Int. J. Aquat. Biol. (2022) 10(1): 21-24
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Short Communication

Abundance and diversity of predominant sulfate-reducing bacteria in the gut of pufferfish

Yusuke Kishi, Yasuyuki Sasaki, Takuya Masuda, Kai Harumi, Shiro Itoi, Haruo Sugita*

Department of Marine Science and Resources, Nihon University, Kameino 1866, Fujisawa, Kanagawa 252-0880, Japan.

Abstract: Sulfate-reducing bacteria (SRB) exist in anaerobic environments, such as marine sediments, and produce hydrogen sulfide, which is toxic to marine animals. However, little is known about the ecology of SRB in the gut of fish. In the present study, we used molecular techniques to analyze the predominant SRB community in the gut of pufferfish inhabiting coastal areas of Japan. The density of the dissimilatory sulfite reductase alpha gene, *dsrA*, derived from SRB and total count of bacteria in guts of pufferfish was 4.4×10^6 - 1.8×10^7 copies/g and 3.5×10^8 - 6.3×10^9 cells/g, respectively, in all specimens. Clones of *dsrA* associated with *Desulfobulbus oligotrophicus*, a dominant SRB species, were detected in all 12 libraries, accounting for 57.7-94.3% of clones in each library. These results strongly suggest that SRB are indigenous bacteria in the gut of pufferfish and that hydrogen sulfide produced by SRB may be a risk factor for fish health.

Article history:
Received 1 December 2021
Accepted 25 January 2022
Available online 25 February 2022

Keywords:
Sulfate-reducing bacteria
Pufferfish
Gut bacteria
Clone library
Real-time PCR

Introduction

Sulfate-reducing bacteria (SRB) are widespread in marine environments and play an important role in the degradation of organic matter in many anoxic ecosystems (van der Wielen and Heijs, 2007; Ehrlich and Newman, 2008). Kondo et al. (2012b) showed the density of SRB in the sediment under fish farms correlated well with the content of organic matter in the sediment. In addition, it has been elucidated that SRB inhabits the intestines of humans and that hydrogen sulfide produced by them causes ulcerative colitis (Deplancke et al., 2000; Kushkevych et al., 2019). This suggests that hydrogen sulfide produced by SRB in the fish gut will cause some damage to fish. However, to our knowledge, the abundance and diversity of SRB in the fish gut have not yet been elucidated in detail, except for spotnape pony fish *Leiognathus nuchalit* (Chen et al., 2012). Therefore, to understand the SRB community structure in fish guts, we investigated the abundance of SRB by real-time PCR technology targeting the *dsrA* derived from SRB, as well as the diversity of the predominant SRB community in guts of the grass pufferfish *Takifugu alboplumbeus*, and tiger pufferfish *T. rubripes* collected in the coastal area, and as the comparison, in the raised tiger pufferfish for the better understanding the microbial community in fish guts.

Materials and Methods

Pufferfish were obtained by fishing in an unpolluted rocky area of Sagami Bay, Japan. The fish included four grass puffers (28.7-40.8 g), and four tiger puffers (21.9-26.5 g). Upon collection, the fish specimens were immediately euthanized by rapid cooling on ice. In addition, juvenile tiger puffers were purchased from Marinetech Co. (Aichi, Japan) and raised in a 50-L glass aquarium with a recirculating water system at 20±1°C; these animals were provided with *ad libitum* access to EP-1 commercial feed (Marubeni Nisshin Feed Co., Tokyo, Japan). Four of these juvenile tiger puffers (7.4-9.3 g) were euthanized by rapid cooling on ice after collection.

Fish guts were removed by aseptically dissecting the animals; the contents were recovered by squeezing out. An aliquot of each content sample was stained with 4′,6-diamidino-2-phenyl indole to determine the total counts of bacteria using a BX50 fluorescence microscope (Olympus, Tokyo, Japan) as described by

*Correspondence: Haruo Sugita
E-mail: sugita.haruo@nihon-u.ac.jp

Table 1. The dsrA densities and total counts of bacteria in the gut of puffer fish.

Fish (library names)	*dsrA* densities (copeis/g)	Total counts of bacteria (cells/g)
Grass puffer No. 1 (GP1)	1.4×10^7	3.5×10^8
Grass puffer No. 2 (GP2)	1.2×10^7	1.4×10^9
Grass puffer No. 3 (GP3)	4.4×10^6	5.8×10^8
Grass puffer No. 4 (GP4)	8.1×10^6	7.9×10^8
Raised tiger puffer No. 1 (RTP1)	1.8×10^7	4.9×10^8
Raised tiger puffer No. 2 (RTP2)	8.9×10^6	6.3×10^9
Raised tiger puffer No. 3 (RTP3)	5.0×10^6	4.1×10^9
Raised tiger puffer No. 4 (RTP4)	7.2×10^6	5.1×10^9
Wild tiger puffer No. 1 (WTP1)	6.5×10^6	5.1×10^9
Wild tiger puffer No. 2 (WTP2)	8.0×10^6	1.1×10^9
Wild tiger puffer No. 3 (WTP3)	6.0×10^6	1.2×10^9
Wild tiger puffer No. 4 (WTP4)	5.6×10^6	7.3×10^8

Porter and Feig (1980).

DNA was extracted from microbial cells in the gut contents using a QIAmp DNA Stool Mini kit (Qiagen, Hilden, Germany). Real-time PCR targeting *dsrA* was performed according to Kondo et al. (2008, 2012a) using a primer set of DSR-1F+ (5'-ACSCACTGGAAGCACGCCGG-3') and DSR-R (5'-GTGGMRCCGTGCAKRTTGG-3'). Real-time PCR cycling was performed in a Piko Real 96 Real-time PCR System (Thermo Scientific, Waltham, MA, USA).

The *dsrA* gene clone libraries were constructed according to the protocol of Kondo et al. (2008). The *dsrA* gene was amplified by PCR using a primer set of DSR-1F+ and PJdsr969R (5'-CATRTCGTCKY KCCAGGT-3') (Pérez-Jiménz and Kerkhof, 2005). The amplicons were cloned into the pGEM T-Easy vector (Promega, Madison, WI, USA) and the DNA inserts were sequenced according to Sugita et al. (2004) using DSR-1F+ and PJdsr969R primers and a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The sequence was resolved on a Model 3130*xl* automated DNA sequencer (Applied Biosystems). Each partial clone sequence (approximately 510 bp each) was compared with all of the sequence data in the DDBJ/GenBank/EMBL databases using the BLAST algorithm. Representative sequences from this study were deposited into the DDBJ/GenBank/EMBL databases (accession numbers: LC633977 – LC633983).

Results and Discussions

The *dsrA* sequences were detected in all 12 fish specimens, with *dsrA* densities ranging from 4.4×10^6 copies/g to 1.8×10^7 copies/g (Table 1). The total counts of bacteria ranged from 3.5×10^8 cells/g to 6.3×10^9 cells/g. Based on the results, and assuming that each cell harbors only one copy of the *dsrA* gene (Kondo et al., 2012b), densities of SRB were estimated to be 4.4×10^6 - 1.8×10^7 cells/g. The proportions of SRB among the total counts of bacteria were estimated to be 0.76 - 4.00% in the grass pufferr, 0.13 - 0.77% in the wild tiger puffer, and 0.12 - 3.67% in the raised tiger puffer.

As shown in Tables 2 and 3, a total of 341 clones in 12 libraries retrieved from the gut contents of grass puffer, wild tiger puffer, and raised tiger puffer were related to Desulfobulbaceae (289 clones), Desulfobacteraceae (23 clones), Desulfomicrobiaceae (23 clones), and Desulfovibrionaceae (6 clones). Clones of *dsrA* related to *Desulfobulbus oligotrophicus* (288 clones) were detected in all 12 libraries, accounting for 57.7 – 94.3% of the clones in each library. These results indicated that *D. oligotrophicus* is the dominant bacterium in all fish specimens examined in this study, and other bacterial species are distributed in 8.3-58.3% of specimens. The predominant sulfate-reducing bacterial flora in wild and raised specimens of tiger puffer also were found to be quite similar.

Table 2. Distribution of SRB species in the clones of the libraries constructed from the gut of grass puffer.

Closest species (accession no.; similarity, %)	No. of clones in each library			
	GP1[a]	GP2	GP3	GP4
Desulfosarcina widdelii (AP021875; 80.9-81.8)	6	1	2	0
Desulfobulbus oligotrophicus (CP054140; 76.0-77.4)	15	23	21	37
Desulfomicrobium salsuginis (AM4936921; 72.4-73.0)	1	0	0	0
Desulfomicrobium thermophilum (AH015806; 71.7-72.4)	3	0	1	2
Desulfovibrio aerotolerans (AY749039; 73.8-74.2)	1	2	0	1
Total	26	26	24	40

[a] Refer to the library name in Table 1.

Table 3. Distribution of SRB species in the clones of the libraries constructed from the gut of wild and raised tiger puffer.

Closest species (accession no.: similarity,%)	No. of clones in each library							
	Wild specimens of:				Raised specimens of:			
	WTP1[a]	WTP2	WTP3	WTP4	RTP1	RTP2	RTP3	RTP4
Desulfosarcina alkanivorans (AP021874;84.4-84.6)	1	0	0	1	0	0	0	0
Desulfatiferula olefinivorans (DQ826725; 71.5)	0	2	0	0	1	0	0	0
Desulfosarcina widdelii (AP021875; 80.9-81.5)	1	2	0	5	0	0	1	0
Desulfobulbus oligotrophicus (CP054140; 71.5-77.7)	24	20	17	24	33	26	31	17
Desulfopila inferna (FJ548990; 80.5)	0	0	0	0	0	0	0	1
Desulfomicrobium escambiense (AB061531; 71.4)	0	1	0	0	0	0	0	0
Desulfomicrobium salsuginis (AM493692; 71.4-73.4)	0	2	2	2	0	0	0	1
Desulfomicrobium thermophilum (AH015806; 70.5-78.5)	2	0	0	1	0	3	2	0
Desulfovibrio aerotolerans (AY749039; 74.2)	0	0	1	0	1	0	0	0
Total	28	27	20	33	35	29	34	19

[a] Refer to the library name in Table 1.

Desulfobulbus oligotrophicus was first described in 2017, when this species was isolated from a municipal anaerobic sewage sludge digester; this bacterium was reported to have an optimal NaCl concentration of 2-5 g/L (Houari et al., 2017), suggesting that it can grow well in marine environments, including fish guts.

Moreover, we previously reported that 32 clones retrieved from the gut of spotnape ponyfish were related to the Desulfobulbaceae (13 clones), Desulfobacteraceae (13 clones), Desulfomicrobiaceae (3 clones), and Desulfovibrionaceae (3 clones) families. These sulfate-reducing bacterial families have been reported to be widespread in sediments along the coast of Japan and in the East China sea (Kondo et al., 2012a; Zhang et al., 2017). Those results, along with the present data, suggest that members of the family Desulfobulbaceae are widely distributed in the fish guts and sediments. In addition, these facts suggest that SRB in the guts of coastal fish may be derived from sediments via seawater and food.

Generally, marine fish drink 10% or more of their body weight in seawater per day for the adjustment of their osmotic pressure, but divalent ions, such as sulfate ions, reach the intestine without being absorbed by chloride cells, and subsequently are excreted with the feces (Wedemyer, 1996). Therefore, sulfate ions are expected to be abundant in the intestinal environment. SRB are obligate anaerobes that use sulfate ions as electron acceptors, and hydrogen or organic acid as electron donors; notably, organic acids may be produced by the gut microbiota. As hydrogen sulfide is toxic to fish (Wedemyer,

1996), we cannot rule out the possibility that hydrogen sulfide produced by SRB in the gut may cause the similar symptoms in fish that are observed in humans, as described earlier (Deplancke et al., 2000; Kushkevych et al., 2019). These results strongly suggest that SRB are indigenous bacteria in the gut of pufferfish and that hydrogen sulfide produced by SRB may be a risk factor for fish health.

Acknowledgment

This study was supported in part by a 2018 research grant from The Toyo Suisan Foundation (*formerly* The Towa Foundation for Food Science and Research).

References

Chen C.H., Sagara K., Itoi S., Sugita H. (2012). Sulfate–reducing bacteria in the intestinal tracts of spotnape ponyfish *Leiognathus nuchalis*. Aquaculture Science, 60: 519-521.

Deplancke B., Hristova K.R., Oakley H.A., McCracken V.J., Aminov R., Mackie R.I., Gaskins H. R. (2000). Molecular ecological analysis of the succession and diversity of sulfate-reducing bacteria in the mouse gastrointestinal tract. Applied and Environmental Microbiology, 66: 2166-2174.

Ehrlich H. L., Newman D. K. (2008). Geomicrobiology, 5th. CRC Press. 628 p.

Houari A.E., Ranchou-Peyruse M., Ranchou-Peyruse A., Dakdaki A., Guignard M., Idouhammou L., Bennisse R., Bouterfass R., Guyoneaud R., Qatibi A-I. (2017). *Desulfobulbus oligotrophicus* sp. nov., a sulfate-reducing and propionate-oxidizing bacterium isolated from a municipal anaerobic sewage sludge digester. International Journal of Systematic and Evolutionary Microbiology, 67: 275-281.

Kondo R., Shigematsu K., Butani J. (2008). Rapid enumeration of sulphate-reducing bacteria from aquatic environments using real-time PCR. Plankton & Benthos Research, 3: 180-183.

Kondo R., Mori Y., Sakami T. (2012a). Comparison of sulphate-reducing bacterial communities in Japanese fish farm sediments with different levels of organic enrichment. Microbes and Environments, 27: 193-199.

Kondo R., Shigematsu K., Kawahara N., Okamura T., Yoon Y. H., Sakami T., Yokoyama H., Koizumi Y. (2012b). Abundance of sulfate-reducing bacteria in fish farm sediments along the coast of Japan and South Korea. Fisheries Science, 78: 123-131.

Kushkevych I., Dordević D., Kollar P., Vítězová M., Drago L. (2019). Hydrogen sulfide as a toxic product in the small–large intestine axis and its role in IBD development. Journal of Clinical Medicine, 8: 1054.

Perez-Jimenez J.R., Kerkhof L.J. (2005). Phylogeography of sulfate-reducing bacteria among disturbed sediments, disclosed by analysis of the dissimilatory sulfite reductase genes (*dsrAB*). Applied and Environmental Microbiology, 71: 1004-1011.

Porter K.G., Feig Y.S. (1980). The use of DAPI for identifying and counting aquatic microflora. Limnology and Oceanography, 25: 943-948.

Sugita H., Nakamura H., Shimada T. (2004). Microbial communities associated with filter materials in recirculating aquaculture systems of freshwater fish. Aquaculture, 243: 403-409.

Wedemeyer G. A. (1996). Physiology of fish in intensive culture systems. Chapman and Hall. 249 p.

Van Der Wielen P.W. J. J., Heijs S.K. (2007). Sulfate-reducing prokaryotic communities in two deep hypersaline anoxic basins in the Eastern Mediterranean deep sea. Environmental Microbiology, 9: 1335-1340.

Zhang Y., Wang X., Zhen Y., Mi T., He H., Yu Z. (2017). Microbial diversity and community structure of sulfate-reducing and sulfur-oxidizing bacteria in sediment cores from the East China sea. Frontiers in Microbiology, 8: 2133.