Role of Na\textsuperscript{+} in Transport of Hg\textsuperscript{2+} and Induction of the Tn2\textit{l} mer Operon\textsuperscript{†}

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The effects of sodium ions on the uptake of Hg\textsuperscript{2+} and induction of the Tn2\textit{l} mer operon were studied by using Escherichia coli HMS174 harboring the reporter plasmids pRB28 and pOS14. Plasmid pRB28 carries mer\textit{RT}' and mer\textit{OS} carries mer\textit{RTPC} of the mer operon, both cloned upstream of a promoterless luciferase gene cassette in pUCD615. The bioluminescent response to 1 \mu M Hg\textsuperscript{2+} was significantly inhibited in E. coli HMS174(pRB28) in minimal medium supplemented with sodium ions at 10 to 140 nM. After initial acceleration, light emission declined at 50 nM Hg\textsuperscript{2+} in the presence of Na\textsuperscript{+}. The mer-lux assay with resting cells carrying pRB28 and \textsuperscript{209}Hg\textsuperscript{2+} uptake experiments showed increased induction and enhanced mercury uptake, respectively, in media supplemented with sodium ions. The presence of Na\textsuperscript{+} facilitated maintenance of bioluminescence in resting HMS174(pRB28) cells induced with 50 nM Hg\textsuperscript{2+}. External K\textsuperscript{+} stimulated bioluminescent response in HMS174(pRB28) and HMS174(pOS14) grown in sodium phosphate minimal medium devoid of potassium ions. Sodium ions appear to facilitate mercury transport. We propose that sodium-coupled transport of mercuric ions can be one of the mechanisms for mercury uptake by E. coli and that the Na\textsuperscript{+} gradient may energize the transport of Hg\textsuperscript{2+}.

Microbial detoxification of mercuric ions in the environment involves uptake of Hg\textsuperscript{2+} into the cytoplasm of bacterial cells. In the process of evolution, living organisms have created a variety of specialized transport systems for different solutes. Some of them are specified by chromosomal genes (mostly for transport of nutrients), and others, such as specific transport systems for toxic inorganic ions, are encoded by plasmids (26, 33). The genes specifying transport of Hg\textsuperscript{2+} are well characterized. However, studies of plasmid-mediated resistance to mercuric ions have shown that mutations and/or deletions of specific transport genes do not prevent uptake of mercuric ions by cells (1, 2, 11, 19, 32, 36).

The mechanism of resistance to Hg\textsuperscript{2+} consists of three steps: the transport of mercuric ions into the cell, the reduction of Hg\textsuperscript{2+} to Hg\textsuperscript{0} catalyzed by the mercuric reductase, and the release of volatile Hg\textsuperscript{0} from the cell. The well-known mer operon of transposon Tn2\textit{l} (from the Shigella plasmid R100) (36) contains six functional genes, mer\textit{RTPCAD}, with mer\textit{TP} (and possibly mer\textit{C}) specifying a transport system for mercuric ions, mer\textit{A} encoding the mercuric reductase, and the products of mer\textit{R} and mer\textit{D} being regulatory proteins. Mer\textit{D} has a minor regulatory role. The characteristic feature of this operon is the Mer\textit{R} protein, which is not a membrane but an intracellular receptor for Hg\textsuperscript{2+} (29). Mer\textit{R} binds the mer operator-promoter region and serves as a repressor and a mercurycoupling transcription activator. Activation of transcription is possible only in the presence of intracellular mercuric ions (28).

In an earlier study (32) we showed that mercuric ions entered into cells in the absence of mer transport proteins. There was no delay in initial induction of the mer operon in Escherichia coli HMS174 harboring pRB28 (carrying mer\textit{RT'}) in comparison with cells carrying pOS14 (containing mer\textit{RTPC}). Moreover, the sensitivity of the mer-lux assay to Hg\textsuperscript{2+} was only slightly increased for HMS174(pOS14) (0.5 nM) over that for HMS174(pRB28) (1 nM) (32). Therefore, the question of how mercuric ions enter into the cell in the absence of specific Hg\textsuperscript{2+} transport proteins to efficiently induce the mer operon arose. Apparently, Hg\textsuperscript{2+} might be transported by other mechanisms that exist for solute uptake.

As was postulated by Mitchell (24, 25), the electrochemical proton gradient can be used by cells to drive energy-consuming processes, such as solute uptake. Although H\textsuperscript{+} is the essential coupling ion in bacterial energy metabolism, Na\textsuperscript{+} is the most important cation linked to endergonic and exergonic membrane reactions. All living cells, including bacteria, establish gradients of Na\textsuperscript{+} and K\textsuperscript{+} ions between their cytoplasm and the surrounding medium. The K\textsuperscript{+} concentration is greater and the Na\textsuperscript{+} concentration is less in the cytoplasm than in the cell environment. K\textsuperscript{+} plays an important role in maintenance of osmotic balance and the cell’s turgor pressure. In the case of E. coli, the intracellular content of K\textsuperscript{+} increases with increasing osmolality of the growth medium. In contrast to K\textsuperscript{+}, the intracellular concentration of Na\textsuperscript{+} is maintained at an essentially constant level (8). Therefore, Na\textsuperscript{+} is constantly extruded from cells by several mechanisms, such as the Na\textsuperscript{+}/H\textsuperscript{+} antiporter (16), a Na\textsuperscript{+} pump driven by decarboxylation (6), NADH oxidation (38), and ATP hydrolysis (14), and intrudes back because of the Na\textsuperscript{+} concentration gradient. One of the major functions of sodium ions is the cotransport of a variety of inorganic and organic solutes (phosphate, organic acids, amino acids, and sugars) across the membrane (7, 12, 18, 20, 31, 34). To the best of our knowledge, sodium-coupled transport of heavy-metal ions has not been reported.

Since the mer operon is an inducible system and induction depends on the entry of Hg\textsuperscript{2+} into the cell, we have found it advantageous to use bacterial luciferase, a sensitive reporter, to study factors that might facilitate or inhibit transport of mercuric ions across cell membranes. In the present study, using E. coli carrying the mer-lux reporter plasmids pRB28 and
pOS14, we examined the effects of sodium and potassium ions on mercury uptake and induction of the Tn21 mer operon.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. E. coli HMS174 [F' recA1 rpoB311 hsdR19 λ IN(rrdD-rrdEY)] (4) harboring plasmid pRB28 or pOS14 (32) was used in all experiments. Luria-Bertani medium and agar plates were prepared according to the methods of Miller (22). The minimal medium (MM) was a modified R medium (32) containing 50 mM potassium or sodium phosphate buffer (pH 7.0), 1 g of (NH₄)₂SO₄ (pH 7.0), R salts mix (400 mg of MgSO₄·7H₂O, 10 mg of FeSO₄·7H₂O, and 20 µl of HCl per liter), a mix of 10 trace elements (10), 3 mg of CaCl₂·2H₂O per liter, and 10 mM pyruvic acid as a carbon source. Cells were grown in the presence of kanamycin (50 µg/ml) at 30°C. Cell growth reached an optical density of 600 nm (OD₆₀₀) of 0.45 units in 16 to 18 h in potassium phosphate MM. However, cells grown overnight in sodium phosphate MM reached an OD₆₀₀ of only 0.2 units.

Cell preparation for mer-lux assays. E. coli HMS174 cells were grown in MM devoid of Na⁺ or K⁺ for 2 days prior to mer-lux assays with daily transfers (1:5 and 1:6 dilutions) into fresh MM. On the 3rd day, when the OD₆₀₀ reached 0.1, the cells were harvested by centrifugation (Sorvall RT6000B refrigerated centrifuge; DuPont Co., Wilmington, Del.) at 3,500 rpm for 8 min at 22°C. After being washed with fresh medium, the cells were suspended in the same volume of the appropriate medium and used immediately in the mer-lux assays.

mer-lux assays. Cells carrying pRB28 or pOS14 were induced with 50 nM or 1 µM HgCl₂ in the appropriate assay medium in the presence or absence of Na⁺ or K⁺. The assays were performed in 7-ml glass scintillation vials at room temperature with 1 ml of the cell suspension in each vial. Monitoring of light emission with the single-photon-count mode of a Tri-Carb 2500 TR liquid scintillation counter (Packard Instrument Co., Inc., Meriden, Conn.) was initiated within 10 min after addition of Hg²⁺ and/or sodium or potassium ions. Analyses were performed for 9 to 11 cycles with a 0.5-min counting period for each sample, and a single cycle lasted 9 to 11 min. Luminescence was expressed as quanta per second per milliliter by transforming photon-per-minute data with the Hastings and Weber standard (13). A single replicate of each treatment was included in each experiment, and experiments were repeated at least twice. The reported trends were fully reproducible, although the kinetics of the response varied slightly from experiment to experiment.

Resting-cell experiments. E. coli HMS174(pRB28), having reached a density of 0.1 units (OD₆₀₀), was induced for 30 min with 50 nM HgCl₂ in potassium phosphate MM in the presence or absence of 140 mM NaCl under growth conditions. Then, the cells were harvested by centrifugation, washed with 50 mM potassium phosphate buffer (pH 7.0), and suspended in the same volume of the buffer. Bioluminescence (mer-lux) assays were initiated within 5 min after addition of fresh portions of 50 nM HgCl₂ and/or 140 mM NaCl or without any supplements.

Radioactive-mercury uptake assays. Cells were grown in potassium phosphate MM to an OD₆₀₀ of 0.3, harvested by centrifugation, and washed in the same medium. Assays were started by addition of 50 nM, 500 nM, or 5 µM ²⁰⁵HgCl₂ (Buffalo Materials Research Center) (specific activity, 1.72 mCi/mg) to 1-ml cell suspensions with or without 140 mM NaCl in 1.5-ml Eppendorf centrifuge tubes. The reactions were stopped by centrifugation at 15,000 rpm for 30 s in an Eppendorf (Fremont, Calif.) microcentrifuge and following washing with 50 mM potassium phosphate buffer (pH 7.0) at various time intervals. Cell pellets were resuspended in 1 ml of fresh buffer and transferred to 7-ml scintillation vials. Opti-Fluor LSC-cocktail for aqueous samples (Packard) was added, and radioactivity was counted after 24 h.

RESULTS

Effect of sodium ions on mer-lux induction in E. coli. Plasmid pRB28 contains the regulatory gene merR, the mer operator-promoter region, and an 87-bp sequence of merT (Fig. 1). In the absence of mer transport proteins, some ion channels may be utilized for nonspecific Hg²⁺ uptake. Two concentrations of Hg²⁺, 50 nM and 1 µM, were used for the study of the effect of Na⁺ on mer-lux induction in HMS174(pRB28). The development of light emission was accelerated in cell suspensions containing 10, 100, or 140 mM NaCl, and the magnitude of bioluminescent response was higher during the first 40 to 50 min after addition of 50 nM Hg²⁺ than that in samples without NaCl (Fig. 2A). However, light emission declined after reaching its optimum in sodium-containing samples. Similar results were obtained with Na₂SO₄ instead of NaCl (data not shown).

A dramatic effect of Na⁺ was observed at the 1 µM concentration of Hg²⁺ (Fig. 2B). Induction with 1 µM Hg²⁺ was inhibited when sodium ions were present in the assay medium. The addition of even 10 mM NaCl decreased the bioluminescent response by 3 orders of magnitude, and light emission in samples with 140 mM NaCl was only slightly higher than that in uninduced controls. We previously reported that the bioluminescent response of HMS174(pRB28) was inhibited in Luria-Bertani medium at Hg²⁺ concentrations above 500 nM (32). The major salt component in Luria-Bertani media was Na⁺ (170 mM). Probably, the inhibition of light emission in the presence of Na⁺ was due to increased influx of Hg²⁺ to a level toxic for the cells.

Production of light by HMS174(pOS14) was not accelerated in the presence of Na⁺. Nevertheless, light production in experiments with 20 nM Hg²⁺ was enhanced 50 min after induction in cells supplemented with 140 mM NaCl (Fig. 3). Light emission was slightly inhibited at 100 nM Hg²⁺ in the presence of sodium ions. E. coli carrying pOS14 was supersensitive to Hg²⁺, similarly to all previously described systems having intact regulatory and transport functions, but devoid of mercuric reductase (9, 27, 36). Maximal induction was achieved with 20 nM Hg²⁺, and the bioluminescent response was decreased at 50 and 100 nM Hg²⁺ in our experiments. The fact that induction by 100 nM Hg²⁺ was reduced in the presence of Na⁺ may indicate that in sodium-containing medium an intracellular concentration of mercuric ions was
cells were triangles

experiments uninduced control. With supplemented MM. to increased from Na+; *, absence of Na+; ▲, 10 mM Na+; ●, 100 (A) or 50 (B) mM Na+; ■, 140 mM Na+; ○, uninduced cells.

Effect of potassium ions on mer-lux induction. HMS174 cells carrying pRB28 and pOS14 were grown in sodium phosphate MM. The mer-lux assay was performed in the same medium supplemented with 50 mM Hg2+ in the absence or presence of 10 mM K+. After induction with 50 mM Hg2+ the magnitude of bioluminescent response in sodium phosphate MM (Fig. 4) was significantly lower than that in potassium phosphate MM (Fig. 2). However, the addition of even 10 mM external K+ increased light emission in cells with both plasmids (Fig. 4). Since our cells were grown with high potassium deficiency, the addition of K+ to the mer-lux assay medium restored physiological K+-Na+ exchange and the Na+ gradient and resulted in stimulation of light induction.

Effect of mercuric and sodium ions on light emission in resting cells of strain HMS174(pRB28). After a 30-min induction of HMS174(pRB28) by 50 mM Hg2+, the light emitted by washed cells in samples that were induced in Na+-supplemented MM was eightfold higher than that in samples induced in unsupplemented MM (Fig. 5). However, light emission declined significantly during the mer-lux assay in the absence of fresh Hg2+ and/or Na+. Addition of 50 mM Hg2+ only slightly retarded the decline of bioluminescence in washed cell suspensions, previously induced in MM devoid of Na+. Interestingly, no decline of light emission was observed in experiments in which fresh portions of Hg2+ and Na+ were added to induced, washed cells. Sodium ions may alter bacterial energetics, providing energy essential for light production in assays performed under nutrient-free conditions.

Radioactive-mercury uptake. To complement bioluminescent assays, we performed a study of 203Hg2+ uptake (17, 27, 37) with strain HMS174(pRB28). The concentration of cell-bound mercuric ions in cells supplemented with 500 nM 203HgCl2 and 140 mM NaCl was threefold higher than that in cells supplemented with 203HgCl2 alone (Fig. 6). A similar
This inhibition is the induction is supplemented with Na+ and/or Na+. △, 50 nM Hg2+; ■, 50 nM Hg2+ and 140 mM Na+; ○, no additions (control); □, cells induced in the presence of 140 mM Na+ with no supplements added to the resting cells after washing.

A pattern of mercury uptake was observed in experiments with lower (50 nM) and higher (5 μM) concentrations of 203Hg2+ (data not shown). Results of mercury uptake experiments support our observations with the mer-lux reporters that Na+ stimulated transport of mercuric ions.

**DISCUSSION**

Using a sensitive reporter system (pRB28), we found a significant effect of sodium ions on the uptake of Hg2+ and the subsequent induction of the mer operon. Sodium ions strongly inhibited induction at 1 μM Hg2+ in HMS174(pRB28) containing the mer operon represented by only merRT' (Fig. 2B). This inhibition increased as the sodium concentration in the assay medium increased. Na+ probably facilitates a rapid increase in the intracellular concentration of Hg2+ to an inhibiting level. At the same time, we found acceleration of induction at a low Hg2+ concentration (50 nM) by Na+. However, this induction declined after 40 to 50 min (Fig. 2A). In resting-cell experiments, induction was increased eightfold in the medium supplemented with 140 mM NaCl (Fig. 5). Since induction is increased as the amount of the intracellular inducer increases, we concluded that more Hg2+ entered into the cells in Na+-supplemented medium. This conclusion was confirmed by studies of 203Hg(II) uptake (Fig. 6) and the effect of K+ on mer-lux induction in HMS174(pRB28) and HMS174 (pOS14) in sodium phosphate MM (Fig. 4). It was shown before in studies with *E. coli* and *Salmonella typhimurium* that the addition of K+ to Na+-loaded cells stimulated uptake of solutes by all Na+-dependent transport systems but not by H+-dependent transport systems. For instance, Na+-dependent uptake of melibiose and proline was stimulated by K+. At the same time, little effect of K+ on α-alanine transport, which is H+-dependent, was observed (3). Therefore, the stimulation of bioluminescence in the mer-lux assay may indicate coupling of Hg2+ and Na+ transport systems. Consequently, we propose that sodium-coupled mercury transport can be one of the mechanisms of mercury uptake.

Earlier findings showed that deletions or mutations in merTP decreased operon induction, and in order to exhibit the optimum response in whole cells the MerR Hg2+ sensor required an intact mercury transport system (11, 19). In our studies we observed that in the absence of a specific mercury transport system, the mer operon could be induced at a Hg2+ concentration almost as low as that required for induction of the operon with intact transport genes. Therefore, mercuric ions can be effectively transported to the MerR receptor by a mechanism(s) other than the mer transport system. The current model for mercury resistance proposes that mercuric ions diffuse across the outer membrane, where MerP binds Hg2+ by the pair of cysteine residues and transfers it to the pair of thiol groups of MerT. Then, Hg2+ is passed through the membrane to another pair of cysteines located on the cytoplasmic part of MerT. Mercuric reductase may receive the Hg2+ directly from MerT and protect cytoplasmic components from the toxic effect of Hg2+ (2, 23). However, it was not clear what mechanism(s) exists for uptake of mercuric ions in the absence of the mer transport genes. Summarizing earlier findings about organization of the mercury resistance genes, Summers concluded that Hg2+ may enter into cells by diffusion (36). Lund and Brown (19) showed that initial induction of transcription of the mer genes by MerR requires neither MerT nor MerP, because enough Hg2+ can cross the inner membrane and reach the MerR receptor. Nevertheless, it is not likely that mercuric ions enter into cells passively, without utilization of energy. Recently, Sahlman and Jonsson (30) described the Hg2+-binding properties of MerP in vitro and raised an important question regarding the energy source for uptake of Hg2+ even in the presence of specific mercury transport proteins. They referred to histidine and maltose transport systems that have a membrane-associated protein which provides the energy necessary for transport by ATP hydrolysis. Such a protein has never been found for the mer system. Sahlman and Jonsson proposed (without experimental support) that transport of mercury could be dependent upon the H+ gradient across the inner membrane. However, it is known that Na+ gradients represent energy reservoirs as well, and in many bacterial systems Na+ gradients can be utilized directly for the purpose of ion, nutrient, and metabolite transport (18). Indeed, both H+ and Na+ gradients represent energy sources for osmotic work such as solute uptake in bacterial cells. Some solutes like glutamate and citrate may utilize both Na+ and H+ gradients for transport. In the case of melibiose permease, which transports a large number of substrates, including monosaccharides, α- and β-galactosides, the transport can be coupled to either Na+ ions or H+ ions, depending upon the substrate. The major proline transport systems use exclusively Na+ as a coupling ion (7). Probably, H+ cotransport systems are more primitive than Na+ systems, because the magnitude and capacity of the H+
gradient are limited by the requirement to maintain a cytoplasmic pH near neutrality (7, 34).

In summary, (i) the microbial mechanism of resistance to mercury involves uptake of Hg\(^{2+}\) into the cytoplasm. However, induction of the mer operon does not require the presence of the transport protein MerP, MerT, or MerC. Sodium-coupled transport of Hg\(^{2+}\) might be an important mechanism of Hg\(^{2+}\) uptake, facilitating the entrance of mercuric ions into cells that trigger initial induction of the mer operon. (ii) Luciferase genes have found wide use as reporters in the study of gene expression (5, 21) and detection of environmental pollutants (15, 32) and various microbial stresses (35). In the present study our initial observations of the possible role of sodium ions in the transport of mercuric ions were obtained by using mer-lux reporters. Thus, sensitive luciferase reporters might be useful in transport studies of other inducible systems as well.

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