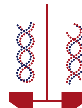

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Effect of intermittent opening of breathable culture plugs and aeration of headspace on the structure of microbial communities in shake-flask culture

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In this study, we found that opening breathable culture plugs for 30 s during periodic and aseptic sampling affects the community structure of cultured soil microbes. Similar effects were observed using an automatic aeration flask system that mimics aseptic opening of the breathable culture plug during sampling, but without interruption in shaking. Thus, the observed changes in the microbial consortia appear to be due exclusively to the intermittent ventilation of the flask headspace. To elucidate the mechanism driving this phenomenon, we monitored CO_2 and O_2 concentrations in both headspace and culture broth using the new system termed as circulation direct monitoring and sampling system. The data show that the CO_2 concentration in the culture broth temporarily decreased with the CO_2 concentration in the headspace, strongly suggesting that the effect of intermittent ventilation of the headspace on the microbial consortia depends on CO_2. Importantly, the data also imply that environmental variables during shake flask culture, especially CO_2 concentration, is important for screening aerobic microorganisms.

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[**Key words:** Aerobic culture; Batch culture; Circulation direct monitoring and sampling system; Denaturing gradient gel electrophoresis; Dissolved carbon dioxide; Dissolved oxygen; Gaseous carbon dioxide; Gaseous oxygen; Sampling; Shake-flask culture]

Soil was reported to contain 2×10^9 bacterial cells/g, of which only 10^5 cells/g or less are dominant, although 8×10^6 different strains may be present (1). Of note, only about 0.001–15 % of all microorganisms in nature can be isolated and cultivated using standard methods (2). Accordingly, several new methods were developed to investigate uncultured microorganisms. For example, a *Nitrospira* microcolony was isolated without cultivation by selective separation on a cell sorter (3,4). Similarly, *in situ* cultures were attempted to mimic the natural environment (5–9). On the other hand, previously uncultivable microorganisms have also been isolated and cultured by simply optimizing variables during standard culture, including duration, medium composition, gelling agent, and signal compounds (10–16). In addition, cultivation of new microorganisms under micro and high gravity was investigated (17,18). Collectively, these studies suggest that several variables during standard culture remain to be exploited in attempts to cultivate uncultured microorganisms.

In this study, we sought to develop a method to search for and isolate uncultured microorganisms by periodic, unobtrusive sampling of environmental specimens cultured in shake-flasks (19), which have been widely used for more than 80 years. Strikingly, we observed that sampling significantly influences the microbial community structure of soil samples cultured at 50°C in YPD medium. To the best of our knowledge, we are the first to report that intermittent, aseptic opening of culture plugs affects the microbial community structure in shake-flasks. We note that sampling is generally believed to have minimal impact so long as contamination is prevented, since the culture plug is opened only very briefly relative to the total duration of culture.

An automatic aeration flask system was also constructed to mimic the aseptic ventilation of the flask headspace during intermittent sampling, but without interruption of shaking. Since periodic aeration was likely to affect gas concentrations in the headspace and in the culture broth, the system was used in conjunction with circulation direct monitoring and sampling system (CDMSS) (20) to measure gaseous and dissolved CO_2 and O_2 in real time, also without interruption of shaking. The goal was to systematically and robustly analyse the effect of intermittent operations on microbial community structures in shake flasks, and to assess the significance of such operations in screening for microorganisms.

MATERIALS AND METHODS

Soil samples A soil sample was obtained 10 cm below the surface, and debris such as stones and plant roots were removed using tweezers. Approximately 2 g of the sample was added to a 500 mL Erlenmeyer flask containing 100 mL distilled water, and shaken for 3 h at room temperature on a rotary shaker operating at 120 rpm and 70 mm shaking diameter. Glycerol stocks for subsequent experiments were prepared by adding glycerol to a final concentration of 40 % v/v, and stored at −80°C.

Shake flask cultures Glycerol stocks (1 mL) were inoculated into 500 mL Erlenmeyer flasks containing 100 mL of autoclaved YPD medium (21,22) pH 7.0, composed of 20 g/L polypeptone, 10 g/L yeast extract, and 10 g/L D-glucose. Flasks were capped with breathable BIO-SILICO N-38 sponge plugs (Shin-Etsu Polymer Co., Ltd., Tokyo, Japan), and handled on a clean bench. The cultures were incubated at 50°C on a rotary shaker operating at 200 rpm and 70 mm (shaking diameter) in the dark.

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Opening of culture plugs and sampling Shake-flask cultures listed in Tables 1 and 2 were grown at the same time and on the same shaker, sampled, and/or opened as indicated. Samples were collected by moving culture flasks to a simple glove box set near the shaker, and withdrawing 1.0 mL through opened plugs, unless otherwise specified. To mimic ventilation due to periodic sampling, culture plugs were simply opened in the glove box, aseptically and without flame sterilization. Shaking was necessarily interrupted during opening and/or sampling through opened plugs, although the timing and duration of the interruption was the same for all flasks. In control conditions, a sample was also collected without plug opening from an Erlenmeyer flask fitted with a highly airtight stopper and a small pipe that did not make contact with the culture broth. However, a 22-gauge needle that could reach the culture broth was also passed through the stopper as sampling port. The tip of the needle exposed to the atmosphere was covered with aluminium foil throughout the experiment, and flame-sterilized with 70 % ethanol before drawing 1 mL of the culture broth via a disposable syringe. We note that the same amount of sample was collected from all flasks, and that the temperature of the culture broth decreased 1°C or less before and after sampling.

Automatic aeration flask system An automatic aeration flask system was constructed to aseptically aerate the flask headspace, and thus mimic the ventilation by opening of culture plugs during sampling while also eliminating manual tasks (Fig. 1). The system was built to operate with the existing shaker and Erlenmeyer flasks, and could be freely set to supply gas from a source for 10 sec–24 h at 0–500 mL/min. The system is fitted to the flask via a 21-gauge needle inserted into the BIO-SILICO N-38 sponge plug, the air permeability of which was about 4000 mL/min. To prevent contamination, the needle was plugged with 0.22 μm hydrophobic filters (Advantec Co., Ltd., Ehime, Japan) (Fig. 1). At 9.75 and 13.75 h of shake-flask culture, the system was used to deliver fresh air into the headspace for 30 s at 500 mL/min without interruption in shaking. Excessive increase in air pressure and contamination were not observed during the course of the experiment. We note that the flask was also connected to CDMSS (20) via bottom and side branches that were beyond the level of the culture broth during shaking (Fig. 1).

Monitoring and sampling by CDMSS Gaseous and dissolved CO_2 and O_2 during shake-flask culture were monitored in real time by CDMSS (20), which also collected 1.5 mL samples at 3, 6, 9, 12, and 15 h of culture, without interruption in shaking (Fig. 2). To minimize changes in culture volume, the sampling volume was selected so that the total volume of all samples collected was <10 % of the initial culture medium. We note that automatically aerated test cultures were initiated with the same CDMSS, Erlenmeyer flask with branches, and culture plug, but within 30 h after the end of control cultures without automatic aeration. We also assumed that long-term cryopreservation of soil specimens between cultures did not significantly impact subsequent experiments.

Analysis of microbial community Samples collected during shake-flask culture were analysed by denaturing gradient gel electrophoresis (DGGE) of 16S rDNA for 18 h at 60 V and 60°C, as previously described (17). The gel contained a gradient of 35–65 % denaturant in the direction of electrophoresis, 100 % denaturant being 40 % v/v formamide and 7 M urea. Several resolved bands were sequenced at Premix Sequence (Takara Bio Inc., Shiga, Japan), and compared by BLAST (23,24) against reference sequences in DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/).

Accession numbers Partial 16S rDNA sequences were deposited in DNA Data Bank of Japan under accession numbers LC314580, LC314581, LC314582, LC314583, and LC314584 for strains AC-0, AC-02, AC-15, CC-16, and AC-21, respectively.

All experiments were performed in duplicate. Results are plotted as means in Fig. 3–8, and were confirmed to be highly reproducible.

RESULTS AND DISCUSSION

As shown in Fig. 3, sampling operations during shake-flask culture altered the microbial community structure in cultured soil samples. Such sampling operations consisted of removing the Erlenmeyer flask from the shaker platform, taking to a clean bench, and collecting 1 mL of culture broth. Thus, the observed changes in microbial structure can be attributed only to the temporary drop in temperature, interruption of shaking, opening of the breathable cultured plug, or decrease in the volume of culture broth. We were able to essentially exclude the effect of temperature, since the decrease in culture broth temperature was only 1°C or less before and after sampling. We were also able to exclude the effect of volume changes by collecting only <10 % of the initial volume over the course of the experiment. We were also able to collect samples without interruption of shaking using automated systems. Thus, we focused on the effect of opening the breathable culture plug, a required step in manual sampling of shake-flask cultures, but one that would also temporarily ventilate the flask headspace. Of note, different enriched communities of the same soil sample were obtained from normal or continuously aerated flasks (25). Accordingly, the volatilization rate constant k_V has been proposed as a quantitative index of the effect of ventilation on aerated fermentation systems (26). For example, k_V was lower for an Erlenmeyer flask plugged with cotton than for a jar fermentor (26).

The microbial community structure of conditions A and D changed in the same culture between 24 and 48 h. But, in the case of the conditions B and C, it did not change (Fig. 4), suggesting that

TABLE 2. Culture plug opening for 30 s and/or sampling of shake-flask cultures in second set of experiments.

Experiment	Culture time (h)					
	3.75	5	7.5	10	11.25	15
A (control)						b
B			a			b
C		a		a		b
D	a		a		a	b

a, opening of culture plug only; b, sampling with culture plug opened.

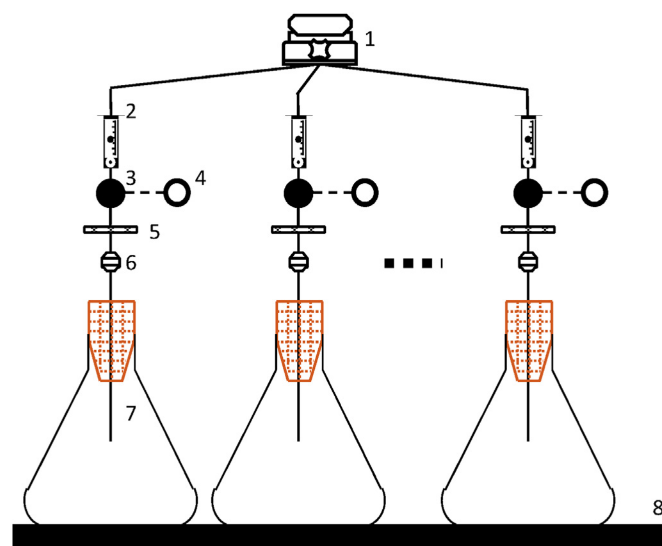

FIG. 1. Schematic diagram of the automatic aeration flask system (AAFS). 1, air compressor; 2, flow meter; 3, solenoid valve; 4, control system; 5, filter; 6, connector; 7, needle; 8, shaking table.

TABLE 1. Culture plug opening for 30 s and/or sampling of shake-flask cultures in first set of experiments.

Experiment	Culture time (h)															
	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
A (control)								c								b
B				a				b				a				b
C		a		a		a		b		a		a		a		b
D	a	a	a	a	a	a	a	b	a	a	a	a	a	a	a	b

a, opening of culture plug only; b, sampling with culture plug opened; c, sampling without opening of culture plug.

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