The nonculturable state of marine bacteria

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ABSTRACT

Indigenous oligotrophic marine bacteria were counted and isolated by an extinction dilution method using 0.22 µm filtered *in situ* raw natural seawater (FRS) as a medium. The "nonculturable state" discussed here is tentatively defined as the physiological condition at which marine bacteria do not demonstrate visible signs of growth in nutrient rich media, such as colonies on agar plates and turbidity in liquid media. As usual, the viable count with VNSS (800 mg C/l) agar plates (3.0×10^2 CFU/ml) was three orders of magnitude lower than the direct microscopic (DC) counts (6.0×10^5 cells/ml) in pelagic seawater collected off Toyama Bay in Japan. However, the MPN values with FRS (2.8×10^5 cells/ml) were in the same range as the DC. The MPN with autoclaved FRS (FAS) were significantly lower than with FRS. This indicated that the marine bacteria in the pelagic seawater required heat-unstable substances in the ambient natural seawater. FRS and FAS were also used for the isolation of indigenous oligotrophic marine bacteria which were predominant in pelagic seawater. Ninety-five percent of 10^5-times-diluted oligotrophic seawater samples (180 tubes) in FRS showed bacterial growth up to more than 10^5 cells/ml after one month incubation at 15 °C and all were nonculturable. These grown cells were transferred to freshly prepared FRS (second transfer). After two weeks, the second transferred bacteria grew up to 10^5 cells/ml again in more than 90% of the culture tubes, and 10% of them were culturable. The longer the storage in filtered natural seawater, the greater the increase in the number of transformed "culturable" marine isolates. *Sphingomonas* sp. strain RB2256 is also an indigenous marine isolate which used to be nonculturable but turned out to be culturable. This strain reverted to a nonculturable state when it went into stationary phase in marine minimum media with 50 µg/l of vitamin B_{12}. The nonculturable state in RB2256 in the batch culture seems to be induced at high cell density.

Introduction

When we count marine bacteria in pelagic seawater using either microscopic or ordinary viable counting methods, great differences often occur between direct counts (DC), with values on the order of 10^5-10^6 cells/ml, and viable counts (VC) on nutrient rich media such as ZoBell 2216E and VNSS, which are often only 1 % of DC. However, we should not consider this difference a discrepancy. As autochthonous marine bacteria in pelagic seawater, which has an average organic concentration of 0.5 mg C/l, may never have experienced the high organic concentrations of nutrient rich media (e.g., VNSS = 800 mg C/l), the large difference between DC and VC using ordinary nutrient rich media might be reasonable.

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A number of explanations have been proposed to explain the large difference between DC and VC results. From an eco-physiological point of view, it has been argued: 1) that there exist obligate oligotrophs that can not grow in any nutrient rich media and which are predominant in pelagic seawater [4, 8] and; 2) that most marine bacteria are starved, injured or dying [e.g.13]. From a technical point of view, it has been proposed that: 1) concentrations, compositions and/or combinations of nutrients in organic media are not suitable for pelagic marine bacteria and; 2) sudden exposure to nutrient rich external conditions induce suicide responses originating from an imbalance between anabolism and catabolism [1].

In this paper, I discuss the nonculturable state of marine heterotrophic bacteria, focusing particularly on pelagic forms, with reference to methodological problems for cultivation and isolation. The phrase "nonculturable state" used here is defined as the physiological condition at which bacteria do not demonstrate visible signs of growth in nutrient rich media, such as colonies on agar plates and turbidity in liquid media.

Viable Counts in Pelagic Marine Bacteria with Various Media

Bacterial counts in pelagic seawater collected off Toyama Bay in Japan were estimated by both direct microscopic and viable counting methods. Six different kinds of media were used for viable counts. Two were nutrient rich media, VNSS agar plates and VNSS liquid medium for MPN. The remaining four media were basically unamended natural seawater: 0.22 µm-filtered *in situ* raw natural seawater (FRS, filtered raw seawater); 0.22 µm-filtered *in situ* autoclaved natural seawater (FAS, filtered and autoclaved seawater); 0.22 µm-filtered autoclaved aged seawater (FAAS) and; FAAS with vitamin B_{12} (final B_{12} concentration 0.05 µg/l). The extinction dilution method (MPN) was employed when using the five different liquid media. In order to check for contamination of filterable small sized bacteria in FRS, the FRS medium, without inoculation of the original seawater sample, was incubated for one month at 15 °C and then tested by microscopy. In addition to the microscopic test, a portion of the incubated FRS medium was inoculated into VNSS liquid medium and incubated for two weeks at room temperature after which the turbidity was also measured. No bacterial growth was recognized in the FRS medium. This means none of the bacterial cells in the original pelagic seawater sample was able to pass a 0.22-µm cellulose ester membrane filter. Some marine bacteria can pass a 0.2-µm polycarbonate membrane, however they can not pass a 0.22-µm cellulose ester membrane due to its open-celled, foam-like structure [9].

As usual, the bacterial count with VNSS agar plates (3.0×10^2 CFU/ml) was three order of magnitudes lower than the direct microscopic count (6.0×10^5 cells/ml; Table 1). In the case of this pelagic seawater sample, MPN value with FRS was ten times higher than that with FAS.

Table 1. Bacterial numbers ($\times 10^3$ cells/ml) by microscopic and viable counting methods in pelagic seawater collected off Toyama Bay, Japan.

Direct Count		**Most Probable Numbers**				
	VNSS-PC	VNSS	FRS	FAS	FAAS	FAAS+B_{12}
600 ± 45*	0.3 ± 0.02*	2.3	280	33	1.3	24

* standard deviation.

Fukami et al. [6] reported that viable bacterial counts using filter-sterilized *in situ* lake water as the medium were significantly higher than those obtained by autoclaved *in situ* lake water. The pelagic marine bacteria in this case also seem to prefer raw (untreated) food (FRS) rather than a prepared (autoclaved) diet (FAS). The majority of the marine bacteria in this sample probably required heat-unstable substrates in the ambient natural seawater. However, this tendency is not always observed in seawater samples. Among 20 seawater samples collected from different water regions and depths, twelve had MPN values with FRS and FAS in the same range (data not shown).

Aged seawater (ASW) used in this study was collected from a different seawater region, filtered through GF/C glass filter and incubated for more than one month in the dark at room temperature. Hence, during the aging in the dark, the organic concentration in the ASW must have decreased. The ASW was filtered through 0.22 µm membrane and autoclaved (FAAS) and then used for the bacterial counts. The MPN value with FAAS was two orders of magnitude lower than that with FRS. The reason for this may be the differences in organic composition and combination and/or too low a concentration of organic substances in FAAS compared to FRS. FAAS with B_{12} showed ten times higher bacterial counts than did FAAS. The addition of B_{12} seemed to enhance the growth of pelagic marine bacteria. As the concentration of added B_{12} is too low (0.05 µg/l) to explain the bacterial increase simply by an increase of organic nutrient, the ten fold increase of bacteria is probably a result of B_{12} acting as a growth factor.

It has been reported that superoxide and free radicals can be easily produced by autoclaving nutrient rich media containing large amounts of reduced organic substances, and that addition of catalase in agar plates enhances the ability of stressed microorganisms in a nonculturable state to form colonies on agar plates [10]. When a mixture of catalase and superoxide dismutase was added to VNSS agar plates, the same rescue effects were observed for ten different pelagic seawater samples. In nine samples, the addition of the enzymes seemed to increase the CFU by only 20-30 %. In the remaining single sample (water depth = 4000 m, sampling depth = 200 m), the CFU value with the enzymes added was significantly (ten times) higher than that without the enzymes. This result indicates that some of the indigenous pelagic marine bacteria are sensitive to the external oxidative substances produced on agar plates and, therefore, can not make colonies.

These results suggest that appropriate technical devises can enable us to isolate and cultivate an increasing number of authochthonas marine bacteria in pelagic seawaters.

Transforming the Nonculturable Cells into Culturable Cells

When 10^5-diluted original pelagic seawater samples, collected off Toyama Bay, were inoculated into VNSS liquid media, no turbidity was observed in any test tubes. After one month incubation in FRS, 95 % of the 180 FRS test tubes inoculated with 10^5-diluted sample showed bacterial growth (>10^5 cells/ml) and all of them could not make colonies on a nutrient rich medium (VNSS). These nonculturable cells were transferred to freshly prepared FRS (second transfer). After two weeks incubation at 15 °C, the second transferred bacteria grew to 10^5 cells/ml again in more than 90 % of the culture tubes, and 10% of them proved to be culturable in VNSS. Although the remaining second transferred FRS tubes still contained nonculturable bacterial populations, there was a tendency for an increase in the number of transformed "culturable" marine bacteria with the length of time of storage in filter-sterilized seawater. It has also been pointed out that cold treatment (5

°C incubation), in addition to length of storage, enhances the change of pelagic marine bacteria from "nonculturable" to "culturable" states [14].

Seventeen bacterial strains were isolated from a natural pelagic seawater (the Kumanonada-sea in Japan; water depth of 3800 m; sampling depth of 0.5 m) by the extinction dilution method using FAAS amended with small amount of trypticase peptone (FAAS-P; final organic concentration of.0.5 mg C/l). These strains showed obligately oligotrophic characteristics, i. e. the ability to grow in unamended natural seawater, but not in nutrient rich medium at first cultivation. However, during several months of incubation in FAAS-P, all of them became culturable in nutrient rich media and were able to be isolated by a traditional streaking method with agar plates. According to conventional taxonomic and biochemical tests, thirteen of these strains were found to be motile, Gram negative and to utilize glucose oxidatively. They belong to the *Pseudomonas* group. In contrast, among isolated bacteria, which made colonies on nutrient rich agar plates at the first cultivation, the percentage of the *Vibrio -Aeromonas* group was relatively higher.

Sphingomonas sp. RB2256 isolated from natural seawater in Alaska by dilution-to-extinction (present in relatively high numbers) was also nonculturable at first cultivation but became culturable after long storage in stationary phase at 5 °C. This strain was also obligately aerobic and its other taxonomic characteristics were similar to those of strains of the *Pseudomonas* group. The predominance of aerobic cells in the nonculturable fraction of the pelagic seawater may be one clue toward solving the problem of cultivation which nutrient rich media have. The pelagic marine bacteria, which can not choose a fermentative life, may induce the imbalance of metabolism easier than facultative anaerobes when they are suddenly transferred from oligotrophic condition to nutrient rich environment. In order to confirm this tendency (predominance of obligately aerobic bacteria in the nonculturable fraction), more data is necessary.

Why does the longer incubation in oligotrophic condition change nonculturable cells to culturable ones? One explanation for this might be the bottle effect. The increased concentration of organic substances near the surface area, but not too concentrated such as is the case in agar plates, may help the gradual adaptation process of nonculturable bacteria to nutrient rich conditions.

Some of the pelagic marine bacteria that are predominant in natural oceanic seawater are normally in the nonculturable state, yet they may be potentially culturable. Some change in cultivation methods can make them culturable as shown in this study. Rehnstam et al. [12] and Pinhassi et al. [11] reported the predominance of potentially culturable marine bacteria in natural pelagic seawaters by using specific DNA probes. Although obligate oligotrophs might compose some parts of nonculturable bacterial populations, it is necessary for us to accumulate more taxonomic and physiological information about obligate oligotrophs to establish their existence.

Nonculturable States in Isolated Marine Bacteria

Sphingomonas sp. RB2256 is an ultramicrobacterium, isolated by the extinction dilution method as described above. This marine isolate is Gram negative, obligately aerobic, yellow pigmented, catalase and oxidase positive, and has little variation in cell volume (0.05 - 0.09 μm^3), a low DNA content (1.0 to 1.7 fg/cell), and a low rRNA operon number [3, 5, 14]. Growing cells of RB2256 are generally as stress-resistant as starved cells [3]. This strain requires B_{12} as a growth factor. Interestingly, in marine minimum medium (MMM; 0.2 % glucose, 9.52 mM NH_4Cl, 1.32 mM K_2HPO_4 in nine salts solution) with 50

µg/l B_{12}, RB2256 grows up to nearly 10^{10} CFU/ml in batch culture and then the CFU drops exponentially. Even after more than a four order of magnitude drop in CFU in full strength MMM with B_{12}, the cells still maintained 60 % of the respiratory activity of the cells in the exponentially growing phase. Thus, this marine isolate seems to lack some mechanism for down regulation in respiration.

The induction of the nonculturable state in RB2256 seems to be dependent on high cell density. When the amount of phosphate, but not other nutrients, in MMM with B_{12} was reduced, the final cell density did not reach the level of nearly 10^{10} CFU/ml due to the limitation of utilizable phosphate, and the decrease of CFU did not occur. The same tendency was observed with other nutrients. It has been reported that *Cytophaga johnsonae* isolated from natural freshwater also loses its ability to grow on agar plates without losing its viability or metabolic activity after long-term cultivation in a glucose-limited chemostat [7]. Höfle [7] suggests that over supply of nutrients on the agar plates make the bacterium nonculturable. Interestingly, this strain is also obligately aerobic, as is RB2256.

The nonculturable state of the marine isolate *Sphingomonas* sp. RB2256, does not seem to correlate directly with the nonculturability of natural marine bacterial populations in pelagic seawater. However, it can be one piece of the puzzle required to solve the problem of nonculturability of pelagic marine bacteria.

Conclusions

The results presented in this paper suggest that authochthonas pelagic marine bacteria seem to prefer filter-sterilized *in situ* seawater (FRS) rather than autoclaved seawater (FAS), and that those bacteria, which are obligately aerobic, can convert into the nonculturable state easier than those which can choose a fermentative life. Although most of the authochthonas pelagic marine bacteria are normally in the nonculturable state they can be transformed into the culturable state after long storage in low-nutrient media such as FRS and FAS. Therefore, some of the predominant marine bacterial population in pelagic seawater must be potentially culturable.

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