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Evidence for facilitated uptake of Hg(II) by *Vibrio anguillarum* and *Escherichia coli* under anaerobic and aerobic conditions

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Abstract

A *mer-lux* bioreporter was used to study uptake of inorganic mercury, Hg(II), at trace concentrations by two facultatively anaerobic bacterial species, *Vibrio anguillarum* and *Escherichia coli*. Uptake of Hg(II) by these bacteria appeared to be facilitated, rather than by passive diffusion. Three lines of evidence support this conclusion. First, under anaerobic conditions Hg(II) uptake was greatly decreased compared with aerobic conditions, even though the chemical composition of the medium was identical except for the lack of oxygen (i.e., no reducing agents were used). Second, the uptake of Hg(II) under anaerobic conditions was not proportional to the abundance of lipophilic Hg species but was dependent on the total concentration of Hg in the samples. Third, at trace Hg(II) concentrations and under anaerobic conditions, Hg(II) uptake was enhanced by the addition of yeast extract and a variety of low molecular weight organic acids. In addition to demonstrating that Hg(II) uptake by these bacteria had the characteristics of facilitated transport, these lines of evidence also support the conclusion that processes under regulatory control of the cell affected Hg(II) uptake. If these findings apply to other bacteria as well, they mean that current models of Hg(II) uptake by microorganisms in aquatic systems, which are based solely on the role of lipophilic Hg species and passive diffusion, will need to be reconsidered.

Mercury undergoes a wide variety of chemical and biological transformations in aquatic ecosystems. Microorganisms play a major role in carrying out the biological transformations, many of which affect the toxicity of mercury. For example, bacteria that contain the *mer* operon can reduce Hg(II) to the volatile and relatively nontoxic Hg^0 (Hobman and Brown 1997). Hg(II) is transformed by bacteria in anaerobic sediments to monomethyl mercury (Compeau and Bartha 1984), which is a more toxic form of Hg(II) and is more readily bioaccumulated in the aquatic food web than inorganic mercury (Mason et al. 1996; Watras et al. 1998). For these reactions to occur, Hg(II) must first enter the cell; therefore, an understanding of the mechanism(s) of Hg(II) uptake by microorganisms is important.

In contrast to other metals (e.g., Nies 1999), current evidence suggests that mercury enters bacterial and algal cells via passive diffusion of lipophilic mercury species, such as $HgCl_2$ and HgS, across the lipid membrane (Mason et al. 1996; Benoit et al. 1999, 2001). Correlations have been found between the calculated abundance of these lipophilic species with (1) rates of diffusion of mercury across artificial phospholipid membranes (Gutknecht 1981; Bienvenue et al. 1984), (2) bioaccumulation and toxicity in a marine algal species (Mason et al. 1996), and (3) rates of production of methylmercury in a sulphate reducing bacterium (Benoit et al. 2001). A limitation of experiments to date has been the inability to work at the trace concentrations ($\leq$10 ng L^{-1}) normally found in aquatic environments, because higher levels were required for a detectable response.

In this study, we used a direct and highly sensitive approach to study Hg(II) uptake into two species of gram-negative bacteria, *Vibrio anguillarum*, an aquatic species, and *Escherichia coli*, an intestinal species. Experiments were

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Table 1. Calculated speciation of Hg(II) in the various defined assay media. All stability constants were from Martell and Smith (2001).[a]

Assay medium (pH 7.1)	Components (all contained 5 mM glucose)	$Hg(NH_3)^{2+}$ (%)	$Hg(OH)_2$ (%)	$HgCl_2$ (%)	HgOHCl (%)
A	2 mM K_2HPO_4, 1 mM NaH_2PO_4, 9 mM $(NH_4)_2SO_4$	98	1.7	1.7×10^{-6}	2.7×10^{-3}
B	4 mM K_2HPO_4, 2 mM NaH_2PO_4, 0.9 mM $(NH_4)_2SO_4$	32	59	5.9×10^{-5}	9.4×10^{-2}
C	4 mM K_2HPO_4, 2 mM NaH_2PO_4, 0.9 mM $(NH_4)_2SO_4$, 3.4 mM NaCl	2.8	5.2	60	28
D	4 mM K_2HPO_4, 2 mM NaH_2PO_4, 9 mM $(NH_4)_2SO_4$	98	1.6	1.6×10^{-6}	2.6×10^{-3}
E	40 mM K_2HPO_4, 20 mM NaH_2PO_4, 9 mM $(NH_4)_2SO_4$, 2 mM NaCl	90	1.1	4.4	3.5

[a] Values used were ones measured at 25°C and ionic strength = 0 or corrected to 0 (HgOHCl, log β = 4.0). Exceptions were stability constants for the ligands $(NH_3)^{2+}$ (I = 1), and HPO_4^{2-} and PO_4^{3-}, (I = 3). Note that log β for $HgPO_4^-$ in Martell and Smith (2001) should be 14, not 9.5 (R. Hudson and R. Smith, pers. comm.).

done at trace Hg concentrations (<10 ng L^{-1}) and at cell population densities similar to those found in aquatic ecosystems (~10^6 cells mL^{-1}, Jordan 1985). This approach uses an intracellular Hg(II) inducible bioreporter (*mer-lux*) (Selifonova et al. 1993; Barkay et al. 1998), which functions as follows: when Hg(II) enters a bacterial cell that contains this plasmid, it binds to the regulatory protein (MerR), the *mer-lux* operon is induced, and the proteins that catalyze light production are synthesized. MerR is highly specific for Hg(II) (Ralston and O'Halloran 1990), and the amount of light produced is proportional to the amount of Hg(II) that enters the cell (Barkay et al. 1997). The mercury transport genes that are present on the native *mer* operon have been deleted from the *mer-lux* construct, so that uptake of Hg(II) can be studied, as it occurs in the majority of bacteria, which do not have the *mer* operon. A constitutive *lux* plasmid was used as a control to test for changes in light emission unrelated to Hg(II) uptake, which may be caused by physiological responses of the host bacteria to the chemistry of different samples that are being assayed (Barkay et al. 1997; Neilson et al. 1999).

Results indicated that facilitated processes for Hg(II) transport were occurring and that the characteristics of transport differed in aerobic and anaerobic conditions. Addition of low-molecular-weight organic compounds enhanced the Hg(II)-dependent response of the bioreporter under anaerobic conditions, demonstrating a previously undescribed role for such compounds in anaerobic environments. These findings are important to our understanding of how Hg(II) is taken up by bacteria, possibly including the gram-negative methylating bacterial species.

Materials and methods

Bacterial strains—*E. coli* HMS174(pRB28) and *E. coli* HMS174(pRB27) have been described elsewhere (Selifonova et al. 1993 and Barkay et al. 1997, respectively). In an effort to create a bioreporter suitable for the study of mercury uptake in natural waters, the *mer-lux*–carrying plasmid, pRB28, and its constitutively expressed derivative, pRB27, were transferred to *V. anguillarum* ATCC 14181, a natural aquatic species. Plasmid DNA was extracted from the original host, *E. coli* HMS174 (Sambrook et al. 1989), and used to transform strain 14181 as described by Chung et al. (1989). Transformants were selected and maintained by growth in presence of 80 μg ml^{-1} kanamycin (Kan), resistance to which is specified by pRB28 and pRB27.

Media—The same three media were used for both the aerobic and anaerobic experiments: (1) Lennox Broth (LB, Difco) was used for initial growth and maintenance of bacterial cultures; (2) glucose minimal medium (GMM) was used to grow cells prior to assays and consisted of 40 mM Na_2HPO_4, 20 mM KH_2PO_4, 20 mM NH_4Cl, 2 mM NaCl, 16.7 mM glucose, trace elements as described by Barkay et al. (1998), 1 mM $MgSO_4$, 3 mM vitamin B_1, and 80 μg ml^{-1} Kan; and (3) assay media were used to study Hg(II) uptake under simple defined conditions and did not include Kan, vitamins, or trace metals (Table 1).

Preparation of anaerobic media and reagents—Media and reagents were prepared for anaerobic work by the Hungate method (Hungate 1969). Addition of reducing agents such as Ti-NTA, Ti-Citrate, and dithionite decreased the response of the *mer-lux* bioreporter to Hg(II) and so were not used in this study. A ChemMets O_2 Kit was used to ensure the reagents and media were oxygen free (O_2 <1 ppb). In a HEPA-filtered air laboratory, culture flasks, Teflon centrifuge tubes, and reagent bottles were washed in 30% H_2S0_4 and rinsed thoroughly with low-Hg (<0.3 ng L^{-1}) Milli-Q water. Reagents used in the growth and assay media were prepared in a class 100 laminar flow hood by use of low-Hg Milli-Q water. There were trace amounts of Hg in the reagents used, but this Hg was not bioavailable to the bioreporter, as measured by blank controls.

Growth of cultures for bioreporter assays—For anaerobic assays, 5 ml of aerobic LB-Kan broth was inoculated with a single isolated colony from a LB-Kan agar plate and incubated at 28°C until late midlog phase (OD_{600} ~ 0.7). All remaining handling of the cultures was performed in an anaerobic Coy glove bag with an atmosphere of 93% N_2/7% H_2, and 50 μl of the culture was transferred to a Balch-Wolfe tube that contained 4.95 ml of anaerobic GMM-Kan. The tube was then sealed with a rubber butyl stopper, crimped with an aluminum cap, and reincubated at 28°C until late midlog phase. The 5 ml anaerobic culture was added to 20 ml of anaerobic GMM-Kan in a 100-ml nominal capacity serum bottle, resealed, and incubated at 28°C until midlog phase. To prepare cells for bioassays, the final 25-ml midlog

culture was decanted into 50 ml nominal capacity centrifuge tubes sealed with an O-ring closure. Cells were washed twice by centrifuging and resuspending in anaerobic, 67 mM phosphate buffer (pH 7.1). A portion of the final resuspension was diluted serially to a final concentration of $\sim 1 \times 10^5$ cells ml^{-1} in the assay media. Cultures for aerobic bioreporter assays were prepared as described for anaerobic assays, except that they were fully aerated throughout.

Bioreporter assays—For anaerobic assays, the assay medium was mixed in scintillation vials with the Hg(II) standards (see below), inside the glove bag, to a final volume of 19 ml. The media that contained Hg(II) were incubated for ~15 min before 1 ml of the final cell suspension of either the inducible (pRB28) strain or the constitutive (pRB27) strain, was added to initiate the assays. The samples were incubated at room temperature in the anaerobic glove bag. After appropriate times, the vials were removed from the glove bag and assayed for light emission prior to aeration to ensure the samples were air free during the induction period. Then vials were opened and shaken, to ensure full aeration needed for the light emitting reaction (Phillips-Jones 1993), prior to scintillation counting (Beckman LS 6500, set in noncoincidence mode). Aerobic *mer-lux* assays were carried out as described for the anaerobic assays except that preparation of media and cells were done on the bench top, and the shaking step was unnecessary.

Hg analyses, standards, and speciation—Water and reagents used in the assay media and anaerobic sediment porewaters were analyzed for total mercury by Flett Research Ltd. by use of a cold atomic fluorescence spectrometer (Brooks Rand, Ltd., Model 2) and EPA Method 1631, as adapted from Bloom and Creselius (1983). Samples were preserved by adding 0.25 ml concentrated hydrochloric acid per 125 ml sample. The primary mercury standard was a 1 $\mu g\ ml^{-1}$ mercury solution that contained 1% BrCl (provided by Flett Research Ltd.). For the anaerobic assays, 0.5 ml of the primary standard was transferred to a Teflon vial and brought into the glove bag. Further dilutions were done by use of anaerobic Milli-Q water and Teflon vials that had been conditioned overnight in the glove bag. For aerobic assays, the primary standard was diluted by use of aerobic Milli-Q water and Teflon vials. Mercury speciation of the assay medium was calculated by use of KINETEQL (R. Hudson, University of Illinois, Urbana-Champaign), which is a MS Excel add-in program that uses the Newton-Ralphson method to solve multicomponent chemical equilibrium problems defined according to the tableau method (Morel and Hering 1993). Stability constants for Hg with some ligands were only available for high ionic strength conditions (see Table 1). The uncertainty introduced was taken into account in interpreting the results (see Discussion).

Sediment porewater samples—Sediment samples were obtained from Lake 114 (Rudd et al. 1986) at the Experimental Lakes Area, Ontario, Canada, by use of an Eckman dredge. Depth of water at the sites sampled was ~1.5 m. Sediments were of high porosity (~98% water) and high organic content (Rudd et al. 1986). Sediment was transferred to overflowing in glass jars that were closed tightly with a screw cap and opened in the laboratory inside an anaerobic Coy glove bag. Porewater was collected by filtration through a 0.45-μm cellulose acetate membrane in the glove bag. The concentration of O_2 in the porewater, measured immediately after filtration, was below the detection limit of the ChemMets O_2 kit (<1 ppb). The collected porewater was supplemented with 5 mM glucose, 2 mM K_2HPO_4, 1 mM NaH_2PO_4, and 9 mM $(NH_4)_2SO_4$ and used the same day for the bioreporter assays.

Results

Aerobic mer-lux *assays*—The typical response of *V. anguillarum* pRB28 to the addition of mercury (0.75–3 ng L^{-1}) in assay media (Table 1) under aerobic conditions begins with an initial lag phase, followed by increased light production, which ultimately reaches a plateau (Fig. 1A). In aerobic assays that used *E. coli* HMS174 pRB28, the same pattern of light production was observed for mercury concentrations in the same range (0.75–3 ng L^{-1}). This pattern has been described elsewhere for *E. coli* HMS174 pRB28 (Barkay et al. 1998) but at higher Hg(II) concentrations (>10 ng L^{-1}).

Under aerobic conditions, light production at any point in time between 60 and 100 min was found to be linear to the concentration of mercury added to the samples (e.g., Fig. 1B). This was important to establish because measurement of light production in the anaerobic assays had to be done at a single time point (see below) rather than by the slopes of induction curves as is used during aerobic assays (Barkay et al. 1998). Control samples in which mercury was not added did not induce light production (Fig. 1).

Anaerobic mer-lux *assays*—Light emission from the constitutively controlled *V. anguillarum* pRB27 clearly showed that anaerobic incubations did not prevent the light-emitting reaction from occurring immediately after the addition of air (Fig. 2A). Also, the amount of light produced was comparable to the light yields in constitutive aerobic assays (Fig. 2A). Therefore, the luciferase system was viable as a reporter for in vivo gene expression under anaerobic conditions.

When the inducible strain *V. anguillarum* pRB28 was tested with Hg(II) under anaerobic but otherwise similar conditions to those used in aerobic assays (medium A), ~100-fold higher concentration of mercury was required for detectable expression (Fig. 2B). The decrease in sensitivity under anaerobic conditions was not due to the loss of Hg(II) in the anaerobic samples, as determined by measuring total Hg (data not shown). The anaerobic assay was repeated with use of the *E. coli* HMS174 bioreporter, to determine whether this lack of a response to low concentrations of mercury was specific to the *V. anguillarum* host. *E. coli* HMS174 pRB28 did not show a detectable response under anaerobic conditions for Hg unless the concentration provided was >50 ng L^{-1}, which was the same as observed with *V. anguillarum* (Fig. 2B). We increased the time of mercury exposure under anaerobic conditions up to 24 h, but no response to mercury concentrations <50 ng L^{-1} were observed with the *V. anguillarum* or *E. coli* bioreporter (data not shown).

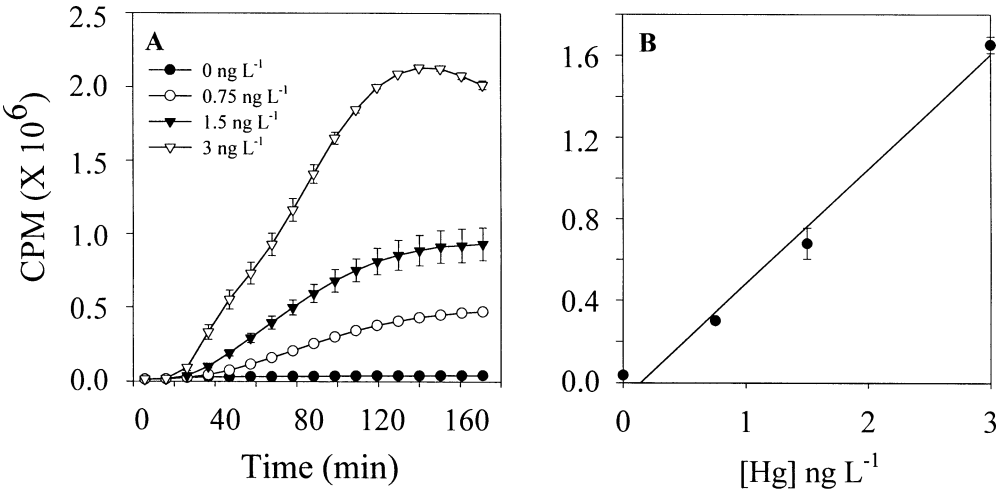

Fig. 1. (A) Aerobic ''real time'' assay and (B) aerobic time-point assay of light production after 100 min of mercury exposure at concentrations ranging from 0 to 3 ng L^{-1} for *V. anguillarum* pRB28. The assay was performed in medium A (see Table 1). Error bars represent the standard deviation of duplicate samples.

The different response under aerobic and anaerobic conditions in these assays (Figs. 1B, 2B) was unexpected because the same assay medium was used in both cases. Thus, the concentration of lipophilic species available for passive diffusion should have been the same. Because the Hg(II) in medium A was present primarily as the charged species $Hg(NH_3)_2^{2+}$, we manipulated the concentration of reagents in the assay medium to increase the proportion of the neutral species $HgCl_2$, HgOHCl, or $Hg(OH)_2$ (Table 1). However, there was still no response at low total Hg (Fig. 3A). At high Hg concentrations, the bioreporter response was generally proportional to the total concentration of mercury and was the same in media B and C (Fig. 3A). Constitutive light production was also the same in media B and C, but higher in medium D (data not shown). Thus, the greater light response to Hg(II) observed in medium D (Fig. 3A) was likely related to the physiology of light production rather than to a higher rate of Hg uptake. The Hg(II)-induced response was not proportional to the concentrations of the neutral species $Hg(OH)_2$ (Fig. 3B) or $HgCl_2$ (Fig. 3C) present in the bulk solution. Thus, uptake at high Hg concentrations did not have the characteristics of passive diffusion. The response was also not proportional to the concentration of $Hg(NH_3)_2^{2+}$ (Fig. 3D).

Mer-lux *experiments with organic supplements*—To investigate whether the simple composition of the defined assay media (Table 1) was limiting some physiological process that affected the bioreporter's ability to respond to trace Hg(II) concentrations under anaerobic conditions, we added a variety of low molecular weight organics. The first organic additions tested were yeast extract and casamino acids. Both enhanced the response, which suggests that components of these supplements increased Hg(II) uptake under anaerobic conditions in both *V. anguillarum* and *E. coli* (Table 2). This was surprising, because yeast extract and casamino acids contain a variety of complex organics, which have been shown to have a high affinity for Hg (II) (Ramamoorthy and Kushner 1975) and would therefore be expected to decrease the bioavailability of mercury.

To identify what components of yeast extract and casamino acids may stimulate the Hg(II)-dependent bioreporter response, we added single amino and carboxylic acids to the anaerobic assay media. Of the amino acids tested, histidine and threonine induced the highest response (Table 2). Unlike the amino acids, the addition of carboxylic acids lowered the pH of the assay media (Table 2). Some of these acids induced an anaerobic *mer-lux* response to low mercury concentrations, and these were the same acids that lowered the pH to the greatest degree (Table 2).

Further investigations into the effect of histidine on Hg(II) bioavailability were undertaken because histidine yielded the largest response of the organic supplements tested. At a constant Hg(II) concentration (10 ng L^{-1}), light induction in *V. anguillarum* pRB28 cells increased with increasing histidine concentration (Fig. 4A). Histidine itself did not induce this response in the absence of mercury (data not shown). Also, varying histidine concentration did not affect the amount of light produced by the constitutive strain (*V. anguillarum* pRB27) after an anaerobic period similar in length to that used for exposure of *V. anguillarum* pRB28 to Hg(II) (Fig. 4A), which confirms that histidine metabolism was not required for light emission.

The aerobic response of the inducible strain to increasing histidine concentration was completely different. Aerobically, the addition of histidine was not required for a Hg(II)-induced light response (Fig. 4B). Hg(II) uptake appeared to be relatively constant or slightly increasing, even though we expect that increasing histidine would result in the formation of a variety of Hg(II)-histidine complexes, on the basis of noncritical stability constants (Van der Linden and Beers 1973). The effect of increasing histidine on light production by the constitutive strain was the same under aerobic conditions as anaerobic—that is, there was no significant effect (Fig. 4A,B).

Anaerobically, in the presence of 1 mM histidine, the

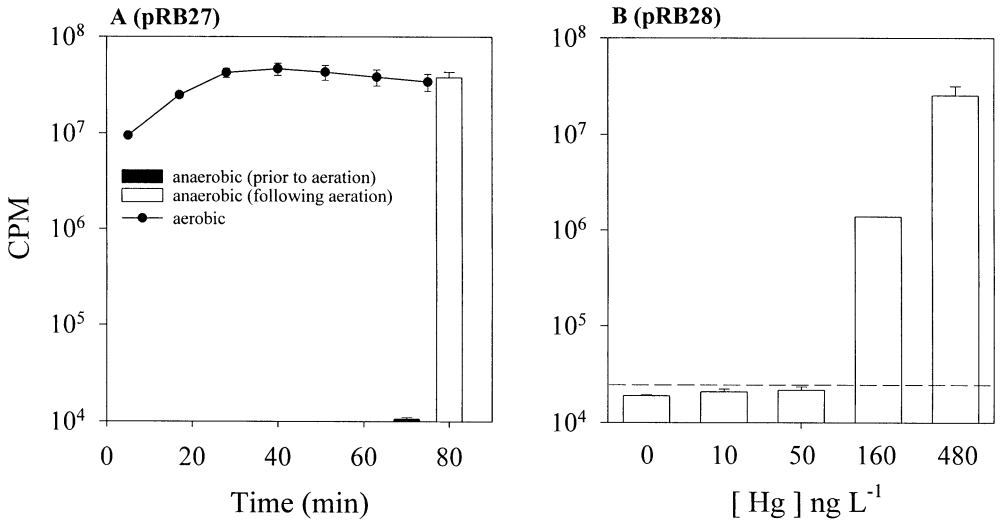

Fig. 2. (A) Light production by *V. anguillarum* pRB27 under aerobic and anaerobic conditions and (B) time-point assay of light production in medium A (see Table 1) after 75 min of mercury exposure under anaerobic conditions for *V. anguillarum* pRB28. Error bars represent the standard deviation of duplicate samples. The horizontal dotted line is the scintillation counter machine blank.

bioreporter response to Hg(II) was linearly related to the concentration of Hg added and induction occurred at trace mercury concentrations (1–2 ng L^{-1}, Fig. 5). This was the same level of sensitivity to mercury concentration as seen in the aerobic assays without histidine (Fig. 1A,B). Therefore, the previous negative results at low Hg(II) concentrations did not stem from some inability of the *merR* regulatory system to function anaerobically. Because of this, and the fact that addition of histidine did not increase light production (Fig. 4A), the most plausible interpretation of the differences seen in expression of *mer-lux* at low Hg concentrations under anaerobic and aerobic conditions was that Hg(II) uptake was present in the aerobic situation but was absent anaerobically.

Anaerobic porewater experiments—The total mercury concentration in the two porewater samples acquired on 12 and 13 September 2000 was 1.0 and 2.3 ng L^{-1}, respectively. The response of the *mer-lux* bioreporter in the porewater without added mercury was never large enough to be considered a positive response (Table 3). Addition of only 10–20 ng L^{-1} of mercury to the porewater samples produced a large response (Table 3). This was a much lower concentration than the ~100 ng L^{-1} needed for expression under anaerobic conditions in the defined assay medium (Fig. 3A).

Discussion

This bioreporter method, which used the *mer-lux* plasmid in facultative bacteria, has enabled the study of Hg(II) uptake for the first time at trace level concentrations under both anaerobic and aerobic conditions. Several workers have hypothesized (Mason et al. 1996; Benoit et al. 2001) that Hg(II) uptake occurs by passive diffusion, and so we begin this discussion by examining this hypothesis. The essential feature of this hypothesis is that Hg(II) uptake is related to the abundance of neutrally charged, lipophilic species in the bulk solution.

First, if passive diffusion were the Hg(II) uptake mechanism for the bacteria we studied, we would expect no difference in the diffusion of lipophilic species across the lipid membrane under anaerobic or aerobic conditions. The concentrations of Hg complexes were the same in the anaerobic and aerobic bioreporter assays, but much higher concentrations of Hg were required under anaerobic conditions for a Hg(II)-induced response (Figs. 1A, 2B). This finding suggests a physiologically regulated mechanism for the transport of mercury. It has been shown for *Bacillus subtilis* (Ye et al. 2000) and hypothesized for *E. coli* (Unden et al. 1995) that >200 genes are expressed differently in anaerobic and aerobic environments. For example, under aerobic conditions Mn^{2+} is required for bacterial protection against oxidative damage, and therefore the expression of the genes responsible for Mn^{2+} uptake is upregulated in the presence of hydrogen peroxide (Kehres et al. 2000). If Hg(II) is ''accidentally'' taken up by this type of essential metal transporter, then its uptake would also differ under anaerobic and aerobic conditions. Facilitated uptake mechanisms have been demonstrated elsewhere for the ''accidental'' uptake of other toxic metals such as Cd^{2+} (Kehres et al. 2000) and Ag^{+} (Solioz and Odermatt 1995) via metal transport systems specific for Mn^{2+} and Cu^{+}, respectively.

Second, if passive diffusion were the predominant mechanism, uptake at high Hg concentrations (100–500 ng L^{-1}) should have been highest in medium C where $HgCl_2$ dominated the bulk solution, followed by medium B, dominated by $Hg(OH)_2$ (Table 1). This did occur in a study that used eukaryotic diatoms (Mason et al. 1996) but was clearly not the case for these bacteria (Fig. 3). There is some uncertainty in the speciation calculations for media B, C, and D, because some of the stability constants were measured at high ionic strength (Table 1). If the $HgHPO_4$ and $HgPO_4^-$ constants ap-

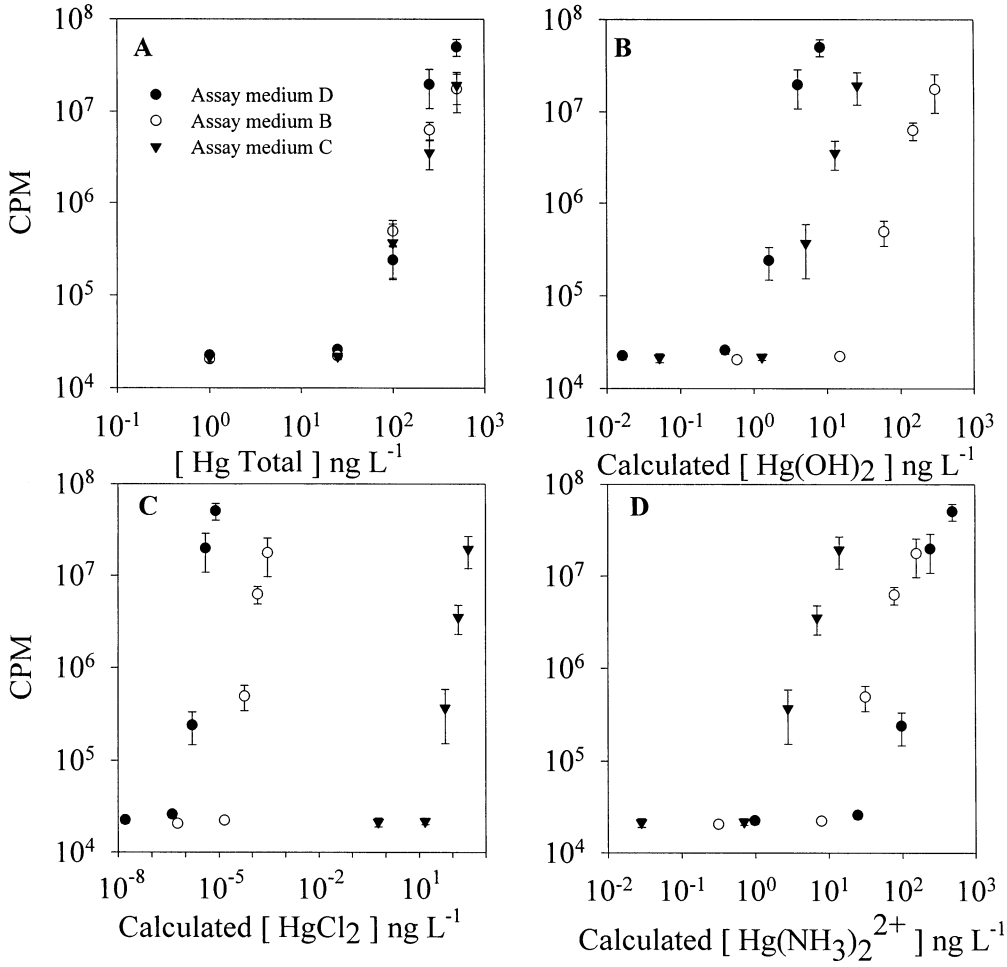

Fig. 3. Effect of Hg(II) speciation on light production after 80 min of mercury exposure under anaerobic conditions for *V. anguillarum* pRB28. Induced light production was compared with (A) total Hg and with the calculated speciation of Hg(II): (B) Hg(OH)2, (C) HgCl2, and (D) $Hg(NH_3)_2^{2+}$, in media B–D (see Table 1). Error bars represent the standard error of duplicate samples of three independent experiments.

propriate for lower ionic strength are smaller, there would be little change in the Hg speciation shown in Table 1. This seems likely, because constants for phosphates and other metals over a wide range of ionic strengths are generally many orders of magnitude smaller than the reported values for Hg (Martell and Smith 2001). We cannot rule out that the appropriate constants are greater, and, if so, the speciation shown in Table 1 would change significantly. However, even if these constants are two orders of magnitude greater, medium C would still have by far the greatest concentration of lipophilic complexes ($HgCl_2$ and HgOHCl), and medium B and D would be dominated by charged complexes. Thus, the interpretation of these experiments would still be that passive diffusion of neutral species from the bulk solution was not the dominant uptake mechanism.

This indiscriminate uptake of $Hg(OH)_2$, $HgCl_2$, and even $Hg(NH_3)_2^{2+}$ observed under anaerobic conditions at high mercury concentration (Fig. 3) has also been demonstrated at low mercury concentration under aerobic conditions (K. Scott pers. comm.). These observations do not coincide with the model of passive diffusion but were consistent with the model of kinetically controlled facilitated uptake proposed by Hudson et al. (1994), in which a variety of labile species interact with an extracellular or surface-associated transport ligand. As long as the affinity of the transport ligand is stronger than the affinity of Hg(II) to the extracellular complex, and provided the rate of entry of Hg(II) into the cell is fast compared with the rate of formation of the Hg(II)-transporter complex (Van Leeuwen 1999; Pinheiro and Van Leeuwen 2001), Hg(II) uptake should be relatively insensitive to Hg(II) speciation in the external medium. The transport ligand could be a protein or could simply be a molecule in the periplasmic space that forms a lipophilic complex with Hg(II) and diffuses across the membrane. In this latter case, the physical process involved could be passive diffusion, but it would still be facilitated by the cell and not controlled by bulk solution chemistry.

The positive effect on Hg(II) uptake of yeast extract, casamino acids, and single amino acids, under anaerobic conditions, was an unexpected finding. Unfortunately, there are

Table 2. Anaerobic induction of *mer-lux* in *V. anguillarum* pRB28 and *E. coli* HMS174 pRB28 after the addition of mercury to the assay medium amended with varying organic supplements.

Bioreporter host, assay medium[a]	Supplement[b]	[Hg] (ng L^{-1})	Response[c]	pH
V. anguillarum				
E	Yeast extract 0.1% (w/v)	25	+	7.1
E	Casamino acids 0.1% (w/v)	10	+	7.1
E	Threonine 8.4	25	++	7.1
E	Arginine 5.7	25	++	7.1
E	Proline 8.7	25	+	7.1
E	Histidine 6.4	25	+++	7.1
E	Leucine 7.6	25	++	7.1
E	Glycine 13.3	20	−	7.1
E	Phenyalanine 6.1	20	+	7.1
E	Asparagine 7.6	20	+	7.1
E	Isoleucine 7.6	20	+	7.1
E	Valine 8.5	20	+	7.1
A	Histidine 1	10	+++	7.0
A	Imidazole 1	10	−	7.1
A	Fumarate 1	10	++	5.7
A	Acetate 1	10	−	6.6
A	Formic acid 1	10	+	6.5
A	Malate 1	10	++	5.8
A	Succinate 1	10	−	6.0
A	Citrate 1	10	−	7.0
A	α-Ketoglutarate 1	10	++	5.8
A	Oxaloacetate 1	10	−	6.5
E. coli				
E	Yeast extract 0.1% (w/v)	25	+	7.1
E	Yeast extract 0.1% (w/v)	50	++	7.1
E	Casamino acids 0.1% (w/v)	25	+	7.1
A	Histidine 1	30	++	7.0

[a] Refer to Table 1.
[b] Data given in mM unless otherwise specified.
[c] Less than 3-fold (−), > 3-fold < 100-fold (+), > 100-fold < 2000-fold (++), and >2000-fold (+++).

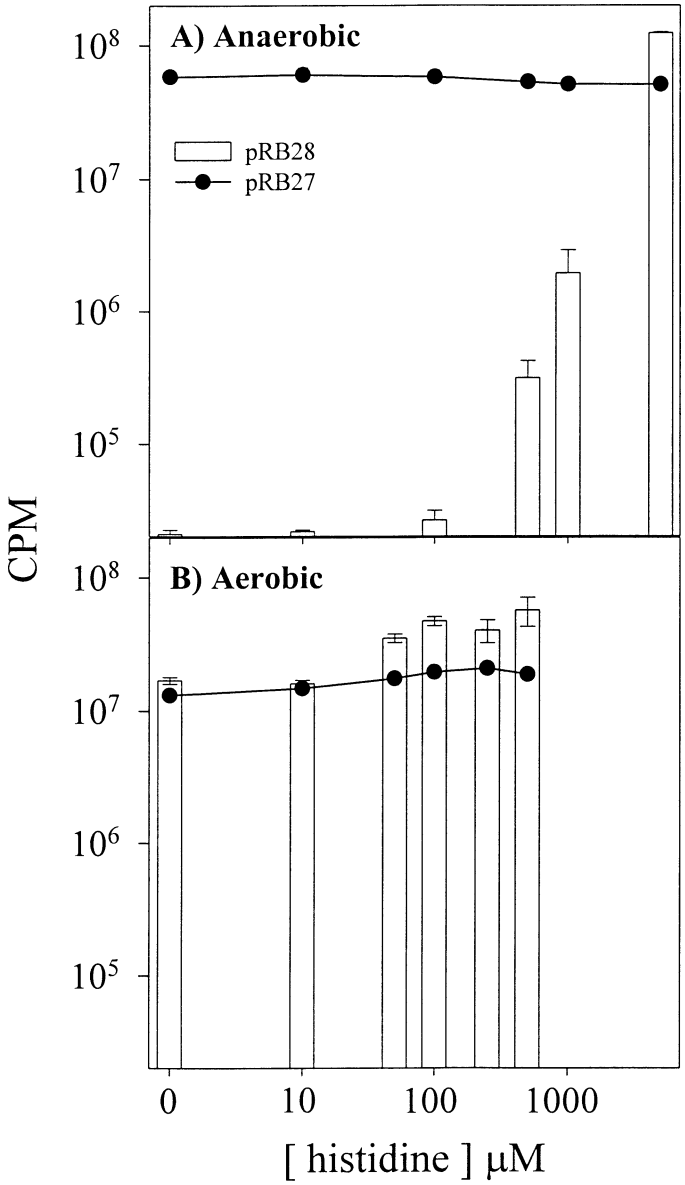

Fig. 4. Effect of increasing histidine concentration on the Hg(II)-induced light response from *V. anguillarum* pRB28 and light production from the constitutive control, *V. anguillarum* pRB27, under (A) anaerobic and (B) aerobic conditions in medium A (see Table 1). Mercury was added to a final concentration of 10 and 2 ng L^{-1} for the anaerobic and aerobic assays, respectively. The cultures were exposed to mercury 80 min prior to measurement. Error bars represent the standard deviation of duplicate samples.

no critically accepted stability constants for complexation of Hg(II) with amino acids (Martell and Smith 2001). On the basis of noncritical constants (Van der Linden and Beers 1973), Hg–amino acid complexes, including neutral species, are likely to form and could be taken up by passive diffusion. However, the increase in Hg(II) uptake when histidine was added anaerobically (Fig. 4B) followed a much different pattern than the calculated increase in $Hg(His)_2$ species ($\log\beta = 22$, Van der Linden and Beers 1973). Thus, perhaps a more likely possibility is that the Hg(II) was cotransported into the cell with the amino acids, because cotransport of Hg(II) with amino acids is known to occur by facilitated means in other organisms (i.e., Clarkson 1994; Cannon et al. 2001). The important finding of this study, however, is that amino acids had a stimulating effect on anaerobic Hg(II) uptake, and investigation into the mechanism involved should be the topic of future study.

Unlike the amino acid additions, the enhancement of Hg uptake by the addition of the carboxylic acids appeared to be related to the lower pH (Table 2). Such an enhancement would be consistent with the behavior of H^+-stimulated metal transport systems such as MntH (Kehres et al. 2000). In relating both the amino acid and carboxylic acid addition experiments to natural anaerobic habitats, it is worth noting that enhanced mercury methylation occurs in flooded environments where high decomposition rates provide an abundance of low-molecular-weight organics (Hecky et al. 1991; Kelly et al. 1997) and in wetlands where the pH tends to be low (St. Louis et al. 1994).

For mercury methylating bacteria, a proposed mechanism of mercury uptake is passive diffusion of mercuric sulfide

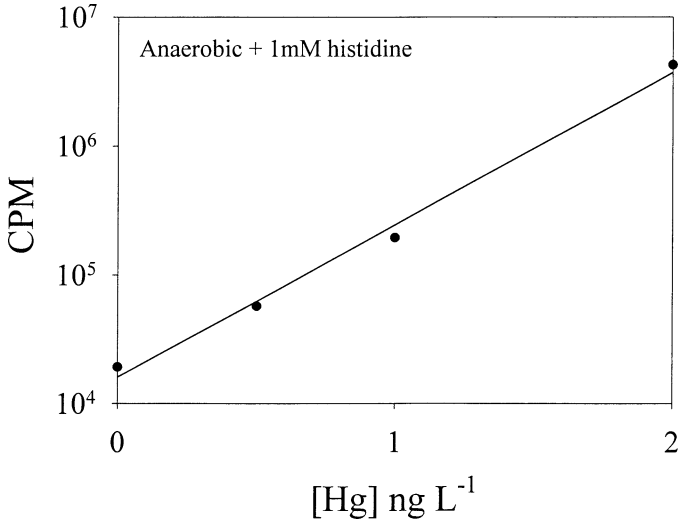

Fig. 5. Effect of 1 mM histidine supplements on mercury-induced light response by *V. anguillarum* pRB28 in anaerobic assay medium A (see Table 1). The cultures were exposed to mercury 120 min prior to measurement.

Table 3. *V. anguillarum* pRB28 in filtered sediment porewater samples.

Date of assay	[Hg] ng L^{-1} added	CPM[a]	Time (min)
28 Jul 2000	—	67,519	75
	20	70,520,921	75
12 Sep 2000	—	45,724	90
	10	212,000,000	90
13 Sep 2000	—	25,482	80
	10	101,000,000	80
13 Sep 2000	0	27,122	80
	0.5	29,744	80
	1	31,516	80
	2	35,248	80
	10	2,193,212	80
14 Sep 2000	—	30,944	120
	—	32,520	120
	10	922,217	120
	10	787,658	120

[a] Average of duplicate samples.

(HgS) across the cell membrane (Benoit et al. 1999). HgS is an important mercury species in anaerobic porewaters because sulfide competes well with large dissolved organic carbon (DOC) molecules for Hg(II), and the large DOC-Hg complexes are unavailable to the cell (Barkay et al. 1997). The very high lipophilicity and stability of HgS make it a better candidate for passive diffusion than other neutral species such as $HgCl_2$ or $Hg(OH)_2$. Alternatively, HgS may be a mercury species available to the cell that is capable of ligand exchange, similar to $Hg(OH)_2$ or $HgCl_2$ as discussed above, whereas increasing sulfide concentration would result in the formation of species such as HgS_2^{2-}, which may bind the Hg(II) too tightly for it to participate in ligand exchange or be repelled from the bacterial cell because of its negative charge.

The ability of the bioreporter to respond to trace mercury concentrations in the anaerobic porewaters used in this study may have been related to the presence of an optimal concentration of sulfide, as proposed by Benoit et al. (1999). A new possibility suggested by this work is that it could have been due to the presence of low-molecular-weight organics (Table 2, Fig. 4). Although we do not have enough information on these porewaters to distinguish between these possibilities, these data show that the combination of bioreporter assays with measurements of porewater chemistry is a promising approach to the examination of bioavailability of Hg(II) in anaerobic sediments.

Much effort has been made in predicting the bioavailability of mercury in aquatic ecosystems on the basis of modeling the chemical speciation of mercury in the bulk solution, together with the assumption that passive diffusion of lipophilic mercury species is the primary uptake mechanism (e.g., Hudson et al. 1994). Although this may be true in some cases, it is obviously not universally true. Hg(II) uptake in the two gram-negative bacteria studied here appeared to occur by facilitated means, and it is worth noting that many methylating bacteria are also gram negative. The existence of facilitated uptake mechanism(s) means that cell physiology can affect Hg(II) uptake, and therefore examination of bulk solution chemistry may be only part of the information needed to determine what portion of total mercury in natural waters is ''bioavailable.'' In addition, we need to know the identity of the transport mechanism(s) with which Hg(II) interacts, factors affecting their regulation, and how they may differ among aquatic microbial species.

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