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Detoxification of Mercury, Cadmium, and Lead in *Klebsiella aerogenes* NCTC 418 Growing in Continuous Culture

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***Klebsiella aerogenes* NCTC 418 growing in the presence of cadmium under glucose-, sulfate-, or phosphate-limited conditions in continuous culture exhibited sulfide formation and P_i accumulation as the only demonstrable detoxification mechanisms. In the presence of mercury under similar conditions only HgS formation could be confirmed, by an increased sensitivity to mercury under sulfate-limited conditions, among others. The fact that the cells were most sensitive to cadmium under conditions of phosphate limitation and most sensitive to mercury under conditions of sulfate limitation led to the hypothesis that these inorganic detoxification mechanisms generally depended on a kind of "facilitated precipitation". The process was coined thus because heavy metals were probably accumulated and precipitated near the cell perimeter due to the relatively high local concentrations of sulfide and phosphate there. Depending on the growth-limiting nutrient, mercury proved to be 25-fold (phosphate limitation), 75-fold (glycerol limitation), or 150-fold (sulfate limitation) more toxic than cadmium to this organism. In the presence of lead, PbS formation was suggested. Since no other detoxification mechanisms were detected, for example, rendering heavy metal ions innocuous as metallo-organic compounds, it was concluded that formation of heavy metal precipitates is crucially important to this organism. In addition, it was observed that several components of a defined mineral medium were able to reduce mercuric ions to elemental mercury. This abiotic mercury volatilization was studied in detail, and its general and environmental implications are discussed.**

Much research has been dedicated to microbial detoxification of mercury salts (8, 23, 25). The mechanisms most often reported (7) involve chemical transformation followed by volatilization either to elemental mercury (13, 14, 16–19, 24) or to methyl mercury (9, 10, 21, 24, 27–29). In most cases, these detoxification mechanisms are plasmid encoded (13–17, 20–22, 24). Among the other mechanisms mentioned are decreased membrane permeability (22) and uptake and intracellular or extracellular precipitation (15, 20, 26). Apart from the latter, these mechanisms were all demonstrated in *Klebsiella aerogenes* (9, 13, 14, 22, 27).

In previous reports (3, 4), it was shown that *K. aerogenes* strains growing in continuous culture adapted to cadmium after a growth lag similar to that found in batch culture. After adaptation, the cells appeared to have accumulated extra inorganic sulfide or phosphate or both in a total molar amount close to that of the accumulated cadmium. The actual cellular contents of cadmium, phosphate, and sulfide, however, depended very much on the particular strain and on the growth conditions. Sulfide dominated under conditions of phosphate limitation, phosphate dominated under sulfate limitation, and under glucose limitation it depended on the strain. This strongly suggests that formation of insoluble cadmium sulfide and that of phosphate are the primary detoxification mechanisms of at least two different strains of *K. aerogenes* and that they are coordinated, as well (3, 4).

Since comparable data were lacking for other metals, it seemed pertinent to extend these investigations to mercury and other heavy metals, such as lead. Therefore, this paper reports our studies on detoxification by *K. aerogenes* NCTC 418 of mercury and lead, in addition to cadmium. It is shown that the relative importance of inorganic phosphate accumulation is decreased and that of sulfide formation is increased in mercury detoxification when compared with cadmium detoxification. No bacterial volatilization of heavy metals was detected. In addition, this paper reports on the abiotic volatilization of mercury due to reduction of the mercuric ion by culture medium components.

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MATERIALS AND METHODS

Organism. *K. aerogenes* NCTC 418 was used throughout this study. It was maintained by monthly subculture on brain heart infusion medium solidified with 1% (wt/vol) agar (4).

Media. For glycerol-limited growth, a modification of a previously described (3) mineral salts medium was used, containing 50 mM NH_4Cl, 5 mM NaH_2PO_4, 5 mM KCl, 2 mM Na_2SO_4, 1 mM citric acid, 0.625 mM $MgCl_2$, 0.5 mM HCl, 0.1 mm $FeCl_3$, 0.05 mM $MnCl_2$, 0.025 mM $ZnCl_2$, 0.02 mM $CaCl_2$, 0.01 mM $CoCl_2$, 0.005 mM $CuCl_2$, 0.004 mM H_3BO_3, 0.0001 mM Na_2MoO_4, and 20 mM glycerol. Glucose-limited medium (see Fig. 1 and 2) contained 10 mM glucose instead of 20 mM glycerol. For sulfate- or phosphate-limited growth, the glycerol concentration was raised to 300 mM, and the concentration of Na_2SO_4 was lowered to 0.1 mM and that of NaH_2PO_4 was lowered to 0.3 mM, respectively. In all cases but glucose limitation (for which glucose was autoclaved separately), 10.5-liter batches of medium were autoclaved for 45 min at 120°C, yielding 10 liters of sterile medium. Heavy metals were added separately to the medium reservoir only, as indicated, from sterile (15 min at 110°C) stock solutions of $CdCl_2$ (300 mM $CdCl_2$ plus 1 mM citric acid), $HgCl_2$, or $PbCl_2$ (30 mM plus 0.1 mM citric acid).

Culture conditions. Bacteria were cultured aerobically in a C-30 Bioflo Continuous Culture apparatus (New Brunswick Scientific Co., Inc., Edison, N.J.). In all cases, the dilution rate was 0.1 h^{-1}, the air flow rate was 25 liters/h, the temperature was 35.0 ± 0.1°C, and the pH was 7.00 ± 0.05.

The latter was achieved by a pH control unit which added 1 M NaOH to the culture whenever the pH dropped below 7.

Sampling. From continuous cultures in steady state (achieved consistently within 48 h at a dilution rate of 0.1 h^{-1}), a 200-ml sample was taken directly from the growth vessel and centrifuged at 4°C (20,000 × *g* for 10 min), and the supernatant was discarded. The bacterial pellet was suspended in distilled water and centrifuged again at 4°C (20,000 × *g* for 10 min). The resulting pellet was lyophilized and stored at room temperature.

Assays. Lyophilized cells were extracted by boiling for 15 min in concentrated hydrochloric acid (for cadmium and mercury) or nitric acid (for lead) and subsequently assayed for metals by flame atomic absorption spectrophotometry on a Perkin-Elmer model 4000 spectrophotometer after appropriate dilution. For mercury assays the Perkin-Elmer MHS-10 mercury hydride system was installed. The cellular contents of sulfide were determined as described previously (4), with the modification that 20 μl of 3% (wt/vol) $NaBH_4$ in 1% (wt/vol) NaOH was added to the reaction mixture before the addition of the *N*,*N*-dimethyl-*p*-phenylenediamine reagent. This modification was essential to determine inorganic sulfide in the presence of mercury, which strongly inhibited the reaction. This addition was only partially effective in overcoming the even stronger inhibition by lead, leading to underestimated values in this case, which could not be avoided. In samples (spiked) with cadmium or mercury or in samples without heavy metals, the borohydride addition had no detectable influence on the accuracy of the determination other than that it decreased the sensitivity of the method about twofold. After 15 min of extraction of lyophilized cells with cold 1 M HCl (6) and subsequent centrifugation (20,000 × *g* for 10 min), P_i was determined in the supernatant by the method of Ames and Dubin (5). Assays were always carried out in duplicate on at least two different batches of cells which were grown under identical conditions and sampled at least 1 week apart. The results were averaged; the standard deviation was as indicated.

Microscopy. Elecron microscopy was performed on unstained cells taken directly from a culture in steady state, as described previously (3).

Electrophoresis. Steady-state cell samples were sonified at 4°C five times for 30 s each time (with alternate cooling) at 50 W output and centrifuged at 4°C for 1 h at 100,000 × *g*. The supernatant was subsequently subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, by the method of Abraham et al. (1).

RESULTS

Fate of mercury in sterile batch media. Recovery of mercury from preliminary continuous culture experiments was so low that detailed investigation of this matter was needed, since the possibility of bacterial mercury methylation could not be discarded (23). However, sterile batches of medium also lost mercury at a high rate (Fig. 1). This rate depended to a large extent on the carbon source. Therefore, it must be concluded that, of all medium components, glucose was quantitatively the most important one that reduced the mercuric ion to elemental mercury, which then disappeared by evaporation. When glucose was substituted by glycerol, which has considerably less reducing capacity, the medium still showed an appreciable residual mercury reduction (Fig. 1), indicating that additional reducing substances must be present. A likely candidate was the Fe^{2+} ion. Originally, the medium contained only Fe^{3+}, but the obvious yellow color that the latter ion gave to the medium largely disappeared

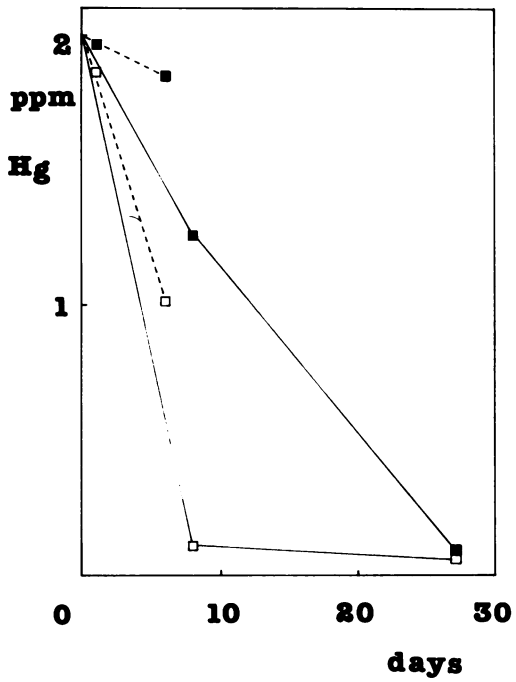

FIG. 1. Decreasing mercury contents of sterile 100-ml batches of carbon-limited medium (pH 7) in cotton-plugged erlenmeyer flasks. Symbols: ■, 0.2% (wt/vol) glycerol; □, 0.2% (wt/vol) glucose; solid lines, autoclaved batches; dashed lines, not autoclaved but sterile due to the presence of mercury salts.

upon autoclaving, suggesting that it was at least partially reduced in the process. Indeed, it turns out that autoclaved media (continuous lines in Fig. 1) exhibited a more rapid decrease of mercury concentration than media that were sterile merely on account of the presence of the mercuric ion (dashed lines in Fig. 1). However, no mercury reduction was found in acidic media (pH 3). These observations enabled a different approach to this unwanted phenomenon of abiotic mercury reduction (Fig. 2). Usually, mercury is determined by the cold-vapor technique in acidified (0.5 M HCl) aqueous samples. Upon addition of $NaBH_4$ in NaOH, the mercuric ion is reduced to elemental mercury, and the resulting vapor is flushed into a quartz flowthrough cell in the optic path of the atomic absorption spectrophotometer by the argon carrier gas. A sharp, transient absorption of reproducible height can then be seen on the recorder tracing (Fig. 2g). The same setup, adding just NaOH (omitting the $NaBH_4$) to acidic (pH 3), mercury-containing medium showed clearly that elemental mercury vapor evolved from neutralized glucose-limited media (Fig. 2a). Figure 2b shows that a substantial part of this nutrient-related mercury reduction derived from the trace metals, but it is clear from Fig. 2c that most of this reduction was due to the glucose present. Figure 2d depicts the control medium without mercury. Additional proof that glucose is indeed the main cause is clear from the large difference between tracings e and f in Fig. 2, showing mercury in acidified water and mercury plus glucose in acidified water, respectively. This finding led us to perform all subsequent experiments in media with glycerol as the carbon source, instead of glucose. Indeed, media containing glycerol (Fig. 2h) showed a much reduced abiotic mercury volatilization in comparison to similar media with glucose (Fig. 2a), although still more than similar media without a

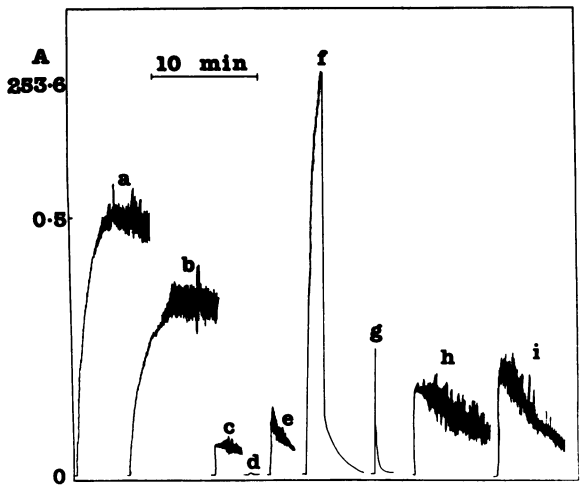

FIG. 2. Mercury evolution due to reduction of Hg^{2+} to Hg^0 by medium components, as measured by the cold-vapor technique upon addition of 1% (wt/vol) NaOH to acidic solutions (pH = 3 in all cases) for 5 s (final pH = 10 in all cases). (a) Complete medium (glucose limited plus 2 ppm [2 μg/ml] Hg^{2+}); (b) complete medium minus trace elements; (c) complete medium minus glucose; (d) complete medium minus mercury; (e) 2 ppm Hg^{2+} in water; (f) 2 ppm Hg^{2+} in water plus 0.2% (wt/vol) glucose; (g) normal reduction of 10 ppb (10 ng/ml) Hg^{2+} by 3% (wt/vol) $NaBH_4$ in 1% (wt/vol) NaOH; (h) complete medium (glycerol limited plus 2 ppm Hg^{2+}); (i) 2 ppm Hg^{2+} in water plus 0.2% (wt/vol) glycerol.

carbon source (Fig. 2c). Similarly, mercury volatilization by glycerol in water (Fig. 2i) was intermediate between that of glucose in water (Fig. 2f) and that of water without a carbon source (Fig. 2e).

Mercury recovery in continuous culture. About 25% of the initially input mercury was removed from the liquid phase (Table 1, sample 2), even at pH 3 and with glycerol as the carbon source. After 29 h at $D = 0.1\ h^{-1}$ the mercury concentration, 93.4% of the input concentration (sample 3), approached the theoretical value ($e^{-Dt} = 0.945$) to a high degree, suggesting that the 25% missing initially was adsorbed to the walls of the culture equipment (silicon tubing, glass vessel, stainless-steel baffles, sensors, etc.) and that this adsorption approached saturation. However, immediately upon activation of pH control (sample 4), the culture mercury concentration started to drop rapidly due to the chemical reduction by medium components and subsequent evaporation described above. In steady state (sample 7), about 20% of the input concentration remained available in the culture vessel. During steady-state growth (sample 8), this value was about 40%. Although the concentrations of all nutrients are changed by bacterial growth, additional new compounds may be excreted into the medium, and appreciable adsorption of mercury to the cells may take place. So the mere fact that mercury retention in the culture vessel doubled upon growth strongly suggests that *K. aerogenes* NCTC 418 accumulates mercury in one form or another and does not utilize mercury reduction or methylation as a detoxification mechanism. The latter was confirmed by mass spectroscopy of organic solvent extracts from effluent gas traps and from the culture fluid.

Detoxification by inorganic sulfide and phosphate. Due to the absence of mercury reduction in *K. aerogenes* NCTC 418, formation of mercury sulfide or phosphate or both had to be anticipated, as in the case of cadmium described earlier (3, 4). In contrast with cadmium, however, mercury inhibited the sulfide assay. As a direct spin-off from the experiments detailed in Fig. 2, it was found that this could be overcome by incorporating some $NaBH_4$ in the assay mixture. Therefore, this was done throughout the assays described in Table 2, even in the controls grown without added heavy metal. It is apparent from Table 2 that the sulfide content of cultures grown under glycerol-, sulfate-, or phosphate-limited conditions was consistently higher in the presence of mercury. Apart from glycerol limitation, when the extra sulfide far exceeded the mercury present in the cells, the molar ratio of extra sulfide and cellular mercury (accumulated in addition to the concentrations found in the control cells grown without added heavy metals) was usually close to 1, suggesting that HgS was formed. The P_i content of cells from mercury-supplemented cultures, however, was not significantly different from that of the control cells (within experimental error).

It is striking, though, that *K. aerogenes* NCTC 418 was most sensitive to mercury under sulfate-limited conditions, whereas it had been most sensitive to cadmium under phosphate-limited conditions (4). To exclude the possibility that this difference was caused by a difference in carbon source, the cadmium experiments (4) were repeated with glycerol. However, the relative sensitivity to cadmium as well as the cellular concentrations of cadmium, sulfide, and phosphate of cultures grown with glycerol (Table 2) agreed well with those of cultures grown with glucose (4).

Additionally, Table 2 shows that no extra P_i is accumulated upon addition of lead to phosphate-limited cultures of *K. aerogenes*, as had been expected. Although the extra sulfide formed under these conditions accounts for only 15% of the intracellularly accumulated lead, it should be stressed here that this is an underestimate which is unavoidable, because the sulfide determination was partially inhibited by lead, even in the presence of $NaBH_4$. The fact that PbS was nevertheless formed is tentatively suggested by the presence of electron-dense granules in these cells (but absent in control cells) which were very similar to the CdS granules described earlier (4). Furthermore, the size of the granules is of the expected order of magnitude (Fig. 3) with respect to the CdS granules (4).

Finally, additional detoxification mechanisms involving volatilization and organic sequestering of metals were not detected, despite extensive mass spectrometry, polyacrylamide gel electrophoresis, and affinity chromatography (12).

TABLE 1. Mercury reduction by culture equipment and growth medium[a]

Sample (pH)	Hg^{2+} concn (% of input)
1. Medium reservoir (3.0)	100.0
2. Culture vessel after autoclaving and filling (3.0)	75.9
3. Culture vessel after 29 h of pumping medium (3.0)	93.4
4. Culture vessel immediately after neutralization (7.0)	85.5
5. Culture vessel 30 min after neutralization (7.0)	72.5
6. Culture vessel 24 h after neutralization (7.0)	16.8
7. Culture vessel 48 h after neutralization (7.0)	21.7
8. Culture vessel after 48 h of steady-state growth (medium plus cells) (7.0)	40.4

[a] Sulfate-limited medium with 0.01 mM $HgCl_2$; after sample 2, the medium pump was set to give a dilution rate of 0.1 h^{-1}; after sample 3, the pH control unit was activated; immediately after sampling, neutral samples were acidified to pH 3 with 1 N HCl and stored frozen.

TABLE 2. Influence of growth conditions in continuous culture on cellular composition of *K. aerogenes* NCTC 418

Limiting nutrient (*n*)	Metal input	Cellular content (mmol/100 g of dry cells) of:		
		Metal	Sulfide	P_i
Glycerol (4)	None	ND[a]	1.18 ± 0.14	5.79 ± 0.69
Sulfate (2)	None	ND	0.93 ± 0.08	9.39 ± 0.04
Phosphate (5)	None	ND	1.40 ± 0.16	1.30 ± 0.57
Glycerol (3)	0.02 mM Hg^{2+}	0.36 ± 0.08	2.16 ± 0.21	5.13 ± 0.85
Sulfate (2)	0.01 mM Hg^{2+}	0.69 ± 0.01	1.78 ± 0.39	11.13 ± 1.82
Phosphate (2)	0.02 mM Hg^{2+}	0.55 ± 0.04	2.04 ± 0.05	1.66 ± 0.28
Glycerol (2)	0.6 mM Cd^{2+}	26.49 ± 3.10	10.49 ± 0.53	23.58 ± 1.41
Sulfate (2)	0.6 mM Cd^{2+}	58.48 ± 4.92	6.21 ± 0.45	53.79 ± 3.17
Phosphate (3)	0.2 mM Cd^{2+}	5.40 ± 0.30	7.52 ± 0.14	0.73 ± 0.12
Phosphate (2)	0.2 mM Pb^{2+}	3.29 ± 0.13	1.98 ± 0.20	0.89 ± 0.16

[a] ND, Not determined.

In this respect, it is clear from Fig. 4 that the effect of a different growth limitation on the composition of the cytoplasmic proteins is much greater than the effect of cadmium. Therefore, if in *K. aerogenes* a soluble protein is induced by cadmium at all, its cadmium-binding potential must be negligible compared with that of the inorganic precipitation mechanisms described above. This is in clear contrast with what has been reported for *Escherichia coli* (12).

DISCUSSION

In continuation of previous work on the adaptation of bacteria to cadmium (3, 4), the present investigation was undertaken to investigate whether similar mechanisms were active in the presence of other heavy metals. Control experiments with mercury in sterile batch cultures showed that even uninoculated mineral media may release appreciable amounts of mercury vapor at neutral pH. In fact, since reduction of Hg^{2+} occurs even in simple, defined media containing just mineral salts, glycerol, and some citric acid, one may wonder whether the same does not happen in the environment and whether the role of bacteria in environmental mercuric ion reduction (8) is not grossly overestimated in relation to (geo)chemical processes. Of course, mercury reduction by sterile controls must always be determined to prevent measuring pH-dependent inorganic mercury reduction rather than bacterial mercury reduction, especially in batch cultures and agar plates. In this respect, our results explain the high mercury losses (sometimes 25% within 24 h) from sterile controls reported by others (9, 14, 23) and show the usefulness of continuous culture as a quantitative research tool to limit and measure mercury recovery losses, which is impossible to achieve by means of batch culture. It is clear, however, that this blank reduction cannot be abolished entirely by modifying the medium, because it was prominent even in the presence of the mercury salt only (Fig. 2e).

A comparison between cadmium-supplemented and mercury-supplemented cultures shows that the correlation between cadmium and extra inorganic sulfide plus phosphate

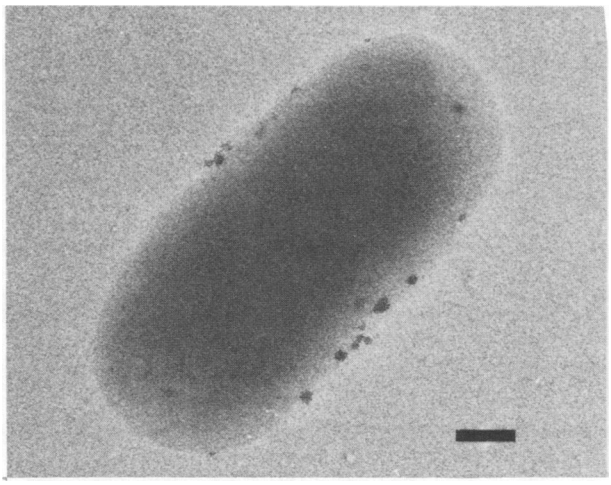

FIG. 3. Unstained *K. aerogenes* NCTC 418 cell grown under phosphate-limited conditions in the presence of 0.2 mM $PbCl_2$. Bar = 0.25 μm.

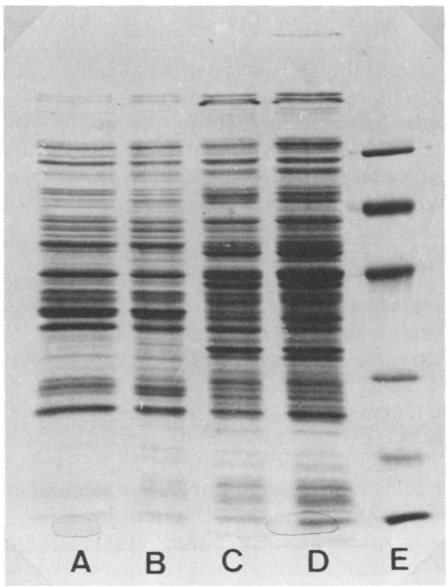

FIG. 4. Gel electrophoresis of soluble proteins of *K. aerogenes* NCTC 418 grown under different conditions. Lanes: A, sulfate-limited cells adapted to 0.6 mM Cd^{2+}; B, sulfate-limited cells; C, glucose-limited cells adapted to 0.6 mM Cd^{2+}; D, glucose-limited cells; E, marker proteins (from top to bottom; 94, 67, 43, 30, 20.1, and 14.1 kilodaltons, respectively).

TABLE 3. Toxicity of heavy metals to *K. aerogenes* NCTC 418 in relation to growth-limiting nutrient

Growth-limiting nutrient	Maximum tolerated level[a] (mM)		Relative toxicity[b] (mercury/cadmium)
	Cadmium	Mercury	
Glycerol	0.6	0.02	75
Sulfate	0.6	0.01	150
Phosphate	0.2	0.02	25

[a] Maximum tolerated input concentration still enabling a steady-state bacterial dry weight of at least 75% of the dry weight without added heavy metals, at a dilution rate of 0.1 h^{-1}.

[b] Ratio of the maximum tolerated levels of mercury and cadmium multiplied by a factor of 2.5 to correct for mercury evaporation, since the cells are actually exposed to only 40% of the mercury input concentration (Table 1).

(in addition to the control) after adaptation is excellent, much better than that between mercury and the inorganic anions. This is probably largely an indirect effect of the much higher toxicity of mercury: the metal, sulfide, and phosphate contents of the cells are all much lower in the presence of mercury and, consequently, the error in all three assays is higher, as can be seen from the relatively higher standard deviation values. The same cause may be responsible for the fact that HgS granules, likely to be present, are probably too small to be detected on electron micrographs. This is in agreement with the relationship between molar metal content and electron-dense granule size if extrapolated from the data available for cadmium and lead (reference 4 and the present report). The data obtained with lead are also in agreement with those of Aickin et al. (2), who found $PbHPO_4$ granules on the surface of a *Citrobacter* species grown in the presence of lead. Although the sulfide contents of cells grown in the presence of mercury were consistently higher than those in the absence of mercury, and the difference is of the order of the mercury contents of the cells, stronger evidence for formation of HgS as a detoxification mechanism derives from the fact that the cells were most sensitive to mercury under conditions of sulfate limitation, whereas, quite in contrast, the cells were most sensitive to cadmium under conditions of phosphate limitation (Table 3). If allowance is made for abiotic mercury reduction, phosphate-limited cells are 25-fold more sensitive to mercury than to cadmium, glycerol-limited cells are 75-fold more sensitive, and sulfate-limited cells are 150-fold more sensitive. So, obviously, mercury is much more toxic to *K. aerogenes* NCTC 418 than cadmium, although the toxicity is of the same order of magnitude as reported for (genetically) resistant strains of this organism (14). Since the present study considered inducible adaptive (3) responses only, it has to be concluded that the classification of strains as either resistant or sensitive is not as clear-cut as it seems. Even when rigorous standardization is adhered to, such classification is easily confused by these kinds of physiological adaptive responses, especially in batch culture. Therefore, a label like sensitive or resistant is useful only when accompanied by a detailed description of the growth conditions.

Clearly, the preference for either sulfide- or phosphate-mediated detoxification (as judged by the relative sensitivity to any metal under sulfate- or phosphate-limited conditions) depends on the particular metal to be detoxified. Although it may be somewhat premature for speculation, in our opinion this suggests that this example of detoxification is one of a more general kind, which may be coined "facilitated precipitation". It seems that metal ions enter the cell (suggested by the complete absence of H_2S excretion reported [11] for all *K. aerogenes* strains) and are precipitated (in some as yet unknown location) and accumulated (near the cell perimeter) merely on account of the high local concentrations of sulfide and phosphate (which are probably replenished by feedback mechanisms). In this way, relative differences in the solubility of their sulfides and phosphates may explain the decreased tolerance to mercury and cadmium under sulfate-limited and phosphate-limited conditions, respectively. The general applicability of this hypothesis might have been tested with lead, but precipitation of lead phosphate in the medium under all conditions except phosphate limitation precluded this validity check. On the other hand, having glycerol-2-phosphate double as a carbon and phosphate source, as is sometimes done (2), will not give unbiased information. Therefore, other metals should be tested, but it is also essential to find out to what extent the formation of heavy-metal precipitates shown by *K. aerogenes* is a general phenomenon among microorganisms. This question deserves our full attention, because the main mechanism of detoxification reported in the closely related organism *E. coli* is induction of a heavy-metal-binding protein (12). However, despite a lot of effort, gel electrophoresis (Fig. 4) and other techniques did not reveal the presence of additional detoxification mechanisms in *K. aerogenes*, other than the formation of inorganic precipitates.

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