

## Optimisation of the microbiological mutagenicity assay based on genetically modified *Vibrio harveyi* strains

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**Abstract.** Recently, we have developed a novel assay designed for detection of mutagenic pollution of the marine environment. This assay is based on the use of a series of genetically modified strains (named BB7, BB7M, BB7X and BB7XM) of a marine bacterium *Vibrio harveyi*. Sensitivity of the *V. harveyi* mutagenicity assay was found to be similar to, or even somewhat higher than, that of the commonly used Ames test. Subsequent studies indicated that this assay may be useful in assessment of mutagenic contamination of the marine environment. Nevertheless, we assumed that improvement of this assay is still possible, and thus we aimed to optimise its procedures. Here we present our research on the optimisation of the *V. harveyi* mutagenicity assay, which indicated that different tester strains used in this assay give the best results depending upon the experimental conditions employed. Incubation of bacteria in a buffer, rather than in a nutrient broth, containing a mutagen, increased the efficiency of the assay with BB7 and BB7M strains, but had a deleterious effect in the case of BB7X and BB7XM. The latter couple of strains revealed higher mutagenicity in the plate assay, as compared to the liquid medium assay. However, the opposite effect was observed for BB7 and BB7M. Low-dose ( $1 \text{ J m}^{-2}$ ) UV irradiation, as well as 30 min incubation in 0.1 M  $\text{CaCl}_2$ , had no significant effect on the efficiency of the assay when using BB7 and BB7M, whereas the number of mutagen-induced mutants of BB7X and BB7XM strains increased about two times under these conditions. Our previous experiments indicated that various tester strains revealed different sensitivity to particular mutagens. Thus, a series of strains should be used in the assay. Results presented in this report show that different conditions should be used for two pairs of the tester strains: BB7 and BB7M, and BB7X and BB7XM.

**Key words:** marine environment, mutagenic pollution, mutagenicity assay, *Vibrio harveyi*.

### Introduction

Mutagenic pollution of the environment is a serious and general problem. It concerns both the artificial urban environment of present-day humans and the natural environment, contaminated with mutagenic pollutants appearing mostly as a result of industrial processes (Heddle 1999; Goldman and Shields 2003; Vargas 2003; Jha 2004). Mutagenic chemicals occurring in various habitats can induce serious diseases, including cancer. The germ line of higher organisms may be also damaged by these compounds, which may lead to fertility problems

and to negative genetic changes in future generations (reviewed by Mortelmans and Zeiger 2000). Therefore, detection of mutagenic pollution of the environment is an important issue. However, this is not a trivial chemical procedure, as there are thousands of known mutagens and they have mutagenic effects usually at very low concentrations.

Because of the problems mentioned above, biological assays for detection of mutagenic agents in environmental samples have been developed. There are various tests in which different organisms are employed, including plants (Kovalchuk et al. 2001), fish (Winn 2001),

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invertebrates (Dixon et al. 2002) and bacteria (Węgrzyn and Czyż 2003). The advantage of microbiological mutagenicity assays is their simplicity and rapidity. However, most of these assays employ species of bacteria that naturally exist in animal gut, rather than those occurring in tested habitats, which causes potential problems (Węgrzyn and Czyż 2003). For example, survival of *Salmonella typhimurium* tester strains, used in the most commonly applied mutagenicity assay – the Ames test (Mortelmans and Zeiger 2000) is dramatically reduced in marine water, as compared to that measured in standard laboratory media (Czyż et al. 2002).

A novel mutagenicity assay for detection of mutagenic pollution of the marine environment has recently been developed in our laboratories (Czyż et al. 2000). This assay is based on the use of a series of genetically modified *Vibrio harveyi* strains. *V. harveyi* is not pathogenic to humans, and thus it is completely safe to work with. This bacterium is naturally significantly more sensitive to mutagenic agents than *E. coli* and the tester strains form mutants in response to their contact with mutagens even more effectively. Moreover, the cellular envelope of *V. harveyi* is considerably more permeable to large molecules than that of *S. typhimurium* (Czyż et al. 2000), which facilitates penetration of mutagens into cells of the tester bacteria.

The *V. harveyi* mutagenicity assay consists of the detection of neomycin-resistant mutants on plates either containing a mutagen in the solid medium or after incubation of bacterial cultures in a liquid medium in the presence of tested compounds or environmental samples. Neomycin is an aminoglycoside antibiotic that interferes with decoding at the ribosomal A site during translation (Dahlberg 1989). Resistance to this antibiotic occurs as a result of various rRNA modifications in the decoding site. Therefore, a large spectrum of mutagenic agents, causing various types of mutations, may lead to the appearance of neomycin-resistant mutants, which can be detected. Sensitivity of the *V. harveyi* assay is equal to or higher than that of the Ames test, depending on the mutagen tested (Czyż et al. 2002). Using this assay, efficient detection of mutagens in marine water samples from different geographical regions was possible (Czyż et al. 2003). Moreover, the assay was also successfully used in laboratory studies on mutagenic and anti-mutagenic activities of various compounds (Piosik et al. 2003).

Previous studies indicated that different *V. harveyi* strains used in the mutagenicity assay respond differentially to various mutagens (Czyż et al. 2000; 2002). Thus, a series of strains should be used in studies of environmental samples rather than a single strain. We observed different growth preferences of these strains, so here we aimed to optimise specific conditions of the assay for all of them.

## Material and methods

### Bacterial strains

A series of *V. harveyi* strains used in the mutagenicity assays is presented in Table 1.

**Table 1.** A series of *Vibrio harveyi* strains used in the mutagenicity assay

Strain	Genotype and/or description	Reference
BB7	Natural isolate	Belas et al. 1982
BB7M	Like BB7 but bearing plasmid pAB91273 <sup>a</sup>	Czyż et al. 2000
BB7X	BB7-derivative, isolated as a UV-hypersensitive strain after mutagenesis with Tn5TpMCS	Czyż et al. 2000
BB7XM	Like BB7X but bearing plasmid pAB91273 <sup>a</sup>	Czyż et al. 2000

<sup>a</sup> Plasmid pAB91273 bears *mucA* and *mucB* genes, whose products enhance error-prone DNA repair.

### Mutagens

2-methoxy-6-chloro-9-(3-(2-chloroethyl)amino propylamino)acridine × 2HCl (ICR-191) and 4-nitroquinolone-*N*-oxide (NQNO) were used as model mutagens, because it was demonstrated previously (Czyż et al. 2002) that all *V. harveyi* tester strains respond to these chemicals.

### Mutagenicity assays

The assays were performed according to previously described procedures (Czyż et al. 2000; 2002). For the *liquid medium mutagenicity assay*, a mutagen was added to bacterial cultures ( $A_{575} = 0.1$ ) growing in the liquid BOSS nutrient medium (per litre: 10 g bacto-peptone, 3 g beef extract, 1 mL glycerol, 30 g NaCl; Klein et al. 1995) at 30°C, and incubation was continued for indicated times. Then, volumes of the cultures containing  $5 \times 10^6$  bacterial cells (estimated by measuring  $A_{575}$ ) were spread onto BOSS agar plates containing neomycin at a final concentra-

tion of 100 µg mL<sup>-1</sup>. Plates were incubated for 48 hours at 30°C and neomycin-resistant colonies were counted. A control experiment was always performed using a part of the same bacterial culture but without addition of a mutagen. The number of spontaneous neomycin-resistant mutants appearing on plates in such a control experiment was subtracted from the number of mutants observed in the experiment with a mutagen added.

For the *plate mutagenicity assay*, volumes of bacterial cultures (growing in BOSS medium) containing 5 × 10<sup>6</sup> cells were spread on the BOSS agar plates containing indicated amounts of mutagens and neomycin at a final concentration of 100 µg mL<sup>-1</sup>. Plates were incubated and analysed as described above. In control experiments, plates with neomycin but without a mutagen were used.

Next, some variants of the assay were tested. In the first variant ('Buffer'), during incubation of bacteria with mutagens, the BOSS medium was replaced by the MM buffer (pH 7.2), consisting of 0.6% Tris, 0.58% maleic acid, 0.25% NaCl, 0.2% KCl, 0.1% NH<sub>4</sub>Cl, 0.1% MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.0132% Na<sub>2</sub>SO<sub>4</sub>, 0.0322% Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O. In the second variant ('UV'), bacteria were UV-irradiated (1 J/m<sup>2</sup>) prior to addition of a mutagen. In the third variant ('CaCl<sub>2</sub>'), bacteria were incubated for 30 min in a solution containing 0.1 M CaCl<sub>2</sub> and a mutagen, prior to incubation in the BOSS medium (total incubation time with a mutagen was 2 h).

## Results and discussion

The *V. harveyi* mutagenicity assay may be performed in a liquid medium (followed by spreading of bacteria on plates containing neomycin) or as a plate assay, analogously to the typical Ames test (Czyż et al. 2000; 2002; Mortelmans and Zeiger 2000). The plate assay is performed by spreading of culture samples on agar nutrient plates containing both a mutagen and a selective agent (neomycin in the case of the *V. harveyi* assay). It appears that due to technical reasons the liquid medium assay may be more useful in analysis of environmental samples than the plate assay (Czyż et al. 2003). The latter version is convenient for testing mutagenicity of known chemicals in the laboratory; it can also be used in environmental studies but samples of tested material (e.g. water samples) must be added to agar plates during their preparation. In this study we used the liquid medium assay as the primary

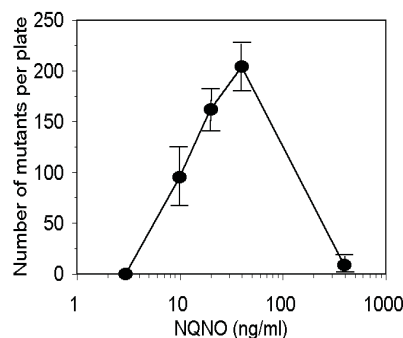


Figure 1. Dose-response correlation in the liquid medium *Vibrio harveyi* mutagenicity assay. The results represent an experiment in which NQNO was added to the indicated final concentrations, and the time of incubation with bacteria was 2 h. Mean values from 5 independent experiments, with bars representing standard deviation, are shown.

test, because it is more likely that this variant will be used in environmental monitoring.

An example of the dose-response experiment is shown in Figure 1. Interestingly, a decreased number of mutants was found at a relatively high concentration of the mutagen (NQNO), which was not

**Table 2.** Selected results of optimisation of the *Vibrio harveyi* mutagenicity assay

Strain	Improvement ratio in relation to standard liquid medium assay <sup>a</sup>			
	Plate <sup>b</sup>	Buffer <sup>c</sup>	UV <sup>d</sup>	CaCl <sub>2</sub> <sup>e</sup>
BB7	0.21	2.29	1.16	1.04
BB7M	0.16	2.90	0.90	0.84
BB7X	2.75	0.07	1.59	1.96
BB7XM	3.75	0.15	1.73	2.16

<sup>a</sup> Ratio of the number of neomycin-resistant mutants observed under the tested conditions to that observed in the liquid medium assay (in which cultivation for 2 h after addition of a mutagen was followed by spreading of 5 × 10<sup>6</sup> cells onto BOSS agar plates containing 100 µg mL<sup>-1</sup> neomycin; mutagen concentration and times of incubation were the same, except the plate assay, where no incubation in the liquid medium containing mutagens was applied).

<sup>b</sup> Plate assay (5 × 10<sup>6</sup> bacteria spread onto BOSS agar plate containing a mutagen and 100 µg mL<sup>-1</sup> neomycin). The results represent experiments in which NQNO was added to a final concentration of 20 ng mL<sup>-1</sup> (in the liquid BOSS medium the incubation time was 2 h).

<sup>c</sup> Liquid medium assay, in which the BOSS medium was replaced with the MM buffer during incubation of bacteria with mutagens. The results represent experiments in which ICR-191 was added to a final concentration of 400 ng mL<sup>-1</sup>, and the time of incubation with bacteria was 2 h.

<sup>d</sup> Liquid medium assay in which bacteria were UV-irradiated (1 J m<sup>-2</sup>) prior to addition of a mutagen. The results represent experiments in which ICR-191 was added to a final concentration of 80 ng mL<sup>-1</sup>, and the time of incubation with bacteria was 2 h.

<sup>e</sup> Liquid medium assay, in which bacteria were incubated for 30 min in a solution containing 0.1 M CaCl<sub>2</sub> and a mutagen, prior to incubation in the BOSS medium (total incubation time with a mutagen was 2 h). The results represent experiments in which NQNO was added to final concentration of 40 ng mL<sup>-1</sup>, and the time of incubation with bacteria was 2 h.

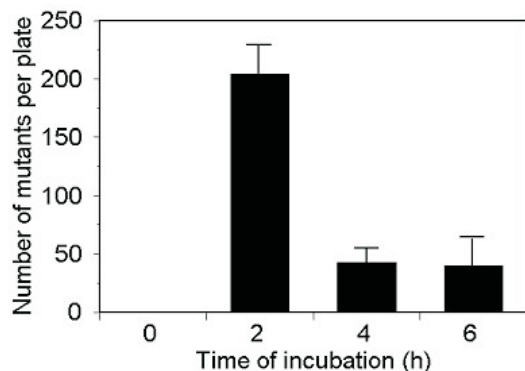


Figure 2. Effect of the time of incubation of bacteria with a mutagen on the liquid medium *Vibrio harveyi* mutagenicity assay. The results represent an experiment in which NQNO was added to a final concentration of 40 ng mL<sup>-1</sup>. Mean values from 5 independent experiments, with bars representing standard deviation,

observed previously in the plate assay (Czyż et al. 2002). This phenomenon may be caused by either toxicity of some mutagens at higher concentrations or by induction of so many mutations that a large proportion of neomycin-resistant mutants contains also additional mutations, which can be deleterious or lethal. Growth of bacterial strains in media containing mutagens at these concentrations was not severely impaired (data not shown), which does not support the toxicity hypothesis.

Efficiency of formation of neomycin-resistant mutants was tested in response to incubation of bacteria in the presence of mutagens for various times. Although it was reported previously that different mutagens required various incubation times to provoke the biggest effects (Czyż et al. 2000), in most cases a relatively short time of incubation was optimal, as exemplified in Figure 2. A putative reason for this phenomenon may be similar to that of the effect of high mutagen concentrations (compare Figure 1).

To compare the efficiency of the liquid medium assay and the plate assay, we performed analogous experiments using these two variants of the assay. We found that the liquid medium assay gives significantly better results when strains BB7 and BB7M are used, whereas BB7X and BB7XM respond more effectively to mutagens in the plate assay (Table 2). The reason for such a difference between these strains remains to be elucidated.

We found that in some cases the efficiency of formation of neomycin-resistant mutants in the liquid medium assay in the presence of relatively high amounts of mutagens can be enhanced by incubation of bacteria with these

compounds in the MM buffer rather than in the BOSS medium (Table 2). This effect was not observed at lower mutagen concentrations (data not shown). We can speculate that the higher number of mutants appearing after incubation of bacterial cultures with a high mutagen concentration in a buffer arises from slower cell metabolism, which allows the bacteria to avoid the negative effects depicted in Figure 1. Alternatively, some compounds of bacteriological media might interact with mutagens (at their sufficiently high concentrations), causing their partial inactivation, which would not be the case when incubating bacteria with these compounds in a buffer. It is worth mentioning that the effects of the incubation in a buffer were observed for all tested mutagens but to a varying extent. Moreover, only strains BB7 and BB7M revealed such a feature, in contrast to strains BB7X and BB7XM, which produced significantly less mutants after incubation in a buffer (Table 2). This may be caused by sensitivity of the BB7X strain to starvation, reported previously (Czyż et al. 2001); note that BB7XM is a derivative of BB7X (Table 1).

The *V. harveyi* tester strains were constructed to be very sensitive to mutagenic agents. We tested whether a short exposure of these strains to UV light (1 J m<sup>-2</sup>) may further sensitise them to mutagens. We found that this procedure has no significant effect on response to mutagens of the BB7 and BB7M strains. However, the number of mutagen-induced neomycin-resistant mutants of BB7X and BB7XM was considerably higher upon stimulation by UV (Table 2). No increase in the number of mutants was detected in analogous experiments without a chemical mutagen (data not shown). It appears that this effect can be caused by impaired DNA repair in BB7X, which has been demonstrated recently (Zielke et al. 2003).

Mutagenic effects of chemicals on cells depend not only on their potential to interact with a genetic material, but also on permeability of the cellular envelope to these compounds. Calcium chloride may considerably affect the permeability of the bacterial cell envelope, a phenomenon that is widely used in procedures of transformation of bacteria with foreign DNA (Smabrook et al. 1989). Therefore, we checked whether transient treatment of the *V. harveyi* tester strains with CaCl<sub>2</sub> can increase their response to chemical mutagens. We found that such a treatment had little effect on the number of mutagen-induced neomycin-resistant cells of BB7 and BB7M strain,

whereas frequency of appearance of such mutants increased about two times in BB7X and BB7XM (Table 2). The BB7X strain reveals many changes in cell structure and physiology in comparison to wild-type *V. harveyi*, including cell morphology (Czyż et al. 2001). Thus it is possible that the cell envelope is also deformed, resulting in higher sensitivity to CaCl<sub>2</sub>.

## Conclusions

Although the *V. harveyi* assay for detection of mutagenic pollution of the marine environment was demonstrated to be relatively sensitive (Czyż et al. 2000; 2002), a further increase in the response of the tester strains to chemical mutagens would be desired because concentrations of active mutagens in natural habitats are usually very low (Czyż et al. 2003). Therefore, we aimed to optimise the assay procedures.

The results of this study indicated that different *V. harveyi* strains, employed in the assay, require different conditions to give the maximal response to known mutagens. At relatively high mutagen concentrations, strains BB7 and BB7M formed significantly more induced mutants when incubated in a buffer instead of a nutrient broth, and the liquid medium assay was more effective than the plate assay, whereas the effect on the BB7X and BB7XM strains was just the opposite. The response of the strain BB7X and its derivative, BB7XM, to mutagens was enhanced by both short UV irradiation at a low dose and transient incubation in a CaCl<sub>2</sub> solution. These treatments had no significant effects on the response of BB7 and BB7M.

It appears that the *V. harveyi* mutagenicity assay may be recommended as a test for detection of mutagenic pollution of marine environment for four reasons: (i) it is sensitive, (ii) it is simple and does not require any special equipment, (iii) it employs strains of a marine bacterium, which survive incubation in samples of marine water, and (iv) it employs bacteria that are not pathogenic to humans, and thus the assay is completely safe to work with. Our further recommendation is using various experimental conditions for particular strains employed in the assay, according to data presented in Table 2. All four tester strains should be used when an environmental sample is tested, because different strains respond with different efficiency to various mutagens, as described previously (Czyż et al. 2000; 2002).

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