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SHORT COMMUNICATION

A Method for Cell Culture and Maintenance of Ammonia-Oxidizing Archaea in Agar Stab

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Abstract Ammonia oxidizing archaea (AOA) are predominantly found and closely linked with geochemical cycling of nitrogen in non-extreme habitats. However, these strains have mainly been investigated using liquid cultures of enriched cells. Here, we provide an agar stab as a simple and reliable means of cultivating and maintaining AOA.

Keywords Ammonia-oxidizing archaea · Agar stab · Cultivation · Immunofluorescence staining

Archaea are conventionally recognized as being able to live in unusual habitats such as hot, acidic and high-salt conditions. This perspective, however, devalues the functional roles of these strains in ecosystems, particularly in the geochemical cycling of various compounds in nature. Recent molecular ecological techniques provides a way of surveying microbial diversity using rRNA sequences retrieved directly from environmental samples. Thereafter, novel archaeal genotypes have been discovered in various habitats using archaea-specific PCR primer sets or enrichment cultures, and then continually found in many diverse non-extreme environments such as soils, marine sediments, marine and deep subsea floor sediments [1, 2]. Thus, a lot of researches are beginning to understand their functional roles in ecosystems. Among them, nitrifying organisms that were previously thought to be restricted to a few groups within the β- and γ-proteobacteria are highly abundant and seem to be mainly related to the non-extremophilic Crenarchaeota (ammonia-oxidizing archaea, AOA) [3]. This result was further supported by the discovery of archaeal *amoA* genes encoding key enzymes of the ammonia oxidation from environmental metagenome studies [4], although the transcription of these genes was relatively less active than those of bacteria [5]. Consequently, much evidence indicates that AOA are also plausible contributors with ammonia-oxidizing bacteria to nitrification of the microcosm. A recent study has reported strong evidence that AOA are real contributors for N_2O production from ammonia oxidation [6].

Currently, the detailed biogeochemical functions of AOA in non-extreme environments remain to be further elucidated. The most direct way to address these questions is to identify factors associated with these functions through a reliable culture method. However, AOA are mainly cultivated through liquid media of enriched cells (marginally co-cultured with few bacterial species), as reported previously [7–9]. Although these methods could provide relatively high levels of purities, an alternative method is still needed to provide more information (physiological and biochemical aspects) from various culture conditions. We here provided a simple method for cultivating AOA using agar stab as a medium. This method was established, based on the fact that favorable growth had been observed when AOA were cultured in static and microaerobic conditions [8, 9]. We presumed that an agar stab could provide these conditions to AOA reliably.

Nitrosopumilus sp. AR, a previously enriched strain from marine sediments, was used to establish the AOA culture in agar stabs. Enriched AR cells were grown microaerobically at 25 °C in artificial seawater (ASW) media [8]. After successive biweekly transfers of enriched cells into the same medium, grown cells were confirmed by

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decreasing the concentration of ammonia (as an energy source) and concomitantly increasing nitrite using a colorimetric assay [8]. These cells were then used as a seed for agar stab culture.

The solid media for stab culture was prepared as follows: pre-warmed 2 × ASW medium was mixed (1:1) with the autoclaved 0.6 % low melting point (LMP) agarose solution at 45 °C. During the mixing process, N_2 gas was sparged for 3 min to reduce the oxygen concentration in the medium. An aliquot (4.5 ml) of the resulting mixture was dispensed into a 5 ml vial (flat-bottom, Wheaton) and allowed to solidify at room temperature for 2 h. Grown cells (2×10^6 cells) in liquid culture were harvested by centrifugation at 6000×*g* for 15 min and re-suspended in 20 µl of ASW medium, then inoculated deeply into the medium of vials using a glass micropipette. As a control, the same number of cells was inoculated into liquid media. Each culture was examined in triplicate.

As generally well known, the cell growth of AR in liquid media was saturated using ammonia (1 mM) as an energy source when incubated for ~15 days, but invisible [8]. Interestingly, colonized cells around the inoculated area in the agar stabs were faintly observed after the incubation for 15 days at 25 °C (Fig. 1a). Growth of cells was reproducibly observed when they were successively transferred from the agar stabs to other stabs. Subsequent analyses showed that about 70 % of ammonia was consumed by cells grown in agar stab. We further attempted to confirm whether the grown cells were AOA or co-existing bacterial cells.

To separate the cells from the agar stab culture (15 days), the agar containing grown cells was mixed with 1 N NaI (13.5 ml) and heated to 55–60 °C for 10 min. The suspended cells were harvested by centrifugation at 13,000×*g* for 20 min and washed twice with a PBS buffer. A phenotype analysis was conducted using fluorescence microscopy by staining the resulting cell with a LIVE/DEAD staining kit (Invitrogen, OR, USA) for 10 min, according to the manufacturer's protocol. Images were observed using U-FBW and U-FGW filters on a BX43TF microscope (Olympus, Tokyo, Japan). As shown in Fig. 1b, over 70 % of the cells from the agar stab fluoresced green (live stain) and 90 % of the cells revealed a relatively small size (<1.0 µm). These characters were identical to those of the cells from the liquid culture and further confirmed by fluorescence in situ hybridization (FISH) using an archaea-specific probe, according to a protocol established previously [8], although the images were partly obscured due to the presence of residual agar and NaI (data not shown). When conducted the same experiment after the pretreatment with agarase or using desalting column such as a centricon (Plus-70, Millipore, Germany), the quality of FISH images was further improved but insufficient. The counting directly by fluorescent activated cell sorter using stained cells also revealed a similar number of cells ($1.26 \pm 0.37 \times 10^7$ cells/ml) to

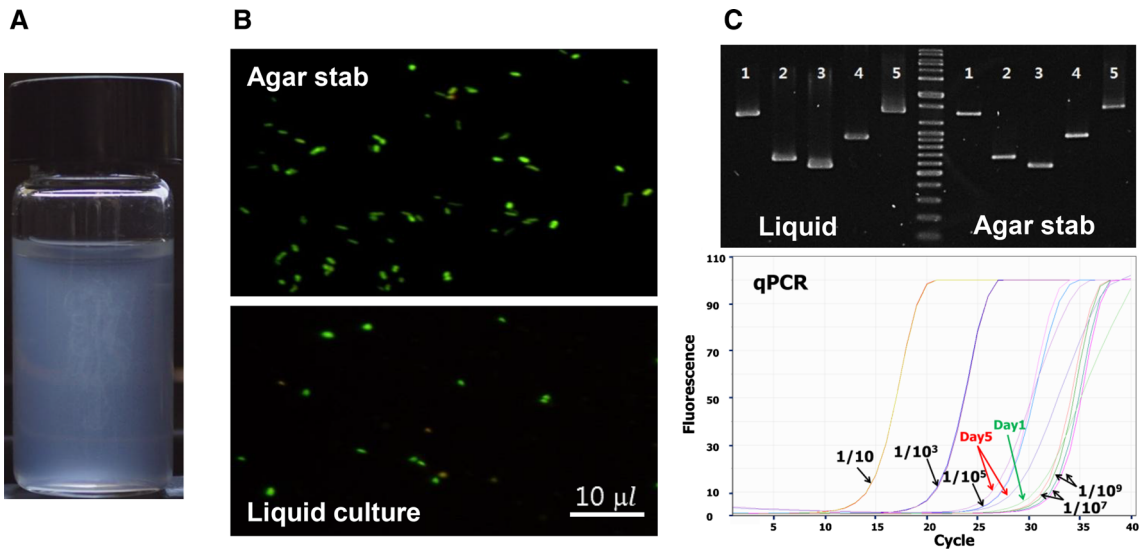

Fig. 1 Microscopic imaging and PCR analyses of archaeal cells grown in agar stab. **a** *Nitrosopumilus* sp. AR cells were cultivated in agar stabs at 25 °C for 15 days. **b** Cells grown in agar stab for 15 days were stained with a mixture of SYTO 9 and propidium iodide for 10 min to analyze the phenotype. **c** PCR with specific primers was conducted using cells grown in agar stab and liquid culture for 5 days (*upper panel*: lane 1, archaeal 16S rRNA; 2, *nirK*; 3, *amoA*; 4, *amoB*; 5, bacterial 16S rRNA), and quantitative PCR was also conducted using cells grown in agar stab for 5 days (*lower panel*). The diluted genomic DNA from cells grown in liquid media and cells inoculated into agar stab were used as a control

that from the same volume of a liquid culture for 15 days. These results provided a possibility of using agar stabs as an alternative culture method for AOA.

We amplified the archaeal 16S rRNA, NirK, AmoA and B genes [8] to further determine the growth of AOA in the agar stabs. To this end, an aliquot (1 µl) of resuspended cells (400 µl) from 4.5 ml of stab culture (5 days, arbitrary selected due to low impurity and distinct cell growth, see also Fig. 2) was used as a template for PCR. The isolated genomic DNA was used as a template when needed. The PCR conditions were as follows: 10 min at 94 °C; 30 cycles of 40 s at 94 °C, 20 s at 55 °C, 1 min 30 s at 72 °C, and 10 min at 72 °C. As shown in Fig. 1c, the amplified band corresponding to archaeal 16S rRNA, *nirK, amoA* and *amoB* genes from the cells grown in stab agar was similar to that of liquid medium. The experiment was repeated

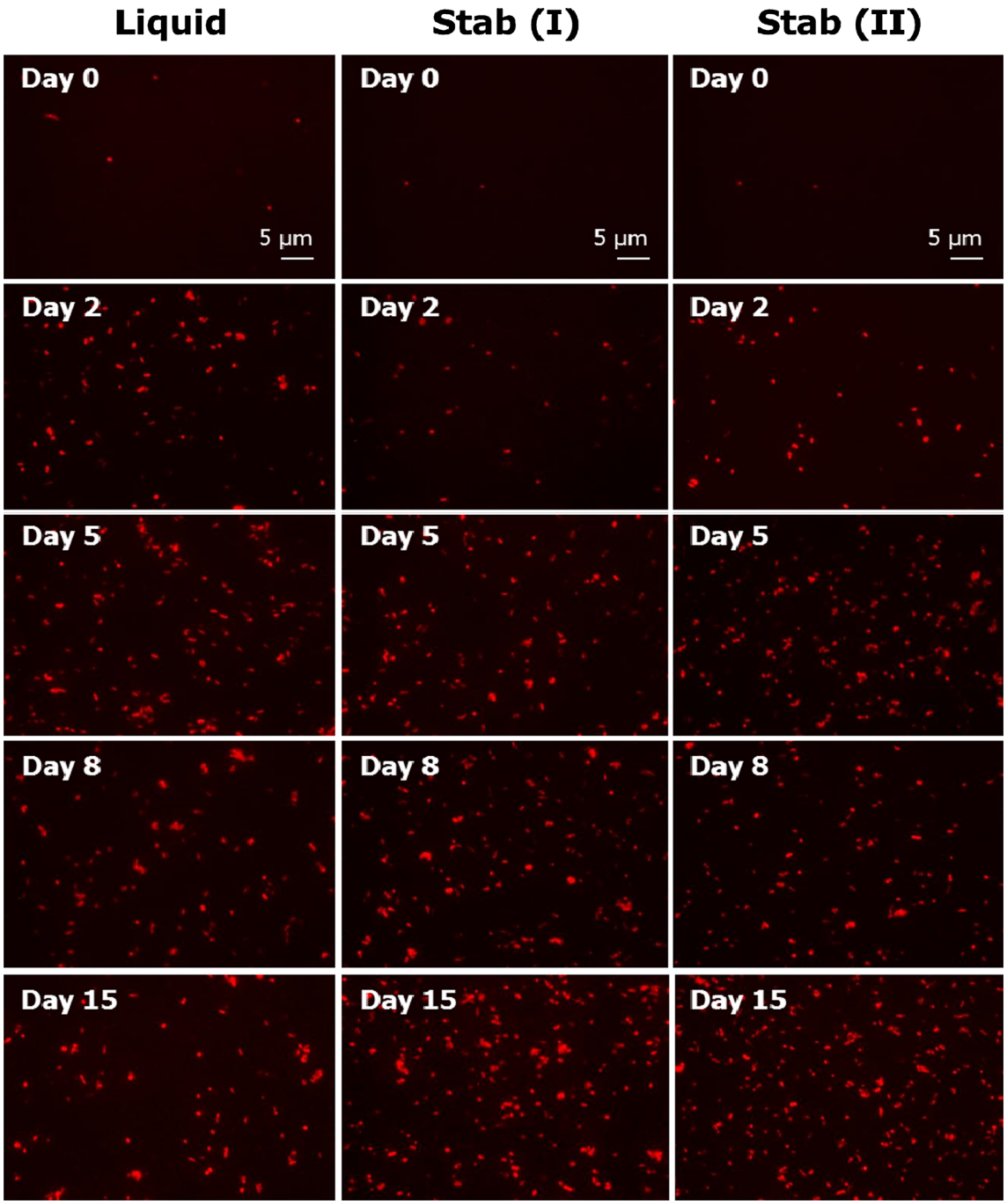

Fig. 2 Microscopic imaging of immunostained cells grown in agar stab. Cells grown in agar stab (0–15 days) were subjected to immunostaining using archaeal specific antibody raised against AmoC and compared to those of grown cells in liquid culture

three times and showed the same results. Further analyses by quantitative real-time PCR (RG-6000, Corbett Life Science, Australia) also supported cell growth in agar stab (Fig. 1c, lower panel).

To provide a more reliable evidence for cell growth in agar stab, we conducted an immunofluorescence staining using an antibody against archaeal AmoC. For the purpose, the polyclonal antibody was raised against AmoC of AR (GenBank Accession No. JX292015), according to a general procedure [10]. The separated cells from stab culture were subjected to immunostaining using rhodamine-conjugated secondary antibody (Goat anti-rabbit IgG, Millipore). As a result, the major portion (70–85 %) of the total cells showed a red fluorescence. In contrast, the control experiments using various bacterial cells did not react with AmoC antibody. We further used this antibody to kinetically measure the cell growth in agar stab and obtained images of stained cells which were quite similar to those of cells from liquid culture (Fig. 2). Taken together, all comparative analyses provide strong evidences that the cultivated cells in agar stab were, at least, comparable to that from liquid cultured cells conventionally used. More detailed kinetic studies (in terms of yields and purities) of AOA grown in agar stab are being under progress.

Because AOA have been recognized as plausible contributors to natural biogeochemical cycles, many attempts have been made to evaluate the utility of various media for isolating and cultivating AOA from various environmental niches [1, 7–9, 11] and mainly accessed through enriched culture in liquid media. A reliable stab culture system, used here, could serve as an alternative route for cultivating AOA strains, because the resulting cultures, including another case of an isolate MYI [11] were successfully transferred to original enriched media (liquid) without a significant loss of cell viability or ammonia-oxidizing activity. These results provide strong evidence that an agar stab is not only a reliable culture method but also a good maintenance system for AOA, although prolonged viability at various temperatures was not assessed currently.

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