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Vibrio harveyi Mutagenicity Assay as a Preliminary Test for Detection of Mutagenic Pollution of Marine Water

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The presence of mutagens in the natural environment is a general problem (for a review, see Mortelmans and Zeiger 2000). Marine habitats are not exceptions. Therefore, detection of mutagenic contaminants in marine water is important. Mutagens elicit deleterious effects on living organisms at extremely low concentrations. Chemical methods for their detection are expensive and time consuming. Moreover, such methods are useful only in assays for particular chemicals. Since there are hundreds of different mutagenic chemicals, it is a problem when one wants to perform a quick and preliminary assay to answer the question of mutagenic contamination in the tested environmental sample. Therefore, biological assays, based on appearance of easily detectable mutants after contact of organisms with mutagens, seem to be more appropriate for such tests than chemical analyses. Using a biological assay, it is possible to detect the presence of various mutagens without complicated chemical analysis.

The most commonly used biological mutagenicity assay is the Ames test (Ames 1971; Ames et al. 1973, 1975; Maron and Ames 1983; Mortelmans and Zeiger 2000). In this assay, a series of *Salmonella enterica* serovar Typhimurium strains is used. However, although the Ames test is excellent for assessing mutagenicity of chemicals under laboratory conditions, the strains mentioned above survive poorly in marine water (Czyż et al. 2002). This may be a serious problem if one wants to test marine water samples.

A new mutagenicity assay, based on genetically modified strains of marine bacterium *Vibrio harveyi*, has been developed (Czyż et al. 2000). As expected, *V. harveyi* strains survive well in samples of marine water taken from different geographical regions (Czyż et al. 2002). Moreover, the *V. harveyi* assay was demonstrated to be significantly more sensitive than the Ames test (Czyż et al. 2002). This is especially important in environmental studies, when concentrations of mutagens is expected to be low. Finally, using only four bacterial strains included in the *V. harveyi* assay, it is possible to detect mutagenicity caused by agents belonging to various groups of chemicals (Czyż et al. 2000). Until now the *V. harveyi* assay was performed only under laboratory conditions with known amounts of specific mutagens. Here, we tested its usefulness in assessing mutagenicity of marine water samples taken from natural habitats.

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MATERIALS AND METHODS

The following *V. harveyi* strains were used: BB7 (wild-type) strain (Belas et al. 1982), its derivative (BB7X) bearing the *cgtA*::Tn*5*TpMSC mutation (Czyż et al. 2000, 2001), and strains analogous to BB7 and BB7X but bearing plasmid pAB91273 (containing *mucA* and *mucB* genes, responsible for enhanced error-prone DNA repair), called BB7M and BB7XM, respectively (Czyż et al. 2000).

The BOSS medium was described previously (Klein et al. 1995) and served as a primary negative control. Artificial marine water was prepared according to MacLeod et al. (1954) and served as a secondary negative control. The BOSS medium containing defined chemical mutagens served as a positive control.

Samples of marine water, taken from different habitats, are listed in Table 1. These samples were collected near shores and transported in plastic bottles. Following sterilization by filtration through 0.22 μl filters (Millipore) the samples were stored at –70°C. Storage and handling of the BOSS medium and the artificial marine water under the same conditions did not change result of the mutagenicity assays with these samples (data not shown). Therefore it is unlikely that the marine water samples were inadvertently contaminated.

Table 1. Samples of marine water

Sample code	Origin of sample (geographical region)
AMW	Artificial marine water (made in laboratory)
AO-A	Atlantic Ocean, Annapolis, MD, USA
AO-L	Atlantic Ocean, Lisbon, Portugal
AS-P	Aegean Sea, Paralia, Greece
AS-T	Adriatic Sea, Trieste, Italy
BS-C	Baltic Sea, Copenhagen, Denmark
NS-O	North Sea, Oslo, Norway
PO-M	Pacific Ocean, Monterey, CA, USA

The mutagenicity assay with marine water samples was performed according to Czyż et al. (2000). Bacteria were cultivated in the BOSS medium at 30°C to mid-log phase of growth. 10 ml of each culture were centrifuged (2000 *g*, 10 min), and the bacterial pellet was resuspended in 2.5 ml of the concentrated BOSS medium (4 x BOSS). Then, 7.5 ml of the artificial marine water or of a sample of marine water taken from the natural environment were added (to obtain a culture in rich medium based on this sample). Cultivation was continued at 30°C for 2 or 4 hours, as these times of incubation in a rich medium were found previously (Czyż et al. 2000) to be optimal for detection of different mutagens. Bacteria were titrated (by spreading of serial dilutions of the culture) on BOSS plates and analogous plates containing neomycin (at final concentration of 50 μg/ml). The number of all living bacteria and the number of neomycin-resistant bacteria were estimated after incubation of plates at 30°C for 48 h, and percentage of neomycin-resistant mutants was calculated.

RESULTS AND DISCUSSION

We aimed to test different marine water samples for their mutagenicity using the *V. harveyi* assay. In control experiments, whose results are shown in Table 2, the BOSS medium containing no mutagens or various concentrations of defined chemical mutagens, was tested. These concentrations were close to detection limits established previously for the BB7 strain (Czyż et al. 2002). The results presented in Table 2 confirm previous findings (Czyż et al., 2000, 2002) that different *V. harveyi* strains produce the highest number of mutants in response to various kinds of mutagens.

Tables 3 and 4 show results of testing marine water samples from different habitats. Artificial marine water (AMW) served as a secondary negative control. Comparison of results for AMW with those for the BOSS medium devoid of mutagens indicates no additional mutagenic effect of AMW relative to BOSS.

Table 2. Assessment of mutagenicity of various mutagens using the *V. harveyi* test; bacterial cultures were incubated for 2 h with water samples.

Mutagen[a]	Percent of neomycin-resistant mutants			
	BB7	BB7X	BB7M	BB7XM
None	0.000450	0.0532	0.0330	0.00333
Dauno. (24)	0.00108	0.148	0.0343	0.00432
SA (6)	0.000495	0.143	0.0825	0.0859
ICR-191 (4)	0.00225	0.186	0.0452	0.00699
NPD (80)	0.000640	0.170	0.122	0.0109
NQNO (20)	0.000720	0.244	0.00495	0.0139
MMS (4)	0.000780	0.436	0.264	0.00832
2-AF (40)	0.00270	0.207	0.0462	0.0136

[a] Abbreviations used are as follows: Dauno., daunomycin; SA, sodium azide; ICR-191, 2-methoxy-6-chloro-9-(3-(2-chloroethyl)aminopropylamino)acridine x 2HCl; NPD, 4-nitro-*o*-phenylenediamine; NQNO, 4-nitroquinolone-*N*-oxide; MMS, methyl methanesulfonate; 2-AF, 2-aminofluorene. Values in parentheses indicate concentrations of particular mutagens in ng/ml.

Table 3. Assessment of mutagenicity of marine water samples using the *V. harveyi* test; bacterial cultures were incubated for 2 h with water samples.

Sample	Percent of neomycin-resistant mutants			
	BB7	BB7X	BB7M	BB7XM
AMW	0.000439	0.0624	0.0322	0.00409
AO-A	0.000299	0.0166	0.00857	0.00814
AO-L	0.00164	0.0085	0.00220	0.0103
AS-P	0.00780	0.0510	0.00380	0.00380
AS-T	0.000804	0.0269	0.0635	0.00665
BS-C	0.000118	0.0201	0.00620	0.0142
NS-O	0.00273	0.0369	0.00242	0.00505
PO-M	0.000417	0.0133	0.00370	0.00498

Table 4. Assessment of mutagenicity of marine water samples using the *V. harveyi* test; bacterial cultures were incubated for 4 h with water samples.

Sample	Percent of neomycin-resistant mutants			
	BB7	BB7X	BB7M	BB7XM
AMW	0.000254	0.0714	0.0119	0.00701
AO-A	0.0000755	0.0230	0.0121	0.00450
AO-L	0.00255	0.00503	0.00137	0.00566
AS-P	0.0157	0.0320	0.00160	0.0118
AS-T	0.000164	0.192	0.00310	0.00362
BS-C	0.0000687	0.0147	0.0000790	0.0391
NS-O	0.000280	0.0130	0.00610	0.00929
PO-M	0.000254	0.00846	0.0149	0.00512

Table 5 shows the mutagenicity of marine water samples assessed using the *V. harveyi* test. Mutagenicity of a given water sample was calculated by dividing the percent of mutants in this sample by the percent of mutants in the artificial marine water. The results obtained generally agree with our expectations. For example, very low (if any) mutagenicity was detected in the water sample taken from the Pacific Ocean (sample PO-M); this sample derives from a clean beach, without expected industrial pollution. Contrary to the PO-M sample, mutagenicity was clearly detected in some other samples, like AO-L, AS-P, BS-C or NS-O. These samples were withdrawn either near large industrial centers (samples AO-L, BS-C and NS-O) or at an intensively explored tourist region (sample AS-P).

Table 5. Summary of the assessment of mutagenicity of marine water samples using the *V. harveyi* test.

Sample	Mutagenicity[a]							
	2 h incubation				4 h incubation			
	BB7	BB7X	BB7M	BB7XM	BB7	BB7X	BB7M	BB7XM
AMW	1	1	1	1	1	1	1	1
AO-A	<1	<1	<1	1.99	<1	<1	1.02	<1
AO-L	3.73	<1	<1	2.51	10.03	<1	<1	<1
AS-P	17.76	<1	<1	<1	61.89	<1	<1	1.68
AS-T	1.83	<1	1.97	1.62	<1	2.68	<1	<1
BS-C	<1	<1	<1	3.47	<1	<1	<1	5.57
NS-O	6.22	<1	<1	1.23	1.10	<1	<1	1.32
PO-M	<1	<1	<1	1.22	<1	<1	1.25	<1

[a] Mutagenicity was calculated by dividing the percent of mutants in a given sample by the percent of mutants in artificial marine water. When the result was below 1, the symbol <1 is shown rather than a specific value.

Results presented in Tables 3-5 indicate appearance of different numbers of mutants for different strains. The most puzzling are values obtained for particular environmental samples that are significantly lower in comparison to artificial marine water (for example, 0.0000790 for BS-C and 0.0119 for AMW in the case of BB7M in Table 4). This may be due either to the presence of anti-mutagenic

compounds (specifically blocking mutagenic activities of certain chemicals) or to the presence of toxic compounds (inhibition of cell growth would prevent establishment of mutations). In this light, different strains of *V. harveyi* used in the mutagenicity assay respond differentially to various chemical mutagens (Czyż et al. 2000, 2002; see also Table 2). Moreover, the genetically-modified *V. harveyi* strains may be more sensitive to cytotoxic agents (Czyż et al. 2000, 2001). Nevertheless, in many cases, an unambiguous increase in the number of mutants after a contact with marine water samples was observed. This indicates the presence of mutagens in these samples. Different proportions of *V. harveyi* mutants in particular samples may suggest various composition of mutagenic pollution in these samples. However, specific composition of pollutants requires further, more complicated analyses.

In each test for detection of mutagens, a threshold value should be defined, above which the results may be considered positive. On the basis of laboratory studies, in which known amounts of various mutagens were used, such a threshold value for the *V. harveyi* mutagenicity assay was determined (Czyż et al. 2002). Namely, a result of this assay was proposed to be considered positive in the case of appearance of at least two times more mutants after treatment with the tested mutagen than in a control experiment (without addition of the mutagen). This corresponds to the mutagenicity value, calculated as described in Table 5, above 2. Analysis of results of our tests presented in Tables 3 and 4 strongly suggest that such a threshold value is reasonable. In our studies, we could expect relatively low mutagenicity in water samples withdrawn from regions of little or not known industrial chemical contamination (like AO-A and PO-M), and high mutagenicity in water samples withdrawn from regions suspected to contain industrial pollution (like AO-L, AS-P, AS-T, BS-C and NS-O). The mutagenicity values for AO-A and PO-M were always below 2, whereas for other samples at least one *V. harveyi* strain gave a value higher than 2. Therefore, we propose that if the mutagenicity value measured using any of the four test strains of *V. harveyi* is higher than 2, the given water may be considered potentially mutagenic. Obviously, with a higher calculated value, greater mutagenic potential of the water is expected.

In conclusion, our results indicate that the *V. harveyi* test may be used as a simple preliminary assay for detection of mutagenic pollution of marine environments. The test seems to be sufficiently sensitive to detect mutagenic agents at concentrations they achieve in marine water. Thus, we hope that the use of the procedure in which water samples (withdrawn directly from investigated regions) might be tested for mutagenic pollution without their concentration or any other preliminary treatment, is possible indeed.

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