intestinal contents, with numbers of bacteria much higher than those in the surrounding water (Cahill 1990; Ringo et al. 2001).

The indigenous microflora of the fish digestive tract has been traditionally investigated by conventional culture-dependent methods including cultivation on selective or non-selective media followed by isolation and phenotypic characterization (Spanggaard et al. 2000; Pond et al. 2006; Hovda et al. 2007; Kim et al. 2007). Culture independent methodologies providing information about non-cultivable bacteria (denaturing gradient gel electrophoresis (DGGE), 16S rDNA sequencing)

are useful tools and these methodologies have widened the knowledge about the intestinal microbiota in fish as not being simple as believed previously (Pond et al. 2006; Hovda et al. 2007; Liu et al. 2008; Ward et al. 2009). Using the polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) method, Yang et al. (2007) found Gram-positive bacteria of the group of low % DNA guanine and cytosine content as the dominant component in the intestine of puffer fish. Dormant, non-active, or viable but non-cultivable bacterial cells of fish may be studied by direct viable count methods, using either fluorescence microscopy or flow cytometry (Hansen and Olafsen 1999; Spanggaard et al. 2000). As mentioned by Hovda et al. (2007), it is difficult to compare all available results as different studies examined different parts of the gut, different fish species, and furthermore, the fish sampling locations and times between feeding and analyses may vary.

Over the past decades, the composition of the intestinal microbiota in wild and cultured marine fish has, however, mostly been based on conventional classification methods of cultivable isolates and the identification of fish microflora has typically relied on phenotypic and biochemical key characteristics using commercial kits (Cahill 1990; Spanggaard et al. 2000; Kim et al. 2007). Although culture-dependent techniques do not compass a correct picture of the complexity in fish gut microbiota, they are still used, especially in extreme environments e.g. in Antarctic region research, as done in our study (Hoshino et al. 1997; Gilbert et al. 2004).

This study was focused on the composition of cultivable Gram-negative, facultative anaerobic heterotrophic bacteria along the digestive tract of four fish species from the family Nototheniidae. There is still little known about the intestinal microbiota of notothenioid fish species (MacCormack and Fraile 1990; Ward et al. 2009). The main objective was the determination whether predatory free-living marine fish of the same family, which occupy comparable habitat in the Antarctic region and showing similar behaviour, have considerable populations of cultivable enteric bacteria in their alimentary tracts during feeding in austral summer. The second objective was focused on evaluation of species identification of enteric bacteria in the gut of fish by using of commercial kits ENTEROtest 24 and Biolog MicroPlate GN2.

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MATERIAL AND METHODS

Notothenioid fish are endemic, dominant fish taxa in the cold continental shelf waters of the Southern Ocean surrounding Antarctica. Gut bacteria were isolated from the intestinal contents of 43 adult fish specimens of the family Nototheniidae, *Notothenia coriiceps*, *Trematomus bernacchii*, *Trematomus hansonii*, and *Trematomus newnesi* (Table 1). They were caught in January/February 2014 in the Weddell Sea at the depth of 12 to 35 m. Randomly selected fish from a group reserved for parasitological investigation were killed by a sharp blow on the head and immediately transported to the laboratory and processed. The steps mentioned below were done in the laboratory of the Czech Antarctic station Johan Gregor Mendel, James Ross Island, Antarctica (63°48'02"S, 57°52'57"W) during expedition in 2014. The peritoneal cavity of the fish was opened aseptically with a sterile blade. Intestinal contents of the digestive tract were aseptically excised from the fish abdominal cavity. Both the intestinal content and piece of gut wall of each fish were sampled and used for culturing by homogenization on surface of Endo and XLDA agar plates (Oxoid, Basingstoke, UK) prepared in-house. The plates were incubated aerobically at 16°C and inspected daily for up to 96 h, in order to obtain the cultivable enteric bacteria present in the intestine. Morphologically different colonies were randomly picked up from selective agars, subcultured and purified on Tryptic Soy Agar (TSA) (Oxoid). Cultivation on TSA has previously been reported to be suitable for bacteria of fish intestine (Hovda et al. 2007). Pure isolates inoculated in agar slats were transported to the Czech Collection of Microorganisms (CCM), Czech Republic.

Afterwards, pure cultures were stored at –70°C in Tryptone Soya Broth (Oxoid) supplemented with 15% (v/v) glycerol as cryopreservant for further analysis. The isolates were initially characterized by Gram-staining and cell morphology, catalase and oxidase reaction, motility and OF test (Weyant et al. 1996). Based on these preliminary tests a moderate quantity of isolates were presumptively identified as enteric bacteria (family Enterobacteriaceae and Aeromonadaceae) and only these further examined using ENTEROtest 24 (Erba Lachema, Brno, Czech Republic) and by Biolog GN2 MicroPlate (Biolog, Hayward, USA) for detection

of fermenting Gram-negative rods in accordance with recommendations from the kit producers.

RESULTS

The species composition diversity of Gram-negative rods present in the intestines of four fish species from family Nototheniidae (*Notothenia coriiceps*, *Trematomus bernacchii*, *Trematomus hansonii*, *Trematomus newnesi*) and obtained from the microflora investigations using traditional microbiological techniques was determined. We have obtained 133 different bacterial isolates inhabiting guts of 43 fish samples (Table 1). The lowest amount of isolates was retrieved from guts of *Trematomus newnesi*. Our results suggested that the dominant bacterial species of Nototheniidae fish intestine belonged to Proteobacteria. Although only Endo and XLDA agars isolation media for enteric bacteria were used in our study, a high amount (56%) of Gram-negative non-fermenting rods (GNNFR) was isolated startlingly by aforementioned agars from fish intestines (Figure 1).

Enterobacteriaceae (48/133 = 36% of the total) and *Aeromonas hydrophila* (6/133 = 5% of the total) comprised the dominant fermenting cultured bacteria isolated from fish intestinal contents (Figure 1). In total, 54 isolates of fermenting rods were tested using commercial kits ENTEROtest24 and Biolog GN2 MicroPlate systems and were phenotypically identified to the species level (51/54 = 94%) with satisfactory discrimination (Figure 2). Various genera within the family Enterobacteriaceae were present and the intestinal microbiota of the studied seawater fish was mainly composed of the bacterial genera *Citrobacter*, *Enterobacter*, *Klebsiella*, *Leclercia*, *Pantoea*, *Rahnella*, *Raoultella*, and *Serratia*. The Aeromonadaceae representatives consisted of only one species – *Aeromonas hydrophila*. There was no significant difference in the identified enteric bacterial flora composition of fish intestine among different fish species.

Table 1. Total numbers of isolates from Nototheniidae fish intestine

Fish species (n)	Bacterial isolates in total	Fermenting isolates
<i>Notothenia coriiceps</i> (10)	34	16
<i>Trematomus bernacchi</i> (3)	6	3
<i>Trematomus hansonii</i> (22)	72	31
<i>Trematomus newnesi</i> (8)	21	4

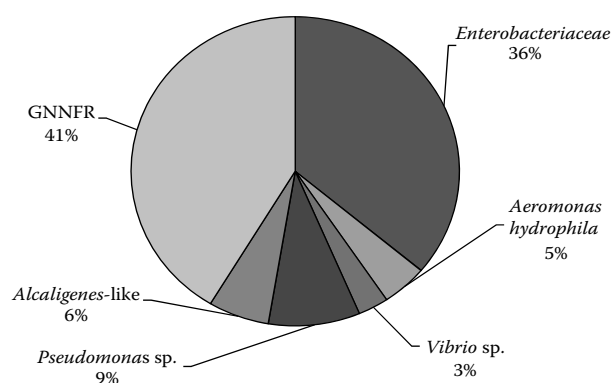


Figure 1. Composition of cultivable bacteria isolated from Nototheniidae fish intestine

GNNFR = Gram-negative non-fermenting rods

Fish intestine isolates classified to the species or genera level are listed in Figure 2. Cultivation-based method detected *Enterobacter* spp. as the predominant taxa in the intestine content of wild Nototheniidae fish. Concretely, the *Enterobacter cloacae* representatives were absolutely dominant ($36/48 = 75\%$ of the Enterobacteriaceae) among the enteric isolates identified. This *E. cloacae* phenon was biochemically slightly heterogeneous but all isolates shared biochemical profiles known for *E. cloacae* species description.

DISCUSSION

Bacterial flora isolates from intestinal tract have been described for a limited number of fish species (Cahill 1990; MacCormack and Fraile 1990; Ward et al. 2009). In the present study, the microflora of marine fish intestinal contents was investigated by a cultivation method. However, only two nutrient media (Endo agar, XLDA agar) and only one incubation temperature (16°C) were used for isolation,

and these conditions might have influenced the results. In respect to the opinion that it may be difficult to identify the representatives from the intestinal microbiota by using traditional morphological and biochemical criteria and without molecular identification (Spanggaard et al. 2000; Kim et al. 2007), we used this approach, due to limited equipment, for a pilot study done in the Antarctic station J.G. Mendel. We did not distinguish between the flora of the gut contents and that intimately associated with the wall of the gastrointestinal tract. Most fermenting isolates have been identified to the species level, which allowed recognition of particular species which may form a resident microflora in wild Nototheniidae fish intestine. The genera present in the gut contents generally seem to be those from the environment or food which can survive and multiply in the intestine tract of fish. Based on results described by Ringo et al. (2001) and Ward et al. (2009), the population abundance and diversity of bacteria in fish intestines is substantially lower, and the microbiota is simpler than that reported for warm-blooded animals, including humans. In our study we confirmed those findings because only about 3 different isolates per fish sample were proved (Table 1). The insufficiency of commercial systems, when testing environmental samples, has been discussed by several authors, and was reviewed by Popovic et al. (2007). As apparent, our study does not support the mentioned data and we have demonstrated that both used identification kits have been applicable for reliable identification of enteric bacteria from the normal intestinal bacterial flora in seawater fish.

The permanent intestinal microflora consisted of bacteria which are also commonly present in the surroundings, but which are able to persist and

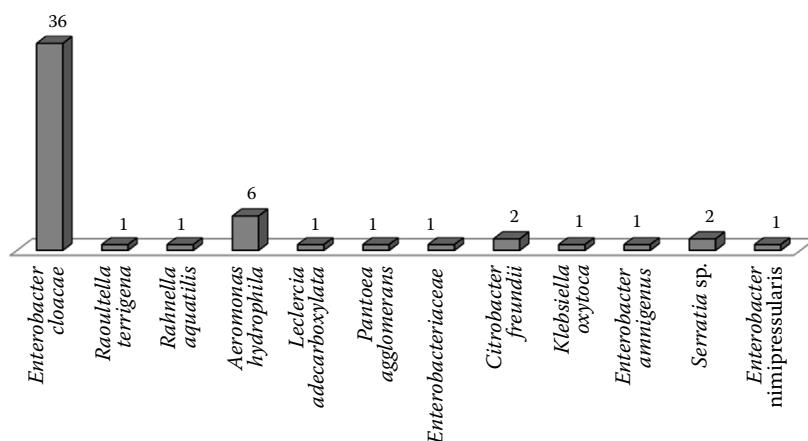


Figure 2. Identification results and numbers of isolated strains

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grow in the environment of the intestinal tract. In early publication (MacCormack and Fraile 1990) describing the gut microbiota of fish, it was reported that bacteria belonging to the genus *Vibrio* were one of the dominant genera. In the review by Ringo et al. (1995) the cultivable intestinal microbiota of salmonids was reported to consist of Gram-negative bacteria, where *Acinetobacter* spp., Enterobacteriaceae, *Aeromonas* spp., *Flavobacterium* spp., and *Pseudomonas* spp. were shown to be most common bacteria. But more recent investigations proved that *Aeromonas* species do not represent the dominant fish gut microbiota as believed earlier (Liu et al. 2008) what is in agreement with our results. It is well known that the intestinal microflora of fresh- and seawater fish harbours different microorganisms and for marine fishes vibrios were found as the dominant species and pseudomonads are also isolated in large numbers (MacCormack and Fraile 1990; Ringo and Strom 1994). In our study we did not prove many vibrios (Figure 1) in intestinal samples of fish probably due to used isolation media which were focussed on primarily isolation of enteric bacteria.

Results of Liu et al. (2008) indicate that *Enterobacter* is isolated from the digestive tract of fish from time to time, but the genus does not seem to belong to the dominant gut microbiota of fish. However our findings are in contradiction with the results of Liu et al. (2008) and clearly demonstrate *Enterobacter* spp. as the most common Enterobacteriaceae species in the intestine of wild marine Antarctic fish having predatory behaviour. We proved *Enterobacter cloacae* phenon as a typical representative of enteric bacteria in the intestine of Nototheniidae fish occurring in a prevailing amount of isolates. Of the 43 studied fish, twenty-five specimens were inhabited by this taxon in the number of 36 different isolates. This discovery is in agreement with the results of Ringo et al. (1995) and Wu et al. (2010) that members of the genus *Enterobacter* are common in the digestive tract of freshwater and marine fishes.

CONCLUSION

In this study a traditional microbial analysis and direct plate isolation methods were used. The intestinal microflora shows evidence of some selection of several species which can multiply in the conditions of this environment to form large

populations of facultative anaerobes. The investigation of the aerobic and facultative anaerobic heterotrophic intestinal microflora of Nototheniidae fish demonstrated that the majority of intestinal bacteria (56%) consisted of non-fermenters including *Pseudomonas* sp. and *Alcaligenes*-like isolates. *Enterobacter cloacae* phenon occurred as a prevailing and typical representative of fermenting rods in the intestine of Nototheniidae fish.

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