Cadmium-specific formation of metal sulfide ‘Q-particles’ by *Klebsiella pneumoniae*

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*Klebsiella pneumoniae* overcomes cadmium toxicity through the ‘biotransformation’ of cadmium ions into photoactive, nanometre-sized CdS particles deposited on the cell surface. The kinetics of particle formation during batch culture growth was monitored by electron microscopy (EM), energy-dispersive X-ray analysis and electronic absorption spectroscopy (EAS). During the deceleration phase of bacterial growth, the presence of CdS particles on the outer cell wall of *K. pneumoniae* (≥5 nm in diameter) was detected by EM. The size of these electron-dense particles continued to increase throughout the stationary phase of growth, with some of the particles reaching a diameter >200 nm. The formation of the extracellular CdS particles contributed to around 3–4% of the total cell biomass. EAS undertaken on these extracellular ‘bio-CdS’ particles suggested that the large ‘superparticles’ observed by EM, e.g. 200 nm, were aggregates of smaller particles termed ‘Q-particles’, ∼4 nm in diameter. Metal sulfide particles were not formed in batch cultures of *K. pneumoniae* grown in the presence of lead, zinc, mercury, copper or silver ions. Growth in the presence of lead ions resulted in the formation of extracellular electron-dense particles containing lead but not sulfide or phosphate. Intracellular phosphorus-containing electron-opaque particles were formed during growth in the presence of copper and mercury. Intracellular electron-dense particles were formed in the presence of zinc ions but these did not contain phosphorus. From these results it was thought that metal sulfide formation in *K. pneumoniae* showed some cadmium-specificity. When cadmium and zinc ions were both added to the growth medium, metal sulfide particles were formed that were predominantly composed of cadmium, e.g. 48-6% cadmium and 0-04% zinc. Similarly, when cadmium and lead ions were both present during growth only CdS particles formed. In both cases analysis of the cells by EAS confirmed the presence of CdS only. These observations suggest that the mechanism of CdS formation is unlikely to occur simply through a cadmium-induced release of hydrogen sulfide by the cells into the external environment. If hydrogen sulfide production was the mechanism of sulfide formation then metal sulfide particles containing lead and zinc ions in addition to cadmium ions should have been produced.

Keywords: cadmium sulfide, heavy metal resistance, energy-dispersive X-ray analysis, *Klebsiella pneumoniae*

INTRODUCTION

Heavy metal ions such as cadmium and lead are highly toxic towards micro-organisms due to their ability to replace functional metals inside cells, leading to the denaturing of proteins and DNA. Many toxic heavy metals are transported into the cell by systems that normally convey an essential metal ion (Gadd &

Abbreviations: ALSA, acid-labile sulfide analysis; EAS, electronic absorption spectroscopy; EDXA, energy-dispersive X-ray analysis; EM, electron microscopy; SEM, scanning electron microscopy; TEM, transmission electron microscopy.
Griffiths, 1978; Hughes & Poole, 1989). For example, cadmium transport occurs through either the Mn$^{2+}$ or Mg$^{2+}$ transport system in many bacteria, including Staphylococcus aureus (Hughes & Poole, 1989). One mechanism of resistance to heavy metals is through the production of hydrogen sulfide ($\text{H}_2\text{S}$) which reacts with many heavy metals to form less toxic insoluble sulfides (Gadd & Griffiths, 1978; Fortin et al., 1994). $\text{H}_2\text{S}$ production as a mechanism of heavy metal ion detoxification is particularly effective for sulfate-reducing bacteria which can precipitate toxic metal ions as crusts of extracellular metal sulfides (Fortin et al., 1994). Slawson et al. (1992, 1994) have reported that the non-sulfate-reducing bacterium Pseudomonas stutzeri detoxified silver ions through the formation of silver sulfide particles via $\text{H}_2\text{S}$ formation. It is possible that P. stutzeri produces $\text{H}_2\text{S}$ in a similar manner to that of Clostridium thermoaceticum (Cunningham & Lundie, 1993). In the presence of cadmium ions, the production of $\text{H}_2\text{S}$ by C. thermoaceticum involves the desulfuration of cysteine to $\text{H}_2\text{S}$ by cysteine desulphhydrase (Kredich et al., 1973).

The first detailed studies of the detoxification of cadmium ions by Klebsiella pneumoniae (formerly K. aerogenes) were undertaken by Aiking et al. (1982, 1984). These studies revealed that in the presence of cadmium ions, under conditions of glucose, sulfate and phosphate limitation in continuous culture, K. pneumoniae exhibited two different detoxification mechanisms: metal sulfide formation and metal phosphate formation. From whole-cell metal and sulfide analysis, Aiking et al. (1982) postulated that cadmium ions were detoxified as extracellular CdS particles. Recently, using the spectroscopic technique of energy-dispersive X-ray analysis (EDXA) (Holmes et al., 1995a), we have established that the composition of these biologically produced particles from K. pneumoniae was CdS in origin. We have also demonstrated that these particles are photoactive and have potential application in light-driven electron-transfer reactions (Holmes et al., 1995b, 1997). UV/visible spectroscopy undertaken on the CdS particles of K. pneumoniae has revealed that the extracellular particles appear to have an electronic absorption profile similar to chemically synthesized 'quantum' semiconductor particles of inorganic CdS, $\sim 4 \text{ nm in diameter}$ (Holmes et al., 1997). 'Bulk' CdS particles ($> 6 \text{ nm in diameter}$) is the expression given to those that absorb visible radiation ($\lambda_{\text{onset}}$) below a wavelength of 515 nm (Henglein, 1989). As the dimensions of the CdS particles decrease below 6 nm there is an observed shift in the $\lambda_{\text{onset}}$ to shorter wavelengths. Particles which show this size quantization are known as 'quantum-particles' or 'Q-particles' (Henglein, 1987, 1989; Weller, 1993; Weller & Eychmuller, 1993).

The 200 nm particles identified on the surface of stationary phase cells of K. pneumoniae by Holmes et al. (1995a) would not have been expected to display properties of photoactive Q-particles. In light of the electronic absorption profile obtained for the extracellular CdS particles in the late stationary phase, the UV/visible profile of the CdS particles at different stages of the batch culture process has been studied. The results presented in this paper suggest that the CdS 'superparticles' observed by electron microscopy (EM) are possibly oligomers of discrete biologically produced CdS Q-particles, $\sim 4 \text{ nm in diameter}$.

In addition to cadmium ions, K. pneumoniae is also resistant to other heavy metal ions such as those of mercury and lead (Aiking et al., 1985). In the case of lead, Aiking et al. (1985) have suggested that the formation of extracellular lead sulfide or lead phosphate as the detoxification mechanism depends upon the limiting conditions in continuous culture. The exact mechanism by which the extracellular metal sulfide particles are formed is unclear. In principle, a similar mechanism to that of sulfide formation in C. thermoaceticum, through the production of $\text{H}_2\text{S}$, could be suggested for the formation of CdS and other metal sulfide particles on the K. pneumoniae cell surface. Metal sulfide Q-particles can be characterized by the valence-conduction band-gap energy ($E_g$) which is dependent on the nature of the metal present and which influences the photoactive properties of the semiconductor (Weller et al., 1995). In the light of the observations of Aiking et al. (1985), an attempt to use K. pneumoniae to synthesize metal sulfide Q-particles of lead, zinc, copper, mercury and silver was also undertaken.

### METHODS

**Preparation of bacterial cultures.** Batch cultures of K. pneumoniae were grown under oxic conditions in sterile conical flasks (250 ml), by inoculating with K. pneumoniae (NCIMB 418) stock solution (100 µl) in the absence of cadmium ions, into growth medium (50 ml) containing (in 1 l) 3.2 g FeSO$_4$.7H$_2$O, 64 mg MgSO$_4$.7H$_2$O, 0.62 g KCl, 0.06 g sodium β-glycerophosphate, 0.96 g (NH$_4$)$_2$SO$_4$ as the main components in tricine (50 mM) buffered to pH 7.6 using NaOH (Holmes et al., 1995a). After autoclaving (2 h), a sterile glucose solution (8.5 mM) was added to the buffered growth medium.

Cadmium, zinc, lead, mercury, silver or copper nitrate was added depending on the experiment (mixtures of cadmium and zinc ions, and cadmium and lead ions were also added) and the flasks were then shaken for at 37 °C (24 h). Na$_2$EDTA (5 mM) was added to the growth media containing lead, mercury and silver ions to prevent precipitation of the metal ions. EM of the growth medium before and after culturing revealed no precipitated metals. Precipitation was also not observed in uninoculated growth medium incubated for 24 h. No difference in cell morphology was observed by EM for cells grown in the absence or presence of the various metal ions.

**Cadmium uptake.** Determination of cadmium uptake by K. pneumoniae, grown in the presence of 0.5, 1 and 2 mM Cd(NO$_3$)$_2$, was achieved using atomic absorption spectroscopy and inductively coupled plasma mass spectrometry (Holmes et al., 1995a)

**Acid-labile sulfide analysis (ALSMA).** Inorganic acid-labile sulfide was determined using a modification of the method described by King & Morris (1967). An aliquot of K.
metal nitrates was added to a test tube containing zinc acetate (500 μl, 2.6% w/v) and NaOH (100 μl, 6% w/v). The tubes were stoppered and vortex-mixed (1 min). N-N-Dimethyl-p-phenylenediamine monohydrochloride (250 μl, 0.1% w/v, in 5 M HCl) was added and the tubes shaken until the solutions became clear. Ferric chloride (100 μl, 0.2% w/v, in 0.6 M HCl) was quickly added to each tube which was then stoppered and vortex-mixed (1 min) and incubated at room temperature (30 min). Finally, Milli-Q water (850 μl) was added to each tube and the suspension mixed and centrifuged (1600 g, 10 min). Samples were measured for methylene blue content at a wavelength of 670 nm against a calibration series containing 0–40 nM of Na2S per sample, on a Pharmacia LKB-Ultraspec III UV/visible spectrophotometer.

**Cysteine desulphydrase assay.** The enzyme cysteine desulphydrase was determined using a modification of the method of Kredich *et al.* (1973). Samples of *K. pneumoniae* were centrifuged (10000 rpm, 20 min) using a Sorvall RC-5B centrifuge. The supernatant was discarded and the pellet was resuspended in phosphate buffer (0.1 M, 1 ml, pH 7.6). Samples were then sonicated at 50 Hz using a Jencons GE-0 ultrasonic processor. Each resuspended pellet was sonicated six times for periods of 10 s with intervals of 30 s. The sonicated samples were centrifuged (10000 g, 20 min), the supernatant removed and the pellet resuspended in phosphate buffer (0.1 M). Hydrogen sulfide was assayed for by incubating the supernatants and pellets with Tris (0.1 M buffered to pH 7.6) and cysteine hydrochloride (100 mM, pH 8.6) at 37°C (15 min). The reaction was terminated with N,N-dimethyl-p-phenylenediamine sulfite (2%, w/v, in 7.2 M HCl). Absorbance was measured at 650 nm and the concentration of sulfide was determined according to a standard sodium sulfide calibration curve.

**EM and EDXA.** Fixing, staining and cross-sectioning for transmission electron microscopy (TEM) was carried out as outlined by Hayat (1989): glutaraldehyde (1 ml 5%, w/v, glutaraldehyde in 0.1 M PIPES, pH 7.2) was added to the bacterial samples (1 ml) in 2 ml Eppendorf tubes and refrigerated for 30 min. Samples were centrifuged (1600 g, 10 min), the supernatant removed, the pellet washed with 0.1 M PIPES buffer (2 ml), left to stand in the buffer for 5 min and then centrifuged (1600 g, 10 min). The samples were then dehydrated in graded alcohols – 30, 50, 70, 90, 98% and absolute ethanol (2 ml, 15 min each). In absolute alcohol each pellet was disturbed and on settling the supernatant was removed and LR (Agar Scientific) medium-grade resin added to each Eppendorf tube (2 ml), the pellets redistilled and left for 30 min before centrifugation and removal of the supernatant. Each Eppendorf tube was then filled with LR medium-grade resin (2 ml) and placed in an oven overnight (60°C). TEM sections (70–90 nm) were prepared on a LKB Nova ultra-microtome. A JEOL model 100CX microscope, operating at 100 kV, was used throughout.

Analytical EM was performed on bacteria and the electron-dense particles using the above microscope, equipped with a thermionic electron gun, a LINK Pentafet windowless X-ray detector and an AN10000 X-ray analysis system capable of measuring all elements heavier than boron. The windows of the elements studied in this work corresponded to their principal L, K and M X-ray lines in the energy range 0–20 keV (S Kα: 2.307, Kβ: 2.648; P Kα: 2.015, Kβ: 2.142; Cd Lα: 3.133, Lβ: 3.316, Lγ: 3.528; Zn Lα: 1.009, Lβ: 1.032, Kα: 8.631, Kβ: 9.572; Pb Mα: 1.246, Mβ: 1.243, Lα: 1.549, Lβ: 1.261, Lγ: 1.262; Cu Kα: 8.041, Kβ: 8.907, Lα: 9.298, Lβ: 9.498; Hg Mα: 2.195, Mβ: 2.283, Lα: 9.987, Lβ: 11.823, Lγ: 11.923; Ag Lα: 2.984, Lβ: 3.151, Lγ: 3.348, Lα: 3.519, Lβ: 3.743 keV). For analysis of samples containing lead, zinc, mercury, silver and cadmium, either copper, aluminium or nylon grids were used. For analysis of samples containing copper, aluminium or nylon grids were used.

For quantitative measurements, spectra were recorded on the above microscope and X-ray analyser with additional JEOL 100CX and ASID (SEM, STEM) quantitative EDXA instrumentation, for a pre-set time sufficient to give the required statistical precision as outlined by Reed (1993). The same probe current was used for both the specimen and the standards, a colloidal dispersion of CdS, metallic Zn and inorganic P on a copper grid, obtained from Micro-Analytical Consultants (St Ives). An X-ray energy window was selected for the elements Cd, S, P and Zn. The analysis process sums the number of X-rays corresponding to each window and quantifies the number of mmol kg⁻¹, taking into account instrument response as calculated from the reference standard. The X-ray emissions for each element in mmol kg⁻¹ and their respective molar ratios was obtained using the program QUANTEM (Oxford Instruments) (Holmes et al., 1995a).

Silver staining of cross-sectioned samples was undertaken according to the method outlined by Hayat (1989). A silver methenamine solution was prepared by adding 2 ml silver nitrate solution (5%, w/v) to 18 ml hexamethylenetetramine solution (3%, w/v). To the silver methenamine solution was added 2 ml sodium tetraborate decahydrate (2%, w/v) and the mixture was filtered twice through a Whatman no. 42 filter paper. Following oxidation of the bacterial sections in periodic acid, sections were rinsed in distilled water and then transferred to the staining solution. Staining dishes were placed in an oven at 60°C (1 h). After adequate staining, sections were transferred to a sodium thiosulfate solution (5%, w/v) for 1 min. Sections were then rinsed thoroughly in distilled water. Sulfide analysis was undertaken on bacterial samples grown in the presence of cadmium and lead nitrate by EDXA. The Ag X-ray energy lines were characteristic to the presence of sulfur.

**Electronic absorption spectroscopy (EAS).** UV/visible absorption measurements were performed on samples of bacterial cultures grown in the presence and absence of metal nitrates. An Hitachi U4001 double-beam spectrophotometer, incorporating a barium sulfate integrating sphere attachment, was used to record the spectra, referenced against bacterial supernatant. The integrating sphere collects the scattered light from turbid samples and redirects it to the detector, hence reducing the loss of light through scattering. Absorption spectra were taken using 1 cm path-length quartz cuvettes.

**RESULTS AND DISCUSSION**

The formation of extracellular CdS particles in batch cultures of *K. pneumoniae* monitored by EM, EDXA and EAS

The formation of extracellular CdS particles in batch cultures of *K. pneumoniae* was monitored by a combination of EM, EDXA and EAS. In the absence of cadmium ions the cultures exhibited a lag phase of 1–2 h and a maximum specific growth rate (μmax) of \( \approx 1:13 \; h^{-1} \). In the presence of 0.5, 1 and 2 mM Cd(NO₃)₂, the lag phase varied between 4–6 h at all cadmium ion concentrations with \( \mu_{\text{max}} \) ranging between 2523
0.8 and 0.9 h⁻¹ (Fig. 1a). Each culture grown in the presence of Cd(NO₃)₂ reached the same level of bacterial cell numbers in the stationary phase as the cells grown in the absence of cadmium ions (~ 10⁹ cells ml⁻¹). Viable count analysis established that the death phase of the cultures grown in the presence of cadmium ions was not reached by 24 h. At this stage maximum cadmium uptake for K. pneumoniae cultured in the presence of 0.5, 1 and 2 mM Cd(NO₃)₂ was found to be 0.25 mmol (g dry wt)⁻¹ (σ = 0.002, n = 24), 0.28 mmol (g dry wt)⁻¹ (σ = 0.002, n = 24) and 0.3 nmol (g dry wt)⁻¹ (σ = 0.004, n = 24), respectively, which represents 3–4% of the total cell biomass.

EM was employed to investigate the formation of extracellular CdS particles during growth in the presence of 1 mM Cd(NO₃)₂ (Table 1). No electron-opaque particles were detected on cell cross-sections in the first 14 h of batch culturing which includes the exponential phase. In the following 7 h, the formation of extracellular electron-dense particles ranging from 5 to >200 nm in diameter were observed. It should be noted that the resolution of the electron microscope used was estimated to be approximately 5 nm; thus particles may have been present in the first 14 h of batch culture growth but were of a size undetectable by EM. In the 2 h between 13 and 15 h growth (the deceleration phase in Fig. 1a), the percentage of cell cross-sections with EM-visible electron-dense particles was found to increase from 0 to 95%. Similar results were also obtained for batch cultures of K. pneumoniae grown in the presence of 0.5 and 2 mM Cd(NO₃)₂ (data not shown).

Chemically prepared CdS particles < 6 nm in diameter are classified as Q-particles and have properties of quantum semiconductors (Henglein, 1989). In the cross-sectioned samples, possible Q-particles were observed after 14 h growth: approximately 28% of the particles had a size distribution between 5–20 nm, with about one-tenth of these being less than 6 nm in diameter (Table 1). The most common size distribution after 15–20 h growth (stationary phase) was between 21–50 nm (33%). The majority of particles observed after the 21–24 h period (late stationary phase) ranged in diameter from 151 to >200 nm (26–31%). Only approximately 1% of the total number of particles observed after 20–24 h growth could be considered to be Q-particles. The particle sizes and numbers reported in Table 1 are for cross-sectioned cells. Hence, it is likely that only a proportion of the total number of particles present on each cell was observed for the results reported above. The assumption has been made that the data obtained from the cross-sectioned samples was representative of the sample population.

It was possible to investigate the formation of extracellular CdS particles by monitoring the electronic absorption profile (A₅₆₅) of samples grown in the presence of 1 mM Cd(NO₃)₂ at various stages of the growth curve (Fig. 2a). No absorption profile characteristic of CdS particles was observed before a time period of 14 h. An electronic absorption profile (λ₊₀₉₅ ~ 400 nm, λₒₙₐₛₜ

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**Table 1. Summary of the number and size of particles observed on cross-sectioned cells analysed by EM**

Cells were from various stages along the growth cycle of K. pneumoniae grown in the presence of 1 mM Cd(NO₃)₂ in a tricine-buffered medium. n = 200 cells for each time period (from three cultures); no particles were detected before 14 h.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Cells with particles (%)</th>
<th>Number distribution (%)</th>
<th>Size distribution (%) of particles (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td></td>
<td>44</td>
<td>26</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>95</td>
<td>3</td>
</tr>
<tr>
<td>16–24</td>
<td></td>
<td>96</td>
<td>4</td>
</tr>
</tbody>
</table>
Metal sulfide formation by *Klebsiella pneumoniae*

inorganic CdS particles ~ 3 nm in diameter. Spanhel *et al.* (1987) also reported that a $\lambda_{\text{onset}}$ of ~ 450 nm corresponded to a particle size diameter of ~ 3.5 nm from experimental data. Furthermore, recent experimental results published by Ogawa *et al.* (1995) for 'Q-CdS' particles on gold surfaces proposed that CdS particles 3 and 4 nm in diameter have the following characteristics: for particles 3 nm in diameter, $\lambda_{\text{max}} = 381$ nm and the energy band gap ($E_g = 3.25$ eV); for particles 4 nm in size; $\lambda_{\text{max}} = 424$ nm and $E_g = 2.9$ eV. A knowledge of $E_g$ is necessary when considering the potential use of these CdS particles to catalyse photochemical and photobiological reactions of the appropriate redox potential.

The size of the extracellular particles predicted from the UV/visible absorption spectra, i.e. ~ 4 nm, is smaller than the particles observed by EM, i.e. 5 to > 200 nm. One explanation to account for this discrepancy between the spectroscopic and microscopic data is that the absorption profiles observed were from microscopically undetectable CdS Q-particles (~ 4 nm in diameter), due to the poor resolution of the electron-microscope with this system (~ 5 nm). A second, perhaps more likely possibility is that the extracellular 'superparticles' observed by EM are composed of aggregated smaller 'bio-quantum dots', ~ 4 nm in diameter. Electron-diffraction studies were undertaken on the 'superparticles' (ca. 200 nm) observed by EM. No diffraction pattern was observed suggesting that the particles were not crystalline but composed of smaller discrete 'amorphous clusters'. This supported the electronic absorption data which suggested the presence of discrete Q-particles of CdS approximately 4 nm in diameter.

If the assumption is made that the particles observed by EM are composed of smaller clusters, then the increase in the mean diameter of the particles as the batch cultures progress from the deceleration phase to the stationary phase is probably due to a 'nucleation effect', where small particles oligomerize to form large multimers observable by EM. This process is distinct from aggregation resulting in formation of bulk CdS. The absorption profiles of the CdS-containing cells did not change when *K. pneumoniae* cultures were stored for a period of four weeks. With many inorganic colloids of CdS there is a red shift in $\lambda_{\text{max}}$ and $\lambda_{\text{onset}}$ during a four week time period, due to particle aggregation, which eventually leads to precipitation of the CdS crystallites as bulk CdS ($E_g = 2.4$ eV) (Weller, 1993). The ‘bio-CdS’ particles were not observed to aggregate as they are held on a ‘bacterial matrix’ and therefore have the advantage over their inorganic counterparts in being stable for several weeks.

**Addition of lead, zinc, copper, mercury and silver ions to *K. pneumoniae***

The confirmation that *K. pneumoniae* can synthesize photoactive extracellular CdS particles with electronic absorption properties characteristic of Q-CdS semiconductor particles raised the question of whether the
organism could also synthesize other metal sulfide Q-particles. This possibility was investigated for lead(II), zinc(II), copper(II), mercury(II) and silver(II) ions which are soft and intermediate metal ions with a propensity for forming a covalent bond with the soft sulfide ligand. If successfully synthesized, these particles would have distinct $E_x$ values and thus possess photoactive properties different to 'bio-generated' CdS particles. Pb(NO$_3$)$_2$ was added to cultures of *K. pneumoniae* prior to growth. The bacterium was able to tolerate lead ion concentrations of up to 6 mM in the tricine-buffered medium, which also contained EDTA (5 mM). EDTA was added to the medium to prevent precipitation of the lead ions from solution. For samples grown in the presence of 5 mM Pb(NO$_3$)$_2$, the lag phase lasted for up to 8 h and $\mu_{\text{max}}$ was $\sim 0.86$ h$^{-1}$ (Fig. 1b).

Extracellular electron-opaque particles were observed on the surface of the cells by EM in the late stationary phase (24 h growth) (Fig. 3a). Initially it was assumed that the electron-dense particles would consist of PbS, in accordance with previous results obtained by Aiking et al. (1985). Direct detection of PbS by EDXA was not possible as X-ray emissions from Pb ($M_{\text{A}}$ and $M_{\text{B}}$) and from S ($K_{\text{A}}$ and $K_{\text{B}}$) could not be differentiated due to peak overlapping (Fig. 3b). To overcome this problem, sulfide identification was achieved by silver staining of the bacterial particles. Silver ions were added to the cross-sectioned samples, where they can bind to the sulfide of extracellular particles, allowing indirect identification of sulfide by analysis of Ag X-ray emissions. To establish the validity of the technique it was first applied to sections of *K. pneumoniae* cells grown in the presence of cadmium ions (Fig. 4a, b). In non-silver-stained sections, the characteristic S and Cd peaks of the CdS particles could be identified (Fig. 4a). In the silver-stained samples the S peak was decreased and an intense...
Ag peak appeared. No silver could be detected in the silver-stained samples prepared from cells grown in the presence of Pb(II) ions (Fig. 4c). This result indicated that the extracellular lead-containing particles were not PbS. No other silver precipitates were also observed by EM.

To confirm the absence of PbS particles, ALSA was also done on cell samples from cultures grown in the presence of Pb2+ ions (Table 2). Analysis of the bacterial samples grown in the presence of 5 mM Pb(NO3)2 revealed that the amount of sulfide present approximated to 30 pM compared to 0.1-0.2 mM observed for growth in the presence of 1 and 2 mM Cd(NO3)2. No phosphorus signal arising from lead phosphate could be detected by EDXA leaving the possibilities that: (i) the Pb2+ ions are converted via a redox reaction into elemental lead \([\text{Pb}^{2+}/\text{Pb}_0, E' = -0.125 \text{ V vs NHE} \text{ (Normal Hydrogen Electrode)} \text{ (Shriver et al., 1990)}]\); (ii) that Pb2+ ions are converted into extracellular PbSO4 particles, which is undetectable by EDXA because of peak overlapping between Pb and S and which is also undetectable by silver staining; (iii) the Pb2+ ions are converted to lead oxide. These three possibilities have not been distinguished in the present study but the conclusion that detoxification of Pb2+ ions by \textit{K. pneumoniae} does not occur through the formation of PbS in this system, as postulated by Aiking et al. (1985) for a continuous culture system is irrefutable.

\textit{K. pneumoniae} was tolerant up to 12 mM Zn(NO3)2 in the tricine-buffered medium. The batch culture growth kinetics of \textit{K. pneumoniae} cultured in the presence of 10 mM Zn(NO3)2 were comparable to that obtained for \textit{K. pneumoniae} grown in the presence of 1 mM Cd(NO3)2 (Fig. 1). EM and EDXA revealed no particle formation on the outer cell walls of the bacterium when Zn(II) ions (10 mM) were added prior to growth. Instead, the zinc was found to be located inside the cell as electron-dense particles (Fig. 5a). From EDXA no other elements, including phosphorus, appeared to be associated with these zinc particles (Fig. 5b). In addition, very little acid-labile sulfide could be detected in cells grown in the presence of zinc ions (Table 2). Therefore, the addition of zinc ions to the bacterial medium prior to growth did not result in sulfide formation, as observed with Cd(II) addition, or the synthesis of zinc-containing polyphosphate particles. The precise chemical composition of the particles could not be determined but they are unlikely to be metallic zinc due to the high reducing power required to convert Zn2+ to Zn0 \([E^0 = -0.763 \text{ V vs NHE} \text{ (Shriver et al., 1990)}]\). It is possible that the electron-opaque particles observed by EM are insoluble hydroxides of zinc, but analysis of O and H by EDXA was not possible.

### Table 2. Summary of ALSA for \textit{K. pneumoniae} grown in a tricine-buffered medium in the presence of cadmium, lead and zinc ions

EDTA was added to samples cultured in the presence of lead ions to prevent metal ion precipitation. \(\sigma_{\text{Cu}}\) values are in parentheses, where \(n = 20\) for each concentration (from three cultures).

<table>
<thead>
<tr>
<th>[Metal ions]</th>
<th>Mean sulfide concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No metal ions</td>
<td>0.003 (0.001)</td>
</tr>
<tr>
<td>0.5 mM Cd(^{2+}) only</td>
<td>0.15 (0.011)</td>
</tr>
<tr>
<td>1 mM Cd(^{2+}) only</td>
<td>0.17 (0.013)</td>
</tr>
<tr>
<td>2 mM Cd(^{2+}) only</td>
<td>0.18 (0.022)</td>
</tr>
<tr>
<td>5 mM Zn(^{2+}) only</td>
<td>0.009 (0.002)</td>
</tr>
<tr>
<td>10 mM Zn(^{2+}) only</td>
<td>0.018 (0.004)</td>
</tr>
<tr>
<td>5 mM Pb(^{2+}) only</td>
<td>0.034 (0.005)</td>
</tr>
<tr>
<td>1 mM Cd(^{2+}) and 1 mM Zn(^{2+})</td>
<td>0.13 (0.003)</td>
</tr>
<tr>
<td>1 mM Cd(^{2+}) and 5 mM Zn(^{2+})</td>
<td>0.19 (0.005)</td>
</tr>
<tr>
<td>2 mM Cd(^{2+}) and 2 mM Zn(^{2+})</td>
<td>0.14 (0.003)</td>
</tr>
<tr>
<td>2 mM Cd(^{2+}) and 5 mM Zn(^{2+})</td>
<td>0.17 (0.006)</td>
</tr>
<tr>
<td>1 mM Cd(^{2+}) and 1 mM Pb(^{2+})</td>
<td>0.11 (0.004)</td>
</tr>
<tr>
<td>2 mM Cd(^{2+}) and 2 mM Pb(^{2+})</td>
<td>0.14 (0.005)</td>
</tr>
</tbody>
</table>
Fig. 6. *K. pneumoniae* cultured in the presence of 10 mM Cu(NO₃)₂, in a tricine-buffered medium (24 h). (a) Electron micrograph (TEM) of a cross-sectional sample. Note the presence of intracellular electron-dense particles (bar, 0.5 µm). (b) EDX (elemental analysis) spectrum of an intracellular electron-dense particle. The sample was held on a carbon-coated aluminium grid.

*K. pneumoniae* was tolerant to up to 15 mM copper in the tricine-buffered medium. Batch cultures of *K. pneumoniae* grown in the presence of 10 mM Cu(NO₃)₂ exhibited a μmax of 1.4 h⁻¹ with a lag phase of 2–3 h (Fig. 1b). These results are comparable to values obtained for *K. pneumoniae* grown in the absence of metal ions (Fig. 1a) and reflects the low toxicity of the copper ions towards the bacterium in the tricine-buffered medium. No extracellular electron-dense particles could be detected by EM at any time-point in the growth curve. EM/EDXA established that copper ions were located inside the cells associated with phosphorus in electron-dense polyphosphate granules that formed in the deceleration phase of growth (Fig. 6a).

Growing *K. pneumoniae* in the presence of silver and mercuric ions was difficult, as these ions were highly toxic towards the bacterium. The maximum concentrations tolerated in the tricine-buffered medium, containing EDTA (5 mM) to prevent metal ion precipitation, were 5 µM Ag⁺ and 2 µM Hg²⁺. The lag phase for *K. pneumoniae* grown in the presence of mercury or silver ions varied for different batches between 24–72 h. EM revealed that no particle formation occurred on the outer cell walls of the bacterium, or in the growth medium, with either metal ion.

**Addition of mixed metal ions to *K. pneumoniae***

The observation that metal sulfide particles were only formed when *K. pneumoniae* was grown in the presence of cadmium ions suggested that the formation of these particles was cadmium ion specific. If CdS was formed by Cd(II) inducing the release of H₂S from the cell, it would be expected that extracellular PbS and ZnS particles would form if mixtures of Cd(II) and Pb(II) or Cd(II) and Zn(II) were present in the growth medium. A range of concentrations of cadmium ions (1–2 mM) and zinc ions (1–5 mM) were added to cultures of *K. pneumoniae* and the extracellular electron-dense particles formed were analysed by quantitative EDXA (Table 3). The results obtained suggest that only a small amount of zinc was incorporated into the extracellular particles which contained around 48% Cd and only ~0.04% Zn. No pure ZnS particles were detected. Intracellular electron-opaque particles were also observed by EM/EDXA and were shown to contain Zn only (not shown). The absence of ZnS suggested that the formation of extracellular metal sulfide particles was specific to Cd(II) ions. If the mechanism of sulfide formation was through the release of H₂S from the cell, then more zinc would have been expected to be incorporated into metal sulfide particles. The signal overlap of Pb and S prevents quantitative EDXA of the extracellular particles on the surface of *K. pneumoniae* cells grown in the presence of both Cd(II) and Zn(II). However, EDXA established that the extracellular particles were of two types; one type was characteristic of CdS particles with no lead incorporated into them (as judged by the absence of the L₃, L₃, and M₃ X-ray peaks from Pb); the second type appeared to contain lead only. The acid-labile sulfide content of cells grown in the presence of Cd(II) and Zn(II) or Cd(II) and Pb(II) were similar to those observed when only Cd(II) was present (Table 2).

In principle, small Q-particles (<5 nm) of ZnS or PbS could be present on the bacterial surface but they are unobservable because of the resolution of the electron microscope used. If such Q-particles were present in large quantities they would be apparent from the electronic absorption spectrum since the band gap energies of ZnS (bulk = 3.6 eV) and PbS (bulk = 0.4 eV) semiconductors are distinct from CdS (bulk = 2.4 eV) leading to a different λonset (Yanagida *et al.*, 1990; Vogel *et al.*, 1994). Absorption spectra were recorded for samples grown in the presence of cadmium plus zinc (Fig. 2b) and cadmium plus lead ions (Fig. 2c). The absorption profiles obtained for both sample types were identical to those profiles obtained for CdS particles on
cells grown with only Cd(II) present (Fig. 2a). This result provided further evidence that metal sulfides other than CdS were not formed.

Conclusions

Although this study is not exhaustive of all heavy metals, the results presented demonstrate a degree of specificity of metal sulfide formation towards the cadmium ion. The results of this study appear to exclude the possibility of extracellular particle formation occurring as a result of H₂S release catalysed by cysteine desulphhydrase as has been suggested for the C. thermoaceticum (Cunningham & Lundie, 1993). Cysteine desulphhydrase activity was assayed in cell-free extracts of K. pneumoniae grown in the absence and presence of cadmium, lead, zinc, copper, mercury and silver ions. Activity was detected and lay in the range 200–400 nmol H₂S produced min⁻¹ (mg protein)⁻¹ in all of the samples. Thus no induction by cadmium was observed. Furthermore, ALSA of culture supernatants during growth in the presence of any of the metal ions studied did not detect release of H₂S. Further evidence that formation of CdS does not occur simply through H₂S being released into the extracellular environment comes from the observation that CdS particles do not display the photochemical properties of bulk CdS but rather to those properties associated with aggregates of discrete Q-particles. With the H₂S release mechanism, as with many sulfate-reducing bacteria, large ‘crusts’ of bulk CdS would be expected to form in preference to discrete Q-particles. In suggesting a testable alternative model for CdS Q-particle formation it is conceivable that cadmium ions enter the cell, perhaps through the magnesium or manganese transport system (Hughes & Poole, 1989) and become bound by a cysteine-containing cadmium-binding protein. This CdS-bound protein could possibly then move to the cell membrane where it is excreted or transported and trapped in the extracellular polysaccharide matrix. These discrete protein capped CdS particles might then oligomerize to form the large electron-dense multimers observed by EM. The presence of a protein cap would prevent the aggregation of the CdS Q-particles into bulk CdS. The precipitation of extracellular Cd- and Ni-binding proteins has been observed previously in other bacteria. For example, Khazaeei & Mitra (1981) reported the presence of an inducible cadmium-binding protein (M, 39000) involved in cadmium detoxification in Escherichia coli. Aiking et al. (1984) have also suggested the presence of a cadmium-binding protein in K. pneumoniae grown under phosphate limitation whilst Kurek et al. (1991) have reported the presence of a cadmium-binding protein (M, 42600) in an axenic bacterial culture. Fortin et al. (1994) have reported that a Desulfotomaculum sp. can convert iron(III) ions into iron sulfide through the release of H₂S. In the presence of nickel(II) ions however, nickel sulfide is prevented from forming due to the production of soluble nickel-binding proteins. The suggestion of a cadmium-specific metal-sulfide formation mechanism leads to the testable prediction that cadmium-sensitive lead-resistant mutants can be isolated.

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