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Inorganic Phosphate Accumulation and Cadmium Detoxification 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, exhibits two different cadmium detoxifying mechanisms. In addition to sulfide formation, increased accumulation of P_i is demonstrated as a novel mechanism. Intracellular cadmium is always quantitatively counterbalanced by a concerted increase in both inorganic sulfide and P_i contents of the cells. This led to the conclusion that production of sulfide and accumulation of P_i are detoxification mechanisms present in *K. aerogenes* but that their relative importance is crucially dependent on the strain and the growth conditions employed.

Microorganisms are known to employ a large variety of mechanisms for adaptation to the presence of heavy metal ions (5). An interesting example of an adaptation mechanism in *Escherichia coli* was described by Mitra et al. (12). These authors claimed that the prolonged lag phase they observed upon addition of cadmium served to induce the synthesis of a specific cadmium-binding protein which was very different from the metallothioneins (9).

In a previous report (1), it was shown that upon addition of cadmium to *Klebsiella aerogenes* S45, growing in continuous culture under conditions of glucose limitation, an adaptation period was observed similar to that found in *E. coli* (12). After adaptation, the cells appeared to have accumulated both cadmium and inorganic sulfide in a molar ratio close to unity. This result was independent of growth conditions. Actual cellular contents of cadmium and sulfide, however, were dependent on growth conditions and varied over a wide range. This strongly suggests that formation of insoluble cadmium sulfide is the primary detoxification mechanism of this organism under conditions of glucose limitation.

It therefore seemed pertinent to determine whether the organism could adapt to cadmium under conditions of sulfate limitation, during which sulfide production would clearly be less favorable. Unfortunately, due to excessive foaming, this particular strain (S45) could not be cultured in continuous culture under any conditions other than glucose limitation (2).

Therefore, this paper reports our studies on detoxification of cadmium by *K. aerogenes* NCTC 418 when grown under conditions of glucose, sulfate, or phosphate limitation. It is shown that P_i accumulation (to our knowledge this is a novel mechanism) plays a role in cadmium detoxification, in addition to sulfide formation, and that the extent to which either mechanism is employed depends both on the strain and on the growth conditions.

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.

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Media. For glucose-limited growth, a previously described (1) 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 10 mM glucose. For sulfate- or phosphate-limited growth, the glucose concentration was raised to 150 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. When glycerol was used as the carbon and energy source, glucose was replaced with 300 mM glycerol. In all cases, glucose was autoclaved (20 min at 110°C) and added aseptically to 9.5-liter batches of the separately sterilized (45 min at 120°C) bulk of the medium. Cadmium was added separately as indicated from sterile (15 min at 110°C) stock solutions of $CdCl_2$ (300 mM $CdCl_2$ plus 1 mM citric acid) as described previously (1).

Culture conditions. Bacteria were cultured aerobically in a C-30 Bioflo Continuous Culture apparatus (New Brunswick Scientific Co., New Brunswick, 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 value 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 5 min in concentrated hydrochloric acid and subsequently assayed for cadmium by flame atomic absorption spectrophotometry on a Perkin-Elmer model 4000 after appropriate dilution. The cellular contents of sulfide were determined as described previously (1), with the exception that $FeCl_3$ was dissolved in 0.6 N HCl instead of 6 N HCl; P_i was determined by the method of Ames and Dubin (3), total phosphate by the procedure of Chen et al. (4), polyphos-

phate as described by Langen and Liss (11), and protein and carbohydrate by the method of Herbert et al. (7). Assays were always carried out in duplicate on at least two different batches of cells, grown under identical conditions, and sampled at least 1 week apart and the results averaged. The relative standard deviation was consistently within 10%.

Microscopy. Electron microscopy was performed on unstained cells, taken directly from a culture in steady state, as described previously (1).

RESULTS

Glucose-limited growth of strain NCTC 418. Upon addition of 0.6 mM cadmium to a glucose-limited continuous culture in steady state, *K. aerogenes* NCTC 418 exhibited an adaptation pattern similar to that of strain S45 (1), i.e., after a lag period of several hours, the bacteria resumed growth and a new steady state could be attained. Under these conditions, strain NCTC 418 exhibited electron-dense granules (Fig. 1B) probably containing CdS as was seen with strain S45 (1). In contrast to strain S45, however, the cellular cadmium content (which amounted to 3.3% of the bacterial dry weight) was not counterbalanced by the cellular sulfide content in strain NCTC 418 (Table 1). In addition to the formation of sulfide, strain NCTC 418 appeared to accumulate an extra amount of cellular phosphate upon adaptation. In fact, 29.6 mmol of cellular cadmium present in 100 g of dry cells was almost perfectly balanced by 6.36 mmol of extra sulfide plus 22.5 mmol of extra phosphate (Table 1).

Sulfate-limited growth of strain NCTC 418. Under conditions of sulfate limitation, the cellular cadmium content increased to about 5.8% of the bacterial dry weight, but the relative importance of sulfide as a counterion for cadmium accumulated by the cells was reduced from 21% under glucose limitation to 6% under sulfate limitation after correction for the sulfide content of control cells (Table 1). Again, this reduction was accompanied by a matching increase in cellular phosphate accumulation. This resulted in 51.5 mmol of cadmium versus 3.02 mmol of extra sulfide plus 49.7 mmol of extra phosphate. Interestingly, cadmium-dependent, electron-dense granules were barely detectable in electron micrographs (Fig. 1C). These few visible granules far from represented all the cellular cadmium, which suggests that cellular forms of cadmium other than CdS are not localized but diffused.

The stoichiometry between cellular cadmium, extra sulfide, and extra phosphate seemingly no longer held under sulfate-limited conditions when glucose was replaced with glycerol as a carbon source, because under these conditions no extra phosphate was accumulated upon addition of cadmium (Table 1). However, in this case, as will be shown below, increased uptake was not necessary, because part of the phosphate already present could serve a different purpose (changed roles) if cadmium were added.

Phosphate-limited growth of strain NCTC 418. When grown under phosphate-limited conditions, the cells became more sensitive and no longer exhibited adaptation to 0.6 mM cadmium. After addition of 0.2 mM cadmium, however, a new steady state was attained within 2 days, which is similar to the results obtained with glucose- or sulfate-limited cultures after addition of 0.6 mM cadmium. The steady-state bacterial dry weight was practically the same before and after addition of cadmium, indicating that the cellular phosphate requirement had increased little. In addition to this observation, even at this decreased concentration of cadmium, the number and size of electron-dense particles increased enormously (Fig. 1E and F). This suggests that under these conditions of growth, formation of CdS is probably the most important mechanism of detoxification in this strain. That this was indeed the case can be seen in Table 1, showing that per 100 g of dry cells, 13.6 mmol of cadmium and 11.83 mmol of extra sulfide, but only 2.7 mmol of extra phosphate, were accumulated.

Chemical form of cadmium-associated phosphate. It is apparent (Table 1) that the cellular polyphosphate content did at no time increase upon addition of cadmium. Moreover, when appreciable amounts of polyphosphate were formed under sulfate-limited conditions with glycerol as the carbon source, addition of cadmium caused a complete disappearance of polyphosphate, which was replaced by a comparable amount of P_i. P_i determinations performed directly on the freeze-dried cell samples show (Table 1) a clear relationship (a ratio close to 1:1) between the amounts of cadmium and P_i present in NCTC 418 cells, whereas under phosphate limitation, a substantial amount of sulfide is present instead. The best correlation is found, in fact, between cellular cadmium content on the one hand and the extra phosphate plus the extra sulfide together on the other hand. This clearly suggests that both P_i and sulfide are counterions for cadmium.

DISCUSSION

When comparing *K. aerogenes* S45 (1) with the low-foaming strain NCTC 418, it seemed at first that both strains have the same mechanism of cadmium detoxification, as judged by the formation of electron-dense granules during glucose-limited growth in the presence of cadmium (Fig. 1B). It soon became apparent, though, that formation of CdS is not the only mechanism of cadmium detoxification in strain NCTC 418, or even quantitatively the most important one (Table 1). Especially under sulfate limitation, cellular sulfide content barely increased upon addition of cadmium. This correlated with the fact that sulfate-limited cultures had practically the same steady-state bacterial density in the presence or absence of cadmium. It also correlated with the fact that under these conditions, electron micrographs revealed merely a few small electron-dense granules of CdS, in addition to some rather electron-dense vacuoles present to the same extent in cells grown without added cadmium (Fig. 1C and D). Interestingly, under both glucose- and sulfate-limited conditions, the cellular cadmium content was quantitatively counterbalanced by the accumulation of both additional phosphate and additional sulfide although the relative amounts of phosphate and sulfide varied widely.

Under sulfate limitation with glycerol as a carbon source, there was no correlation between cellular cadmium (36.3 mmol) versus extra sulfide (3.93 mmol) and extra phosphate (negative). However, in accordance with the literature (6), analyses revealed that such cells grown without added cadmium contained a substantial amount of polyphosphate (22.6 mmol per 100 g of dry bacteria), but on addition of cadmium, polyphosphate became undetectable (<1 mmol per 100 g of dry bacteria). Since the data under consideration merely reflect steady-state situations, this finding does not necessarily imply actual breakdown of polyphosphate into discrete phosphate molecules; all we may conclude is that there is no net synthesis of polyphosphate in the presence of cadmium. In all other situations, however, addition of cadmium resulted in an increase in total cellular phosphate accompanied by a parallel increase in P_i. Thus, the data available strongly suggest involvement of P_i in cadmium

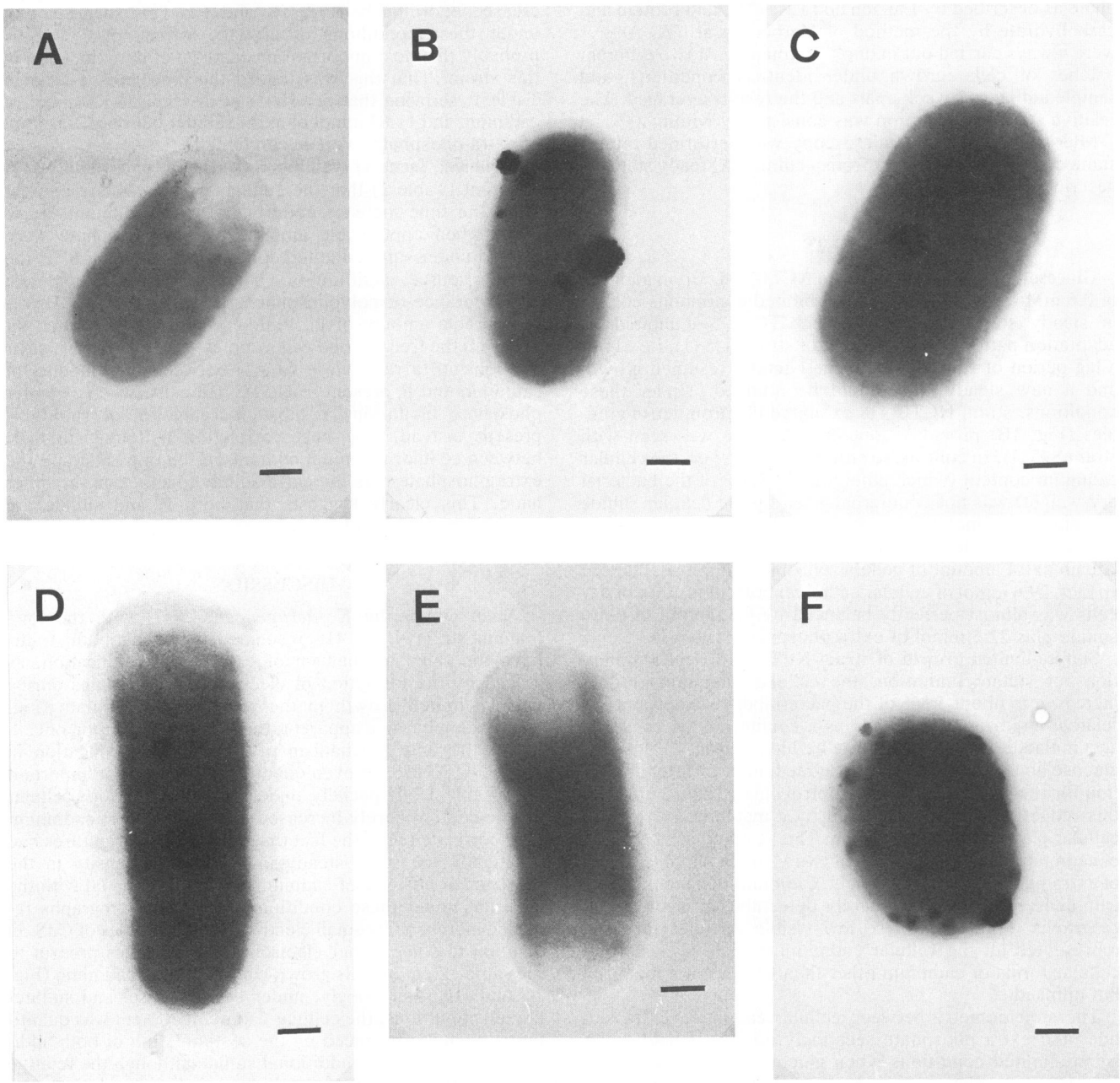

FIG. 1. Unstained *K. aerogenes* NCTC 418 grown under glucose limitation in the absence (A) or presence (B) of 0.6 mM cadmium, grown under sulfate limitation in the absence (C) or presence (D) of 0.6 mM cadmium, or grown under phosphate limitation in the absence (E) or presence (F) of 0.2 mM cadmium. Bar, 0.25 μm.

detoxification as a second mechanism distinct from sulfide production, although the mechanisms operate coordinately. This hypothesis is reinforced by the fact that phosphate-limited cells proved more sensitive to cadmium than do either sulfate- or glucose-limited cells.

The presence of polyphosphates in the absence of cadmium and their absence in the presence of cadmium requires comment. The three main hypotheses on the role of polyphosphate in bacteria are that it is (i) an energy storage polymer (phosphagen hypothesis), (ii) a phosphorus reserve polymer, and (iii) a regulator of metabolic processes, mainly through control of the energy charge (6, 10). In relation to the second hypothesis, our results seem to suggest a specific role for polyphosphates as a phosphate storage polymer, namely as a defense reserve against heavy metals, as has been suggested before (10). Its disappearance upon addition of cadmium, as indicated above, suggests at least that whatever its role it can be dispensed with in the presence of cadmium. On the other hand, with respect to the third hypothesis, it should be noted that addition of cadmium induced a sharp decrease in carbohydrate content under sulfate limitation (both with glucose and with glycerol) and a large increase in protein under phosphate limitation. The former seems to indicate that addition of cadmium increased

TABLE 1. Relationship between growth conditions[a] in continuous culture and cellular composition of *K. aerogenes* NCTC 418

Growth conditions			Cellular content/100 g of dry cells						
Limiting nutrient	Carbon source	Added cadmium	Cadmium (mmol)	Sulfide (mmol)	P_{tot}[b] (mmol)	P_i[b] (mmol)	P_p[b] (mmol)	Protein (g)	Carbohydrate (g)
Glucose	Glucose	None	<0.1	0.78	70.8	3.2	<1.0	66.0	3.44
Glucose	Glucose	0.6 mM	29.6	7.14	93.3	29.1	<1.0	58.3	3.34
Sulfate	Glucose	None	<0.1	1.00	75.6	3.2	3.2	51.1	29.55
Sulfate	Glucose	0.6 mM	51.5	4.02	125.3	38.7	<1.0	49.5	9.79
Sulfate	Glycerol	None	<0.1	0.72	126.6	6.5	22.6	52.1	20.51
Sulfate	Glycerol	0.6 mM	36.3	4.65	122.4	35.5	<1.0	51.6	9.67
Phosphate	Glucose	None	<0.1	0.91	31.9	1.9	<1.0	45.3	9.88
Phosphate	Glucose	0.2 mM	13.6	12.74	34.6	2.3	<1.0	63.6	9.56

[a] Cells were grown aerobically at 35°C, pH 7.0, and a dilution rate equal to 0.1 h^{-1}.
[b] Intracellular total phosphate (P_{tot}), P_i, and polyphosphate (P_p) are expressed as mmoles of P_i.

the cellular energy requirements. The latter might indicate synthesis of a specific cadmium-binding protein under phosphate limitation, as has been described for *E. coli* by Khazaeli and Mitra (8). The presence of this kind of additional mechanism seems likely in relation to the increased cadmium sensitivity of phosphate-limited cells.

Phosphate accumulation may be considered as a detoxification mechanism since cadmium phosphate is poorly soluble and a cadmium phosphate precipitate either inside or attached to the bacteria would be anticipated, as is the case with CdS (Fig. 1B and F). Under conditions of sulfate limitation, however, electron microscopy did not reveal the presence of such large precipitates (Fig. 1D). In our view, three possibilities remain: (i) cadmium phosphate is present in the cells as a diffuse, finely dispersed, colloidal suspension; (ii) a polymeric cadmium phosphate is formed as described by Kulaev for other metals (10); or (iii) cadmium phosphate is not actually formed, but some other relationship exists between cadmium adaptation and phosphate accumulation. This last possibility, however, seems less likely because it would imply the presence of large amounts of free P_i in the cells, with a concomitant increase in osmotic pressure. Further research is clearly needed to elucidate the nature of the relationship between cadmium and phosphate, but the existence of such a relationship has unequivocally been established.

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