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Cell-Density-Dependent Sensitivity of a *mer-lux* Bioassay

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The sensitivity of a previously described assay (O. Selifonova, R. Burlage, and T. Barkay, Appl. Environ. Microbiol. 59:3083–3090, 1993) for the detection of bioavailable inorganic mercury (Hg^{2+}) by the activation of a *mer-lux* fusion was increased from nanomolar to picomolar concentrations by reducing biomass in the assays from 10^7 to 10^5 cells ml^{-1}. The increase in sensitivity was due to a reduction in the number of cellular binding sites that may compete with the regulatory protein, MerR, for binding of the inducer, Hg^{2+}. These results show that (i) the sensitivity of the *mer-lux* assay is sufficient for the detection of Hg^{2+} in most contaminated natural waters and (ii) *mer*-specified reactions, Hg^{2+} reduction and methylmercury degradation, can be induced in natural waters and may participate in the geochemical cycling of mercury.

The fraction of inorganic mercury (Hg^{2+} and various Hg[II] complexes) in environmental samples which is bioavailable is a critical parameter in the geochemical cycling of mercury and consequently in the control of mercury contamination. Inorganic mercury is the substrate for microbial methylation, and methylmercury (MM) is the form of mercury which is accumulated through the food chain to levels that pose a health threat to consumers of fish and shellfish (4, 10, 19). Furthermore, Hg^{2+} induces the mercury resistance (*mer*) operon whose functions, the reduction of Hg^{2+} to Hg^0 (by the *merA* gene product, the mercuric reductase) and degradation of MM (by the *merB* gene product, the organomercurial lyase), are beneficial as they reduce the net accumulation of MM (1). Thus, the detection and quantitation of bioavailable Hg^{2+} would enable evaluation of the potential for some of the most important reactions that determine MM accumulation. Available analytical methods measure trace amounts of organic and inorganic mercury (3, 9), but they cannot distinguish Hg^{2+} from other pools of mercury in environmental samples. The need to specifically quantitate Hg^{2+} motivated us (14) and others (17, 18) to develop indicator bacteria containing gene fusions between the promoter of the *mer* operon and *lux*, the genes specifying luminescence. The *mer* promoter is activated when Hg^{2+} binds to the regulatory protein, MerR (15, 16), and light is emitted in such fusions when mercury in this specific form is present in the cytoplasm of the indicator bacteria. Because the cytoplasm is the location where MM is synthesized (6) and *mer* is induced, light production is likely to indicate availability of Hg^{2+} for these processes.

With the exception of the *mer*-controlled production of firefly luciferase (18), all luminescence fusions developed to date respond to nanomolar concentrations of Hg^{2+}. This sensitivity was sufficient to detect Hg^{2+} in highly contaminated waters (14) but would be too low for less polluted and pristine waters. In the latter, MM is often accumulated in fish to levels that render them unsafe for unrestricted consumption, although total mercury concentrations in the water are in the picomolar range (8). We report here an increase in the sensitivity of a *mer-lux* assay that was achieved by decreasing the number of cells in the bioassay and show that sensitivity is compromised at higher cell densities by binding of Hg^{2+} to the bioindicator cells.

Bacterial strains, growth, and cell preparation. The strains used in this investigation were *Escherichia coli* HMS174 (F^- *recA1 rpoB331 hsdR19* λ^- IN[*rrnD-rrnE*]*1*) (5) and two of its derivatives, one with the *mer-lux* plasmid pRB28 (14) and the other with pRB27, a *lux* constitutive mutant of pRB28 (2). Cultures were maintained in Luria-Bertani medium with kanamycin (50 $\mu g\ ml^{-1}$) for selection of plasmids. Growth and preparation of cells for *mer-lux* assays were as described by Selifonova et al. (14). The optical density of the cell suspension in 67 mM phosphate buffer (pH 6.8) was adjusted to 0.4 (Spectronic 21; Milton Roy Co., Rochester, N.Y.) at 660 nm, which equaled approximately 2.2×10^8 cells ml^{-1}.

***mer-lux* assays.** The assay medium consisted of pyruvate (5 mM), NaK-phosphate buffer (67 mM PO_4, 34 mM Na, 33 mM K [pH 6.8]), and $(NH_4)_2SO_4$ (0.091 mM). Note that the molarity of $(NH_4)_2SO_4$ had been reduced substantially from that given by Selifonova et al. (14). Assays (final volume of 2 ml) were mixed from stock solutions immediately prior to use, in 20-ml glass scintillation vials, and the assays were performed as described by Selifonova et al. (14). Appropriate dilutions (0.1 ml) of the cell suspension were added to each scintillation vial. Assays were initiated by the addition of 10-fold dilutions of a mercury stock solution [5 μM Hg^{2+} as $Hg(NO_3)_2$ in 0.2 N HNO_3] in distilled water (freshly made to minimize loss of Hg due to sorption or volatilization). Tested Hg^{2+} concentrations ranged from 6.25 pM (1.25 ng $liter^{-1}$) to 70 nM (14 $\mu g\ liter^{-1}$). Assays were performed at cell densities of 5×10^4, 1×10^5, 1×10^6, 1×10^7, and 3×10^7 cells ml^{-1}. Light emission was measured as described by Selifonova et al. (14). *mer-lux* expression factors ([log quanta] $minute^{-1}$) were calculated from the slopes of curves depicting increased light production with time as described by Barkay et al. (2).

Cell-density-dependent sensitivity of the *mer-lux* assay. The range of Hg^{2+} concentrations at which *mer-lux* expression by strain HMS174(pRB28) was induced was directly related to cell density. When expression factors, calculated from light induction curves, were plotted versus Hg^{2+} concentration, the typical ultrasensitive or threshold activation of the *mer* promoter (12, 13) was demonstrated (Fig. 1). Thus, light production was induced between 1 and 70 nM Hg^{2+} with 3×10^7 cells

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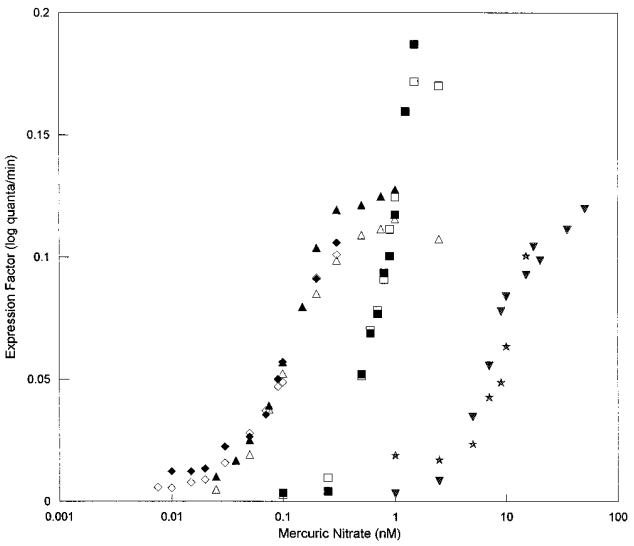

FIG. 1. The effect of cell density on the sensitivity of the *mer-lux* response. *mer-lux* assays were performed (see text for details) at the indicated Hg^{2+} concentrations with 3×10^7 cells ml^{-1} (stars and inverted triangles; duplicate experiments), 1×10^7 cells ml^{-1} (squares), 1×10^6 cells ml^{-1} (triangles), and 1×10^5 cells ml^{-1} (diamonds), and expression factors were calculated for reactions showing induction of light production.

ml^{-1}, but the range of ultrasensitive increase in promoter activity was between 3 and 30 nM Hg^{2+}. Light was induced between 0.1 and 3 nM Hg^{2+} (ultrasensitive response at 0.3 to 1 nM) with 10^7 cells ml^{-1} and between 0.007 and 3 nM Hg^{2+} (ultrasensitive response at 0.03 to 0.3 nM) with $\leq 10^6$ cells ml^{-1}. At each cell density, there was a range of Hg^{2+} concentrations sufficient for the induction of light but below the concentration that triggered the ultrasensitive response. At the upper range of concentrations, the ultrasensitive response was saturated. Decreasing cell density below 1×10^6 to 1×10^5 (Fig. 1) and 5×10^4 (data not shown) did not improve the assay sensitivity any further. The typical sigmoidal response (Fig. 1) indicated that under the employed assay conditions light production was determined by the Hg^{2+}-dependent activation of the *mer* promoter rather than by factors relating to the synthesis and activity of the luminescence system.

Previous in vivo (7, 12) and in vitro (13) assays detected induction of *mer* when Hg^{2+} concentrations were at least 1 to 10 nM with the midpoint of the sigmoidal response at 10 nM (16). In nature, such high concentrations are found only in highly contaminated waters (1), casting doubt on the significance of *mer*-specified functions, the reduction of Hg^{2+} and the degradation of MM, in the geochemical cycling of mercury. Our results suggest induction of *mer* at much lower Hg^{2+} concentrations. Induction of *mer-lux* was detected at 7 pM Hg^{2+} (Fig. 1; low cell densities). This finding may explain the presence of *merA* transcripts in RNA samples collected at a site with background concentrations of total mercury (11), and it implies that *mer*-specified functions should not be limited to highly polluted environments but, rather, may occur in less contaminated, or even pristine, locations.

Binding of Hg^{2+} to cellular material compromises the sensitivity of the *mer-lux* assay. Increasing concentrations of the nonluminescent strain HMS174 (from 0 to 10^7 cells ml^{-1}) were added to 10^6 cells ml^{-1} of the *mer-lux* strain HMS174(pRB28) to test if cell biomass limited availability of Hg^{2+} for the induction of *mer-lux* by 0.25 nM Hg^{2+}. These conditions were selected because at this Hg^{2+} concentration *mer-lux* induction reached saturation, with respect to Hg^{2+}, with 10^6 cells ml^{-1} and threshold levels with 10^7 cells ml^{-1} (Fig. 1). Light emission was reduced by 90 and 99% when 2×10^6 and 4×10^6 cells ml^{-1}, respectively, of HMS174 were added relative to assays that did not contain this strain (Fig. 2A). Light production was not detected when 6×10^6 or more HMS174 cells ml^{-1} were added. Because the total number of cells in assays was increased with the gradual addition of strain HMS174, it was possible that light output was masked by excess biomass in the assays. This possibility was rejected by repeating the experiment with the constitutively luminescent indicator, strain HMS174(pRB27). No reduction in light output was noted when the same cell ratios as for HMS174(pRB28) were assayed with the nonluminescent strain, HMS174 (Fig. 2B). Thus, the reduction in light output by HMS174(pRB28) in presence of additional cell biomass was likely due to a decline in *mer-lux*

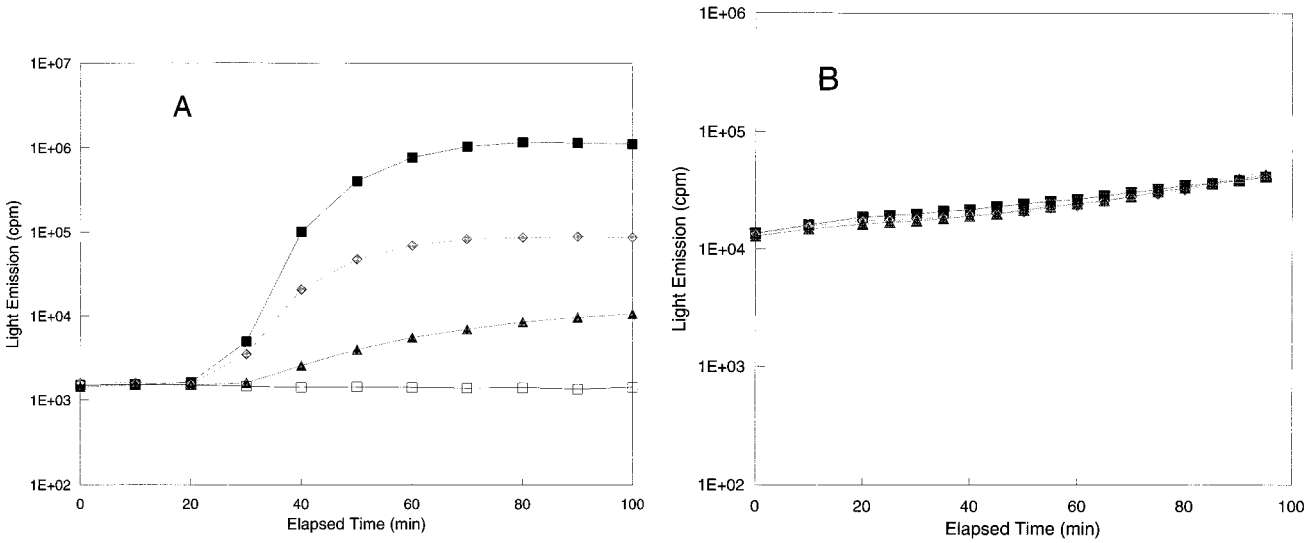

FIG. 2. The effect of the addition of nonluminescent cells on light production. Strain HMS174 was added at 2×10^6 (diamonds), 4×10^6 (triangles), or 6×10^6 (open squares) cells ml^{-1} or not added (dark squares) to 10^6 cells of HMS174(pRB28) (A) and HMS174(pRB27) (B) ml^{-1}.

induction as the availability of Hg^{2+} to MerR was reduced. A competition between MerR and cellular binding sites may be responsible for this phenomenon. Such sites could include negatively charged groups and other ligands on the outer surface of the cell or intracellular sequestration with sulfhydryl-containing reducing agents such as glutathione. Further work is necessary to identify and characterize the specific ligands that control availability of Hg^{2+} to MerR.

At its most sensitive range (10 pM), with low cell densities, the assay could be used to measure bioavailable Hg^{2+} in many contaminated waters and in some pristine environments (see Table 1 in reference 8). However, accumulation of MM in aquatic biota has been reported in numerous locations where total mercury was in the <10 pM concentration range. As Hg^{2+} is likely to be only a fraction of total mercury, the sensitivity of the assay needs to be improved by further modifications of the existing bioindicator and the development of more sensitive ones, to allow detection and measurement of Hg^{2+} in all waters in which mercury contamination poses a risk to public health.

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