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Short Communication

Oxygen tolerance of *Cetobacterium somerae* isolated from the gut of freshwater fish and their environments

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Abstract: *Cetobacterium somerae* is a predominant bacterium found in the gut of freshwater fish. However, being an anaerobic bacterium, its survival is believed to be prevented in oxygen-rich environments. Therefore, in this study, we investigated the oxygen tolerance of 83 *C. somerae* strains collected from the guts of freshwater fish, rearing water, and sediment of culture ponds and tanks. When placed in sterile bottles containing common carp- and goldfish-rearing water, *C. somerae* showed a 1-log decrease after 24 hours, suggesting that this organism faces challenges in growing in rearing water. Subsequently, we inoculated the bacterial strains onto agar plates and exposed them to air for 12 hours to measure the oxygen inhibition index (OII). The OII values ranged from 0.01 to 4.65 among different strains, indicating significant variation in oxygen tolerance within the bacterium. Furthermore, the OII values varied considerably depending on the isolation source, with sediment, rearing water, and gut samples showing increasing values in that order. This suggests that oxygen tolerance plays a substantial role in the ecological behavior of *C. somerae*.

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Introduction

Trust et al. (1976) reported the presence of obligate anaerobic bacteria, including *Actinomyces*, *Bacteroides*, *Eubacterium*, *Fusobacterium* and *Peptostreptococcu*s, in the gut of rainbow trout, *Oncorhynchus mykiss*, grass carp, *Ctenopharyngodon idella* and goldfish, *Carassius auratus.* Sakata et al. (1980, 1981) also discovered anaerobic bacteria widely distributed in the gut of several freshwater fishes, such as Nile tilapia, *Oreochromis niloticus*, common carp, *Cyprinus carpio*, goldfish, and ayu, *Plecoglossus altivelis*, and they provisionally named it *Bacteroides* type A. Furthermore, Finegold et al. (2003) isolated a novel gram-negative, microaerotolerant, non-spore-forming, rod-shaped bacterium from human infant feces and proposed it as *Cetobacterium somerae*. Subsequently, Tsuchiya et al. (2008) confirmed that *Bacteroides* type A was, in fact, identical to *C. somerae.* Since then, studies using culture-independent methods such as the clone library analysis and next-generation sequencing (NGS) have revealed that *C. somerae* is predominant in the gut of many freshwater fishes (Sugita and Mizuki, 2012; Yi et al., 2016).

On the other hand, it is known that fish are generally sterile inside their eggs, and after hatching, they grow by acquiring various bacteria from the environment, including water, bottom sediment, and food, ultimately establishing an adult-type gut microbiota (Sugita et al., 1988; Cahill, 1990; Kurosaki et al., 2021). This suggests that anaerobic bacteria surviving in an oxygenated environment invade the gut of fish by some route and become established there. However, since the predominant anaerobic bacterium in the gut of freshwater fish is *C. somerae*, which does not form spores, oxygenated environments are considered unsuitable for this bacterium. In this study, therefore, we investigated the oxygen tolerance of *C. somerae* isolated from the gut of freshwater fish, rearing water, and bottom sediments to better

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Table 1. Changes in dissolved oxygen (DO) concentrations and viable counts of *Cetobacterium somerae* in the water from common carp- and goldfish-rearing tanks during 48 hr-incubation.

Fish	Time (hr)	DO mg/L	Aerobes CFU/mL	*C. somerae* CFU/mL
Common carp	0	3.20	7.6×10^5	8.0×10^2
	24	2.71	1.3×10^6	2.0×10^1
	48	2.58	1.6×10^6	2.0×10^1
Goldfish	0	3.15	4.8×10^4	5.0×10^2
	24	2.62	1.2×10^5	3.8×10^1
	48	2.07	1.1×10^5	7.0×10^1

understand the ecology of gut bacteria in fish.

Materials and Methods

Behavior of *C. somerae* in fish rearing water: Two pairs of sterile100 mL-brown glass bottles were each filled with 80 mL of rearing water from common carp and goldfish culture tanks (800 L) using the recirculated aquaculture system, and placed in an incubator at 25°C under aerobic conditions for 48 hours. Dissolved oxygen in one set of rearing water was measured using a YSI 85 Series dissolved oxygen meter (YSI Inc., Yellow Springs, OH, USA). For the other pair, water samples were collected at 0, 24 and 48 hours after incubation and examined bacteriologically. That is, the water was serially diluted with anaerobic diluents, GAM (Gifu anaerobic medium, Eiken Chemical, Tokyo, Japan) broth, inoculated onto TSA (trypticase soy agar, BD, MD, USA) and 1/3 NBGT (neomycin-brilliant green-taurocholate) blood agar medium (Sakata et al., 1980a), and incubated at 25°C for 5 days under aerobic and anaerobic conditions, respectively. After incubation, the grown colonies were counted and viable counts (CFU, colony forming units/mL) were determined. For the 1/3 NBGT blood agar medium, 20 colonies per sample were picked up and classified into simple categories based on their growth ability under aerobic conditions, Gram staining, cell morphology, and spore-forming ability as reported previously (Sugita et al., 1988). Anaerobic conditions were established by evacuating the atmosphere of an anaerobic jar containing steel wool activated by acidic cupric sulfate, and replacing it with a 20% CO_2–80% N_2 gas mixture as reported by Sugita et al. (1988).

Oxygen tolerance of *C. somerae* strains: Eighty-three strains of *C. somerae* were used from the culture collection of our laboratory. These strains were obtained from the gut of Nile tilapia, common carp, goldfish, grass carp, and Amur catfish, *Silurus asotus*, as well as from the rearing water and bottom sediments of their culture tanks and ponds. To evaluate the oxygen tolerance of *C. somerae*, each strain was inoculated into GAM broth, and incubated under anaerobic conditions at 25°C for 24 hours. The cell density of cultures was then adjusted to an optical density of 0.1 at 655 nm with GAM broth. Subsequently, the bacterial cultures were further serially diluted in GAM broth and inoculated on five sets of GAM agar medium. Bacterial cells on these agar media were exposed to the atmosphere for 0-48 hours and then incubated again anaerobically at 25°C for 5 days to determine viable counts.

Results and Discussions

Behavior of *C. somerae* in fish-rearing water: The behavior of aerobic bacteria and *C. somerae* when common carp-rearing water was exposed to atmospheric conditions for 48 hours is shown in Table 1. Dissolved oxygen concentration decreased from 3.2 mg/L at the beginning of the experiment to 2.7 mg/L after 24 hours and 2.6 mg/L after 48 hours. Aerobic bacteria (including facultative anaerobic bacteria) increased from 7.6×10^5 CFU/mL at the beginning of the experiment to 1.3×10^6 and 1.6×10^6 CFU/mL after 24 and 48 hours, respectively. In contrast, *C. somerae* decreased from 8.0×10^2 CFU/mL at the beginning of the experiment to 2.0×10^1 CFU/mL after 24 and 48 hours, respectively. Furthermore, the dynamics of

Table 2. Distribution of OII values in strains of *Cetobacterium somerae* isolated from the fish guts, water and sediments.

OII range	*Cetobacterium somerae*		
	Guts	Water	Sediments
0.00-0.99	5	14	24
1.00-1.99	10	7	5
2.00-2.99	6	4	2
3.00-3.99	3	1	0
4.00-4.99	1	1	0
Mean±STD*	1.90±1.11[a]	1.13±1.16[b]	0.65 ± 0.64[b]

*Data with the different superscript significantly differ from each other (P<0.05).

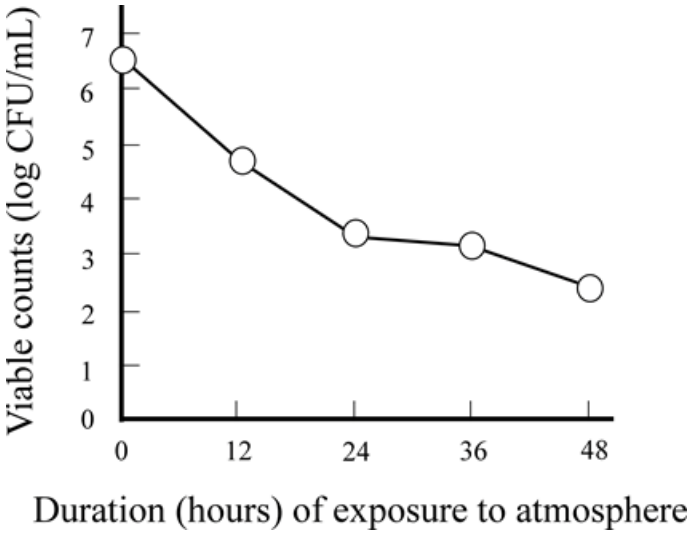

Figure 1. Viable counts of *Cetobacterium somerae* i43 strain on GAM agars exposed to the atmosphere for 48 hours.

dissolved oxygen concentrations and the density of aerobic and obligate anaerobic bacteria in the bottle containing goldfish-rearing water were similar to those in the common carp-rearing water.

These experiments showed that in water with 2-3 mg/L of dissolved oxygen, aerobic bacteria increased by an order of magnitude after 24-48 hours, while *C. somerae* decreased by an order of magnitude after 24 hours. The results suggest that obligate anaerobes such as *C. somerae* have difficulty maintaining populations in common carp-and goldfish-rearing water unless they are supplied by another source, such as fish feces. Sugita et al. (1985, 1998) also reported the significant impact of fish feces on bacterial communities in rearing water.

Oxygen tolerance of *C. somerae* strains: The *C. somerae* i43 strain was inoculated on GAM agar medium, and viable counts immediately before exposure to the atmosphere (0 hours) was 4.0×10^6 CFU/mL, which decreased to 5.2×10^4 CFU/mL after 12 hours of exposure (Fig. 1). This fact strongly suggests that 98.7% of viable cells were damaged by intracellularly generated reactive oxygen species such as superoxide radical, hydroxyl radical and hydrogen peroxide after 12 hours of air exposure and were dead or in the process of dying (Madigan et al., 2017). The percentage of dead cells increased with increasing duration of exposure to the atmosphere. However, since the percentage of viable cells varied widely among strains, this study used the oxygen inhibition index (OII) as a measure to determine the oxygen tolerance of each strain.

To determine the OII, the logarithm of the viable count (log CFU/mL) after 0 hours–exposure to the atmosphere was subtracted from the logarithm of the viable count (log CFU/mL) after 12 hours–exposure. Strains with lower OII values indicate higher oxygen tolerance. The OII values of the 83 strains of *C. somerae* tested ranged from 0.01 to 4.65, with a mean of 1.18±1.10 (±STD), indicating significant variation in oxygen tolerance among the strains. When these values were grouped according to their sources, the results were summarized as shown in Table 2. The OII values averaged 1.90 (range: 0.05-4.02) for the strains derived from fish guts, 1.13 (range: 0.01-4.65) for those from rearing water, and 0.65 (range: 0.04-2.19) for those from sediment. This suggests that OII values generally increased in the order of bottom sediment, rearing water, and gut, indicating a decreasing level of aerotolerance.

The results strongly suggest that among the *C. somerae* cells excreted into the rearing water along with feces, less aerotolerant cells rapidly perish, while aerotolerant cells remain in the water and sediment for

a certain period of time and occasionally spread to other fish to become established there. Furthermore, if environmental conditions such as reducing conditions and organic matter concentrations are met, these cells may proliferate to some extent in the bottom sediment.

Finegold et al. (2003) reported *C. somerae* as microaerotolerant; nevertheless, the observed broad range of oxygen tolerance in this bacterium indicates that it should be regarded as encompassing a spectrum from microaerotolerant to obligate. This suggests that the cellular-level oxygen tolerance of *C. somerae* might have a substantial influence on its distribution within aquaculture environments, a notion that is exceptionally intriguing.

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