Evaluation of Methods for Storage of Marine Macroorganisms with Optimal Recovery of Bacteria

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Marine macroorganisms are a potential source for new bioactive substances. In many cases marine microorganisms—especially bacteria—associated with these macroorganisms are actually producing the bioactive substances. One often is not able to immediately isolate microorganisms from collected macroorganismic materials; we therefore evaluated different methods for storage of such material, e.g., on board research vessels. These methods were the following: storage of macerates in sintered glass beads and 5% trehalose at -20°C (SGT method); storage of sections in 5% dimethyl sulfoxide at -70°C (SD method); storage of macerates at -20°C using the commercial ROTI-STORE system (RS method); storage of macerates at -20°C in 50% glycerol (GC method); and storage of macerates covered by mineral oil at 4°C (MO method). The SGT and SD methods resulted in numbers of and especially diversity of recoverable bacteria that were higher than for the other methods. Data for the RS method indicated its potential usefulness, too. The MO method resulted in growth during storage, thereby enriching a few selected microorganisms; the GC method resulted in a survival and diversity of recovered bacteria that was too low.

Natural products are used as starting material in many cases during the search for new bioactive compounds; this strategy probably is the most successful for the discovery of new medicines (24). The oceans cover ca. 70% of the surface of our world and represent 95% of our biosphere (2). Yet, only some 10,000 natural compounds have been isolated from marine sources (8). This number is low compared to >100,000 known natural compounds, and therefore marine habitats are increasingly recognized as potentially important sources for natural compounds (2, 9, 12, 13, 15, 23, 25).

It is becoming increasingly clear that in many cases marine microorganisms—living associated with marine macroorganisms—are producing bioactive compounds (25). These substances can have various activities, such as acting as antibiotics, cytotoxins, or neurotoxins or possessing inactivation ability towards viruses, to name only a few (17). The search for new marine microorganisms therefore is increasing in intensity and might be more successful when using material from new habitats (e.g., deep-sea areas, etc.). One problem arising in this respect stems from the fact that, at least in some cases, macroorganisms obtained during collection journeys have to be stored on board ships for a certain time before attempts to cultivate microorganisms therefrom can be undertaken in laboratories. In this study we tested several different methods for storage of macroorganismic material on board ships or at remote locations and evaluated how each performed with respect to survival and recoverable diversity of bacteria.

MATERIALS AND METHODS

Macroorganismic material. Since we wanted to compare different methods of storage we had to use macroorganismic samples of sufficient size containing a

great amount and a high variety of different microorganisms. Marine sponges fulfill these criteria (14) and were chosen as starting material in this project because they also seem to contain a large fraction of a constant microbial community (10). For the experiments presented here, two specimens of the breadcrumb sponge *Halichondria panicea* were used. They were collected at Middlefart, Denmark (Kattegat, Baltic Sea), transferred at 4°C within 24 h to Regensburg, Germany, and stored there overnight at 6°C in an aquarium containing 30 liters of sterile artificial seawater (SME) (22).

In addition to *H. panicea*, we used the sponges *Axinella damicornis*, an *Ircinia* sp., and *Petrosia ficiformis* for the same kind of comparison of different storage methods; the results were very similar in tendency to those reported here. Since the total numbers of microorganisms in the macerates were highest for *H. panicea*, only results for this species are given here.

Storage methods. Five different methods were compared, including the MO (storage of macerates covered by mineral oil at 4°C) and GC (storage of macerates at -20°C in 50% glycerol) methods (11), the RS method (storage of macerates at -20°C using the commercial ROTI-STORE system [Carl Roth GmbH, Karlsruhe, Germany]), and the SGT (storage of macerates in sintered glass beads and 5% trehalose at -20°C) and SD (storage of sections in 5% dimethyl sulfoxide [DMSO] at -70°C) methods developed by us.

Prior to preparation of material for storage, the macroorganisms were soaked three times for 30 min each in 1 liter of sterile SME, resulting in at least a 10^4 -fold reduction of cultivatable bacteria from the medium compared with original seawater (data not shown). To avoid problems of survival of microorganisms arising from repeated freezing and thawing, a total of four samples were prepared for each storage method in parallel and used only once for each time point analyzed.

For the SD method, an aliquot of the sponge was cut off and soaked for an additional 30 min in 500 ml of sterile SME, containing 5% DMSO. From this DMSO-impregnated material, a core of 6-mm diameter was removed with the use of a flame-sterilized cork borer. Two pieces of ca. 5-mm total length were placed into a 2-ml screw-cap vial containing ca. 3 mm of crushed dry ice. The vial, placed into a box containing crushed dry ice, was filled to the top with crushed dry ice to result in rapid freezing. The frozen vials were stored in a deep-freezer at -70°C .

For the SGT, RS, GC, and MO methods, one single macerate was prepared from the SME-washed sponge by cutting the material with the aid of a sterile scalpel into <1-mm pieces and recovering most of the liquid (>10 ml) by gentle pressure. Coarse debris was removed by filtering through sterilized cotton, and this macerate was used for subsequent preparation of the SGT, RS, GC, and MO samples.

In the case of SGT samples, 500 μl of macerate was transferred into a vial containing 500 μl of filter-sterilized 10% trehalose in SME and ca. 20 sinter glass

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spheres of 2- to 3-mm diameter (SIRAN-Carrier; ca. 10- μ m pore size; Schott Engineering GmbH, Mainz, Germany). The number of glass spheres was adjusted to result in complete coverage after mixing; the samples were stored at -20°C .

In the case of GC samples, 500 μ l of macerate was added to a vial containing 500 μ l of sterilized glycerol; after mixing, storage again was at -20°C .

For preparation of RS samples, 500 μ l of macerate was added to one ROTI-STORE vial and mixed. The protocol recommended by Roth Company asks for removal of the liquid contained in the vials and storage of dry glass beads in the vials. We could not detect a difference in survival if the glass beads were used in the recommended dry form or if the cryopreservation liquid added by the supplier was not removed. For both cases storage was at -20°C .

In the case of MO samples, 500 μ l of macerate was overlaid by 1 ml of heat-sterilized (120 min at 160°C) paraffin oil, and samples were stored at 4°C .

Methods for analyzing survival and diversity of bacteria. Survival of bacteria in stored macroorganismic material was measured by plating 0.1 ml of different dilutions (in sterile SME) in duplicate onto two different media. Marine broth plates (MB plates) were made from a commercially available preparation (Marine Broth 2216; Oxoid, Becton Dickinson, Sparks, Md.); with respect to organics, this is a rather rich medium containing 0.5% peptone and 0.1% yeast extract. Plates more closely representing available organics in seawater (OM plates) were prepared from SME to which 0.01% peptone and 0.005% yeast extract were added. Plates were incubated at 17°C for 1 (MB plates) or 2 (OM plates) weeks; survival was calculated by counting colonies, taking into account the dilution factors. Though media reduced in nutrient contents resulted in lower recovery of total isolates, they also have been shown by others to allow isolation of microorganisms which would be lost by the use of rich media (due to overgrowth by fast growing cells; see, e.g., references 4 and 19); this was the rationale for us to include OM plates.

Diversity of bacteria was determined by various physiological tests and by analyzing restriction fragment length polymorphism (RFLP) of PCR-amplified 16S rDNA. For these tests, well-separated colonies were used from MO or OM plates showing a maximum of 100 colonies and purified by streaking on MB plates. Care was taken not to preselect for similar colonies by eye, e.g., by using as many different sized and colored colonies as possible from both plate types.

Differentiation of the purified isolates with respect to physiology was by the following criteria: (i) color and size of colonies; (ii) morphology of cells in liquid media; (iii) inhibition of growth in liquid YEPSME medium (0.5% yeast extract plus 0.1% peptone in SME) by antibiotics (streptomycin at 2 and 20 $\mu\text{g/ml}$; tetracycline at 0.5 and 5 $\mu\text{g/ml}$; erythromycin at 2 and 20 $\mu\text{g/ml}$; rifampin at 0.5 and 5 $\mu\text{g/ml}$; chloramphenicol at 1 and 10 $\mu\text{g/ml}$; and penicillin at 1 and 10 $\mu\text{g/ml}$); and (iv) growth in liquid YEPSME medium at different temperatures (4, 10, 17, 20, 25, 37, and 43°C). The first and second criteria together possessed much less differentiation power than the third and fourth criteria, especially for samples stored for more than 1 day. Data for the first and second criteria were checked by two experiments, while results for the third and fourth criteria were verified at least three times.

Differentiation of the purified isolates by RFLP was as follows. (i) Genomic DNA was prepared from colonies by a well-established procedure (3). (ii) PCR amplification of 16S rDNA therefrom used the universal primers (6) 8F (GRG TTGATCCTGGCTCAG) and 1512R (ACGGHTACCTGTGTACGACTT) (*Escherichia coli* numbering). (iii) PCR products were checked for purity on 0.7% agarose gels, and bands of the expected size were purified using the QIAGEN II gel extraction kit. (iv) Single restrictions were performed using enzymes *RsaI* and *TacI* (*MspI* restrictions resulted in too few bands to be useful). (v) RFLP was checked by separating the *RsaI*- and *TacI*-generated fragments on 2% agarose gels. Restriction patterns were analyzed and grouped by eye; only clear differences were counted. RFLP data in general had a lower resolving capacity than physiology data.

RESULTS AND DISCUSSION

Survival of bacteria. The SD method was the only one which allowed isolation of eukaryotic microorganisms that were not further characterized, like flagellates (tetramastigonema-type) and bodonea-type eukaryotes from material stored for 1 or 6 months. All other storage methods resulted only in survival of bacteria; the data presented in Tables 1 and 2, therefore, refer to bacteria.

Survival of bacteria in differently stored macroorganismic

TABLE 1. Survival of bacteria after various times using five different storage methods

Storage method	Titers after 1-day storage ^a		Titers after 1-month storage		Titers after 6-month storage	
	MB plates	OM plates	MB plates	OM plates	MB plates	OM plates
SD	8.5×10^3	5.5×10^3	2.1×10^4	7.0×10^3	1.2×10^4	1.1×10^3
SGT	4.2×10^5	1.3×10^5	6.2×10^4	4.1×10^4	1.1×10^4	7.7×10^3
RS	4.3×10^5	1.8×10^5	3.4×10^4	1.3×10^4	1.2×10^4	1.7×10^3
GC	2.0×10^4	3.0×10^3	1.5×10^3	1×10^2	3.8×10^3	8×10^2
MO	3.9×10^5	5.7×10^5	1.3×10^8	8.3×10^5	8.5×10^6	1.6×10^6

^a Titer before storage was 1.3×10^6 .

materials is compared in Table 1. The titer in the original macerate was determined by two independent experiments to be 1.4×10^6 and 1.2×10^6 , indicating that at maximum a 10% inaccuracy exists for the values we obtained. The first freezing step reduced surviving bacteria by ca. 70% for the SGT and RS methods. The GC method resulted in a ca. 20-fold reduction of surviving bacteria, indicating its limited usefulness. In the case of the MO method, growth could be observed already after 1 day of storage (at 4°C), which was much more pronounced after 1 month; this method of storage therefore should not be used. Since we used one and the same original macerate for the SGT, RS, GC, and MO methods, we can directly compare the data for these methods.

For the SD method, a certain toxic effect of DMSO resulted in reduced recovery of bacteria after 1 day of storage compared to the other methods; such an effect had been noted already earlier (1, 11). This difference is reduced after 1 month of storage and cannot be seen after 6 months of storage. The reduction varied with the time of DMSO treatment and the species of macroorganisms being analyzed; after 1 month of storage such differences, however, were in a range to be negligible. The SD method used a protocol of sample preparation that was different from the other four methods (impregnation with DMSO and subsequent storage of macroorganismic material versus preparation of a macerate from macroorganisms); therefore, the SD samples contained starting materials different from those of the samples prepared for the other four methods. The differences we observed for recovery of bacteria on MB and OM plates (containing 0.6% and 0.015% organics, respectively) were consistent in all experiments, with higher numbers of bacteria being recovered on MB plates. OM plates

TABLE 2. Percentage of different bacteria (physiological and/or RFLP differences) isolated after storage, using five different methods

Storage method	Different clones after 1-day storage ^a		Different clones after 1-month storage		Different clones after 6-month storage	
	MB plates	OM plates	MB plates	OM plates ^b	MB plates	OM plates ^b
SD	32	ND ^c	25	16	23	9
SGT	35	ND	25	12	17	15
RS	26	ND	18	14	11	9
GC	23	ND	8		6	
MO	24	ND	6	9	2	3

^a 58% different clones were observed before storage.

^b Numbers are additive to those of MB plates; e.g., in the case of SGT storage for 1 month, a total of 37 different clones had been recovered.

^c ND, not determined.

on the other hand allowed isolation of additional isolates. These data are corroborated by results of other studies (4, 7, 19, 21, 26) which have tested the importance of "poor media" for the isolation of certain marine bacteria.

Diversity of isolated bacteria. A crucial question for comparing the different methods asks for the diversity of isolates which can be isolated from the macroorganismic material after storage. Diversity was defined here as the number of colonies possessing differences in physiology and/or RFLP. Since we used many more physiological criteria (growth under 19 different conditions plus morphology) than genetic criteria (presence of recognition sites for two restriction enzymes in 16S rDNA), the most differences were found for physiological data. These tests were performed in 96-well microtiter plates for each method and storage time, and therefore the numbers given in Table 2 represent differences in percentages. We could identify a total of 58 different isolates in the original (not frozen) macerate using the same criteria as for the samples stored according to our different methods (and times). The observed reduction to 41 and 37% different isolates after 1 month of storage (SD and SGT methods, respectively) therefore indicates that the diversity of bacteria that was recovered was reduced to ca. two-thirds of the original diversity by using these methods (Table 2).

It has to remain open as to what extent the number of isolates we could recover and differentiate reflects the absolute percentage of these isolates in the original macerate, since we tried to use as many different colonies as possible in the differentiation process. The objective of this study was not to determine the diversity of bacteria present in our starting material. We rather wanted to evaluate which storage method resulted in the greatest variety of recovered isolates, because each different colony might be a potential producer of bioactive compounds. In this respect differences in physiology are of prime interest, and differences in genetics (RFLP) are of secondary interest.

Evaluation of the different storage methods. A critical question asks for reliability (statistics) of our data; as mentioned above, only data for the sponge *H. panicea* are given here. We did obtain similar results for other macroorganisms (i.e., growth after storage by the MO method, lowest survival after GC storage, equal and highest diversity recovered after SD and SGT storage, and somewhat reduced diversity after RS storage) with the relative numbers of diversity differing by a maximal 25%; titers of bacteria recovered after storage differed to a greater extent, due to different starting titers. The main conclusion from those combined results, however, is that the same tendency was observed for all data sets, allowing us to draw the following conclusions.

The MO method (as expected) should not be used for storage of macroorganismic material, because it very clearly selects for a few phylotypes of recoverable bacteria. Selection will take place by growth of bacteria during storage, which clearly was observed (compare survival numbers of MO samples in Table 1). The MO method, indeed, was included in our study only to be able to analyze which influence a selection force might exert on the diversity of recoverable bacteria.

The GC method did result in a survival of bacteria which is roughly 10% compared to that for the other methods. In addition, the number of different colonies we could isolate from

samples stored that way also was low, which clearly speaks against the use of the GC method for the purpose of recovering a great diversity of bacteria.

The RS method resulted in the recovery of 32 and 20% different bacteria recovered after 1 and 6 months of storage, respectively. These numbers are lower than those for the SGT (37 and 32%) and SD methods (41 and 32%), but still the RS method allowed us to recover many more different isolates than the GC and MO methods. Therefore, the RS method well might be considered to be used for storage. With respect to handling, slightly less hands-on time is required for the RS method and the SGT method (2 h) than for the SD method (2 h versus 3 h).

The SD and SGT methods had been developed by us with the rationale that addition of a cryoprotectant to biological samples should aid in survival of microorganisms contained therein. The SD method uses a quick-freezing protocol to further reduce cell damages; a certain inhibitory effect of DMSO, however, was observed (see also above). Trehalose—which we used in the SGT protocol—is another widely used cryoprotectant, not only for preservation of microorganisms (5, 16, 18) but also to stabilize protein preparations (20). In the case of the SGT method, the addition of the SIRAN-Carrier glass spheres was essential; in parallel experiments, survival of microorganisms was reduced by at least fivefold if no glass spheres were added (data not shown). Both the SD and SGT methods were more or less equally efficient with respect to recovery of diverse bacteria and also did not show great differences with respect to survival of bacteria after storage (but note that only the SD method allowed us to recover lower eukaryotes). By macroscopic observation (difference in colony types), the SD method seemed to result in the highest diversity of bacteria to be recovered; this was also reflected to a certain degree by the 1-month storage value (Table 2). It has to be taken into account, however, that the SD method asks for a more complicated handling of samples and their storage at -70°C . The SGT method in this respect is much less demanding by asking for normal freezer temperatures of -20°C .

We conclude that the SD and SGT methods for storage of macroorganismic material result in equal or higher survival of total bacteria and allow recovery of a higher diversity of bacteria than the other three methods tested here. The choice of which of these two methods should be used probably will depend on the equipment available during collection of macroorganisms.

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