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Effect of Cadmium on the Morphology, Membrane Integrity and Permeability of *Pseudomonas putida*

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Cadmium-adapted *Pseudomonas putida* exhibited a long lag phase (6 h) on incubation in a defined medium containing 3 mM-Cd^{2+}. During this time extensive blebbing of the outer membrane was observed by electron microscopy and polyphosphate granules containing Cd^{2+} were present in the cells. Cells from exponential-phase cultures of cadmium-adapted *P. putida* were found in clusters. They were much smaller than control cells grown without cadmium, and contained electron-dense aggregates. Cadmium-adapted cells released more lipopolysaccharide and protein into the external medium than did control cells; the addition of Ca^{2+}, but not Mg^{2+}, to the medium prevented this increased release. Cadmium-adapted cells also showed greatly increased sensitivity towards certain antibiotics, including the aminoglycosides, cyclic polypeptides and doxycycline. It is suggested that this is related to changes in membrane structure.

INTRODUCTION

We have recently described the isolation of metal-resistant strains of *Pseudomonas putida* from natural sources and discussed the mechanisms involved in their adaptation to growth in a chemically-defined medium containing 3 mM-Cd^{2+} (Higham *et al.*, 1985). Cadmium was actively accumulated by the cells, and the synthesis of cadmium-binding proteins was also induced (Higham *et al.*, 1984). This is in contrast to the mechanism in Gram-positive bacteria (Tynecka *et al.*, 1981; Laddaga *et al.*, 1985), where cadmium resistance involves a cadmium efflux mechanism.

The long lag phase which occurred on subculturing cadmium-adapted cells into fresh cadmium-containing medium was particularly notable. We therefore decided to investigate the morphology of cadmium-adapted and control cells during this phase of growth and also during the late-exponential phase. In this paper we report the cadmium-induced changes in membrane morphology, cell permeability and the release of lipopolysaccharide (LPS) and protein into the medium. Similar effects have been observed in Gram-negative bacteria exposed to polymyxin B or EDTA and in LPS-defective mutants (Eagon & Carson, 1965; Irvin *et al.*, 1975; Storm *et al.*, 1977). A model for the disruptive effect of cadmium on the outer membrane structure is proposed and discussed in relation to the increased antibiotic sensitivity of cadmium-adapted cells. The effects of cadmium on cell morphology are discussed in terms of the overall pattern of cadmium resistance in this organism.

METHODS

Growth medium. Cadmium-adapted cells were cultured as described previously (Higham *et al.*, 1985) in a defined medium containing $CdCl_2.2{\cdot}5H_2O$ (3 mM), $ZnSO_4.7H_2O$ (60 μM), and (mM) $(NH_4)_2SO_4$ (0·76), $MgSO_4.7H_2O$

Abbreviations: KDO, 2-keto-3-deoxyoctanoic acid; LPS, lipopolysaccharide.

(0·8), NaCl (17·24), KCl (13·5), NH_4Cl (18·7), glucose (27·8), sodium β-glycerophosphate (3·1), and the following L-amino acids – alanine (1·9), arginine (0·57), methionine (0·67), phenylalanine (0·48), serine (2·09), and valine (1·02) – buffered with 50 mM-Tris/HCl, pH 7·2. Control cells were cultured similarly but without added cadmium or zinc (Higham *et al.*, 1985).

Electron microscopy. Cadmium-adapted cells were harvested during the lag phase (0–6 h), or towards the end of the exponential phase (14 h), by centrifugation (6000 ***g***, 15 min, 4 °C). Control cells were also harvested during the lag phase (0–3 h) and near the end of the exponential phase (8 h).

Cell pellets (1–2 mm thick) were fixed in 1% (w/v) aqueous OsO_4 overnight at 4 °C, embedded in 2% (w/v) agar, dehydrated through a graded ethanol series, and embedded in Epon 812 resin. Thin sections were cut on a Reichert OMU2 ultramicrotome and stained at 4 °C in 2% (w/v) aqueous uranyl acetate followed by Reynolds' lead citrate (Reynolds, 1963). Micrographs were taken on a Philips EM300 microscope operated at 80 kV. Cells were counted directly on the microscope screen, and the occurrence of features quoted is based on counting 500–1000 cells per sample.

Albert's stain was used to identify metachromic granules (Norris & Swain, 1971).

LPS release, extraction and separation. Cadmium-adapted and control exponential-phase cells of *P. putida* were harvested and resuspended in fresh defined medium (10 mg cells wet wt ml^{-1}) containing one of the following: (*a*) 3 mM-Cd^{2+}, 60 μM-Zn^{2+}; (*b*) 3 mM-Cd^{2+}, 60 μM-Zn^{2+}, 20 mM-Ca^{2+}; (*c*) 3 mM-Cd^{2+}, 60 μM-Zn^{2+}, 20 mM-Mg^{2+}; or (*d*) no added metal ions. Zinc was included in the medium to provide optimum growth conditions for cadmium-adapted cells (Higham *et al.*, 1985). Duplicate samples were incubated at 30 °C for 6 h and then centrifuged. The supernatants were lyophilized and analysed for 2-keto-3-deoxyoctanoic acid (KDO) as described below.

LPS and protein released into the medium from an equal weight of control or cadmium-adapted cells were separated by gel filtration chromatography on a Sepharose 4B (1 × 30 cm) column, equilibrated with ammonium bicarbonate buffer (50 mM, pH 8·2). Fractions (2 ml) were collected and analysed for KDO (see below). Protein was detected by absorbance at 278 nm using an LKB Uvicord detector. Total LPS was extracted from 1 g wet weight of control and adapted cells using the aqueous butan-1-ol procedure described by Morrison & Leive (1975). The extracts were chromatographed as above.

Analysis of LPS. Lyophilized residues or fractions were dissolved in 10 mM-H_2SO_4 and hydrolysed at 100 °C for 30 min, and then assayed for KDO using the thiobarbituric acid assay (Weissbach & Hurwitz, 1959). The absorbance at 548 nm was read immediately, due to the instability of the chromophore.

Antibiotic sensitivity tests. These were done on a defined medium (1·5%, w/v, Difco-Bacto Agar) using plates with the same thickness of agar (15 ml per plate). A sample from a late-exponential-phase culture containing about 10^7 cells was flooded onto the plates and allowed to dry. Commercial antibiotic discs (Whatman) were used. The discs contained the following doses of antibiotics: 25 μg tetracycline, 25 μg oxytetracycline, 25 μg demethylchlortetracycline, 25 μg chlortetracycline, 25 μg doxycycline, 30 μg nalidixic acid, 10 μg gentamicin, 10 μg neomycin, 30 μg kanamycin, 10 μg fusidic acid, 10 μg streptomycin, 50 μg colistin, 100 units polymyxin B, 200 μg nitrofurantoin, 5 μg erythromycin, 25 μg chloramphenicol. In addition, the concentrations of chloramphenicol, erythromycin, kanamycin and ampicillin (Sigma) were varied by spotting onto blank antibiotic discs. The discs were placed on the surface of agar plates and the diameters of the zones of inhibition were recorded after incubation overnight at 30 °C.

RESULTS

Morphology of lag-phase cells

A number of morphological changes were observed in cadmium-adapted cells which were not observed in control cells. After 1 h incubation, about half of the adapted cells had protuberances ('blebs') on their outer membrane of up to 100 nm in diameter (Fig. 1*a*). These blebs were surrounded by a clearly defined triple layer, typical of a unit membrane. In some cells, this membrane was continuous with the outer membrane (Fig. 1*b*, *c*). Some blebs were clearly separated from the main cell by a single layer of membrane (Fig. 1*b*, *c*). Many blebs appeared to be densely packed with membranous material, whereas others were simply long loops (Fig, 1*a*). Membrane fragments and detached blebs were present in large numbers. The sizes and frequency of occurrence of these blebs reached a maximum after 3 h incubation, and then declined.

Electron-dense granules were observed in the cells after 1 h incubation (Fig. 1*a*). These granules stained red with toluidine blue (Norris & Swain, 1971), and were identified as polyphosphate following extraction and purification, using the method of Herbert *et al.* (1971). The polyphosphate extract from cadmium-adapted cells was analysed by atomic absorption

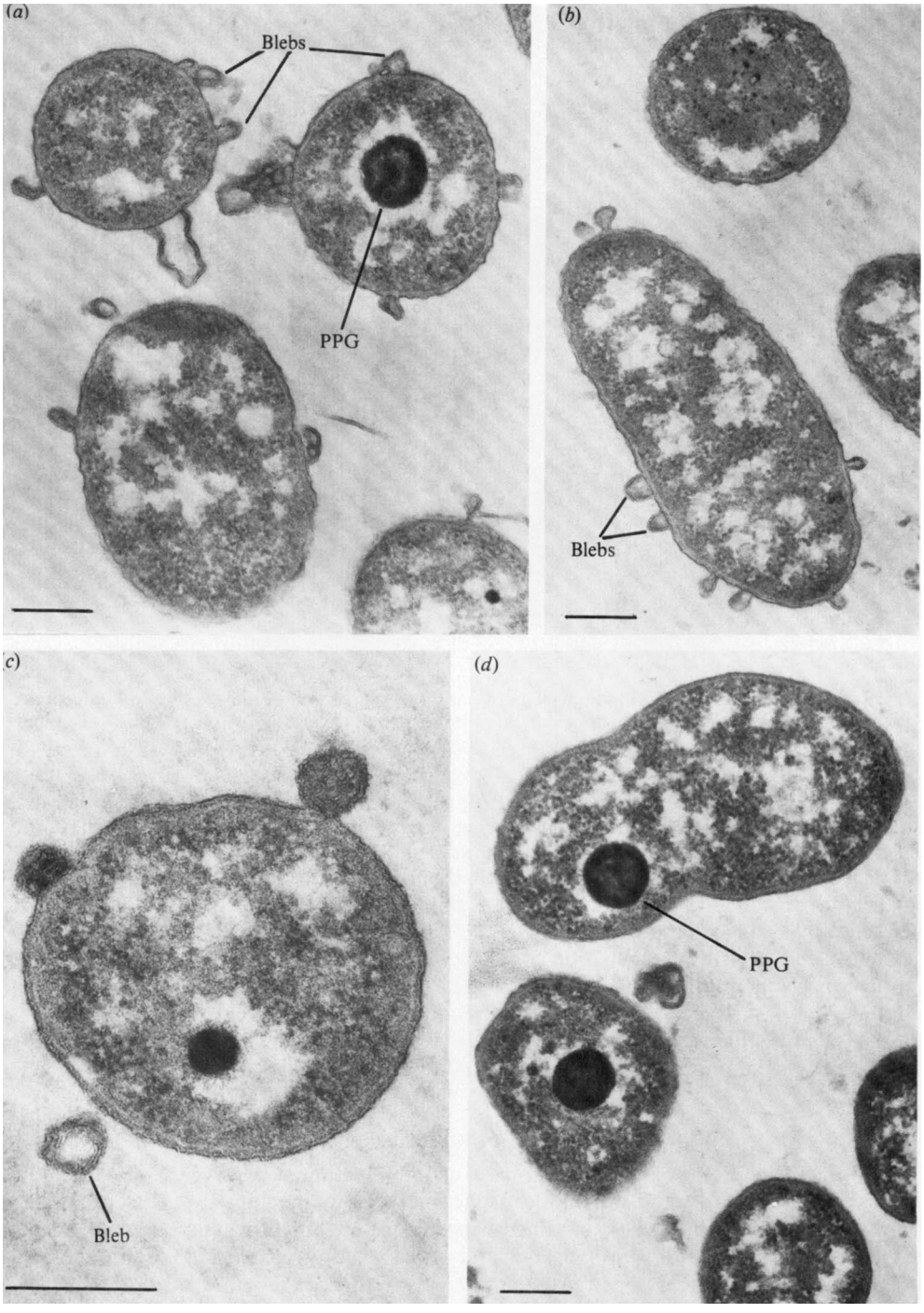

Fig. 1. Electron micrographs of thin sections of cadmium-adapted cells after 1 h (*a*), 2 h (*b*), 3 h (*c*) and 4 h (*d*) incubation in medium containing Cd^{2+}. Polyphosphate granules (PPG) and extensive blebbing of the outer membrane of cadmium-adapted cells are notable. Bars, 0·2 μm.

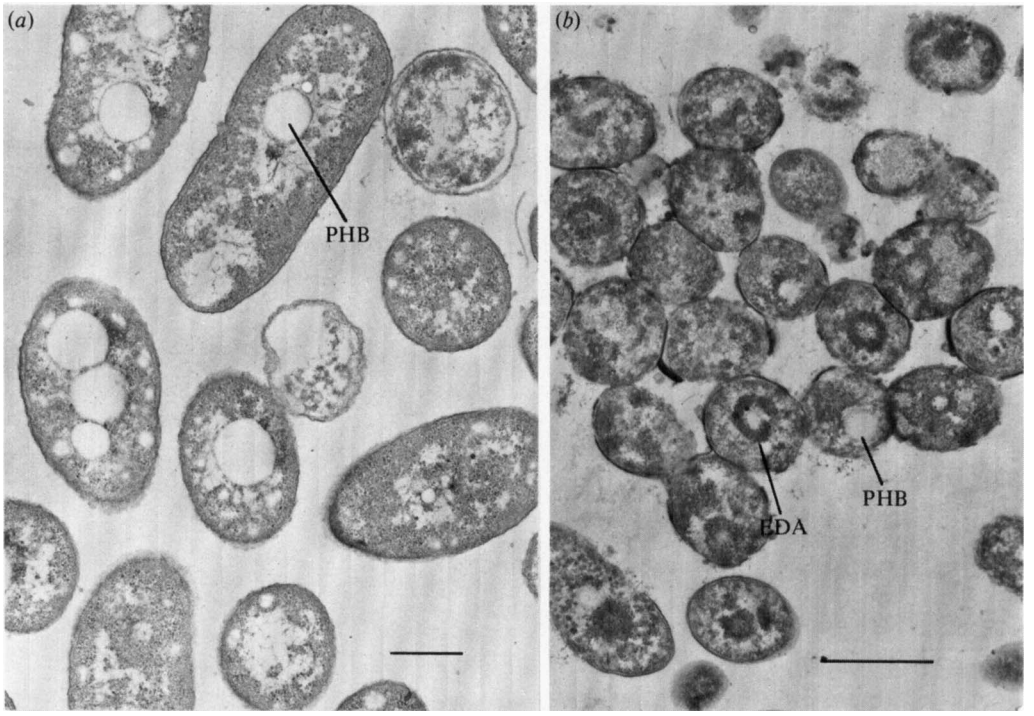

Fig. 2. Electron micrographs of thin sections of control (*a*), and cadmium-adapted (*b*) mid-exponential-phase *P. putida* cells. Poly-β-hydroxybutyrate (PHB) granules are present in both types of cell, but electron-dense aggregates (EDA) only in the cadmium-adapted cells. Cadmium-adapted cells form chains and clusters. Bars, 0·5 μm.

spectroscopy and found to contain 8·56 mg Cd per g polyphosphate, representing 15% of the total cadmium content of lag-phase cells. The number of polyphosphate granules peaked at 3–4 per cell after 3 h incubation.

Control cells after 1·5 h incubation contained a similar number of polyphosphate granules as mid-lag-phase cadmium-adapted cells. However, they showed no signs of membrane disruption, nor did they show the clustering, nor the presence of electron-dense aggregates, that were found for exponential-phase cadmium-adapted cells (see below). A few control cells did have very small blebs (<5 nm diameter) on the membrane near the mid-point of the cell perimeter.

Morphology of late-exponential-phase cells

Towards the end of the exponential phase, cadmium-adapted cells were approximately half the size of control cells, and were almost spherical. Cadmium-adapted cells were non-motile. Both control and adapted cells contained deposits of poly-β-hydroxybutyrate (identified after extraction and hydrolysis to crotonic acid, and confirmed by proton NMR). Cadmium-adapted cells had accumulated only one-third as much of this storage polymer as control cells.

Exponential-phase cadmium-adapted cells contained electron-dense aggregates (Fig. 2*b*), many of which appeared as rings with an electron-transparent centre. These cells showed thickened areas of membrane and were joined together in long chains and clusters (Fig. 2*b*). Close examination of electron micrographs suggested that these cells had not divided completely. Similar features were also observed in cadmium-adapted cells grown in medium containing 1 mM-Cd^{2+} in the absence of added Zn^{2+}. Control cells showed none of the latter features (Fig. 2*a*).

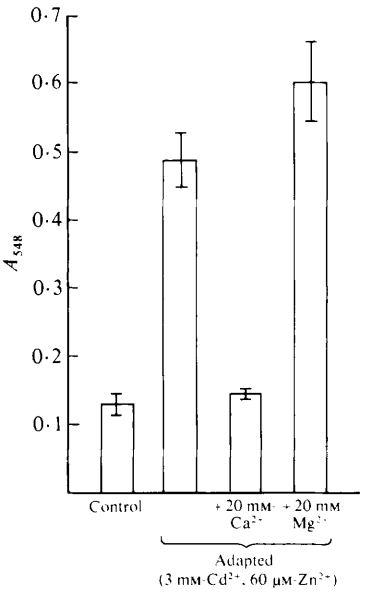

Fig. 3

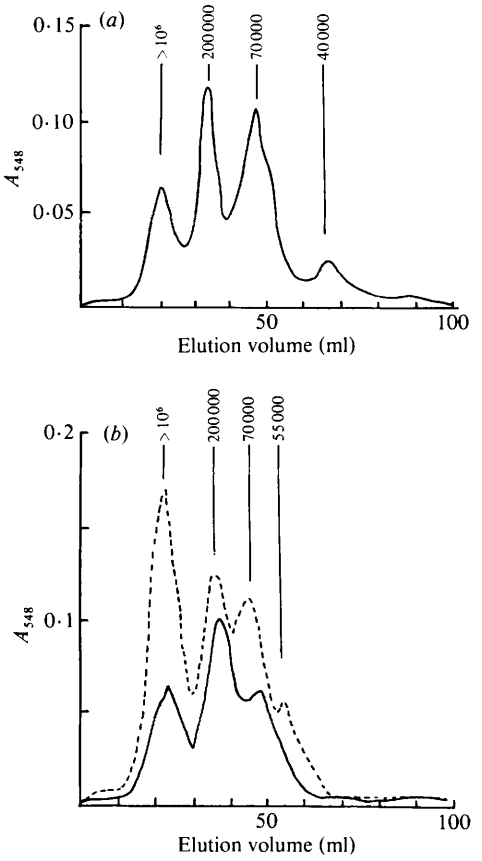

Fig. 4

Fig. 3. Effect of Cd^{2+}, Mg^{2+}, and Ca^{2+} on the release of LPS from lag-phase cells as measured by the absorbance of KDO at 548 nm. Zn^{2+} was added to promote optimum growth of cadmium-adapted cells as described previously (Higham *et al.*, 1985). The results are means of three determinations; the bars indicate the range.

Fig. 4. Sepharose 4B elution profiles of (*a*) LPS from the culture medium of cadmium-adapted cells, and (*b*) LPS isolated from the walls of control (-----) and cadmium-adapted (——) cells. The column was calibrated with the following M_r standards: carbonic anhydrase (30000), bovine serum albumin (67000) and β-galactosidase (110000).

Release of LPS and protein induced by Cd^{2+}

The electron micrographs suggested that Cd^{2+} had perturbed the membrane structure, with the consequent release of membrane fragments into the medium. The release of LPS, which is found almost exclusively in the outer membrane of Gram-negative cells (Nikaido & Nakae, 1979) was examined using chemical tests for KDO as a marker for LPS. Although some LPS was released by control cells, more than three times as much was released by cadmium-adapted cells (Fig. 3). The addition of 20 mM-Ca^{2+} to cadmium-adapted cells reduced the release of LPS to almost the control level, whereas the addition of 20 mM-Mg^{2+} slightly increased LPS release. The release of LPS was not a result of autolysis, as the viable cell count did not alter significantly over the time course of the experiment (6 h).

The material released into the medium was further characterized by gel filtration chromatography. Elution profiles of LPS were compared with those of LPS extracted from whole cells. Four major LPS fractions were separated from the medium (Fig. 4*a*). The LPS released into the medium was of similar composition to that extracted from the cadmium-

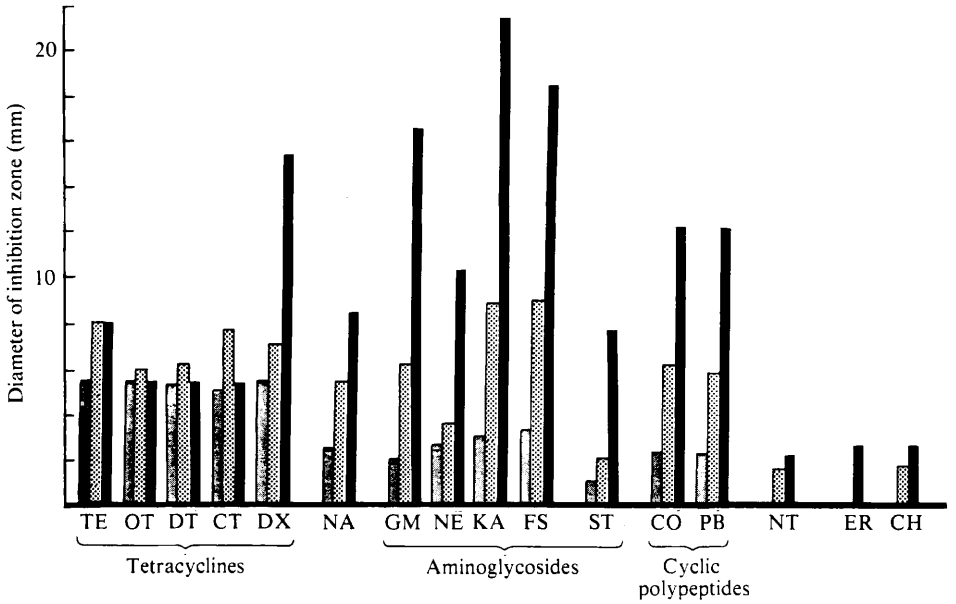

Fig. 5. Effect of Cd^{2+} on the sensitivity of *P. putida* towards antibiotics, measured as the diameter of the zone of inhibition of bacterial growth on 1·5% Difco-Bacto Agar. ▭, Control cells; ▨, cadmium-adapted cells; ■, cadmium-adapted cells cultured on Difco-Bacto Agar plus 3 mM-Cd^{2+} and 60 μM-Zn^{2+}. Antibiotics: TE, tetracycline; OT, oxytetracycline; DT, demethylchlortetracycline; CT, chlortetracycline; DX, doxycycline; NA, nalidixic acid; GM, gentamicin; NE, neomycin; KA, kanamycin; FS, fusidic acid; ST, streptomycin; CO, colistin; PB, polymyxin B; NT, nitrofurantoin; ER, erythromycin; CH, chloramphenicol. The values are means from three experiments.

adapted cells themselves (Fig. 4*b*), except that it contained an additional low-M_r (40000) peak (Fig. 4*a*). The elution profile of LPS extracted from control cells was very different, showing a much larger proportion of high-M_r LPS (Fig. 4*b*).

Permeability of control and cadmium-adapted cells to antibiotics

Control cells were resistant to penicillins, cephalosporins and erythromycin but sensitive to tetracyclines, aminoglycosides and cyclic polypeptides. Cadmium-adapted cells showed slightly increased sensitivity to aminoglycosides and cyclic polypeptides when exposed to these antibiotics even in the absence of Cd^{2+} (Fig. 5) and in addition were sensitive to chloramphenicol and nitrofurantoin. In the presence of 3 mM-Cd^{2+}, adapted cells showed increased sensitivity to aminoglycosides, cyclic polypeptides, nitrofurantoin and chloramphenicol, and were now also sensitive to erythromycin, to which they were previously resistant. The greatest increase in sensitivity was towards the aminoglycosides, cyclic polypeptides and, anomalously, doxycycline (Fig. 5). There was only a small or no increase in sensitivity towards the other tetracyclines. The sensitivity of adapted cells was five- to sixfold greater than that of control cells towards erythromycin, chloramphenicol or kanamycin. Cadmium-adapted cells were, in contrast, more resistant to ampicillin.

DISCUSSION

Immediately after inoculation into fresh Cd^{2+}-containing medium, cadmium-adapted *P. putida* cells take up a large amount of Cd^{2+}. Intracellular concentrations of approximately 16 mM are reached. These decrease gradually during the lag phase (Higham *et al.*, 1985). Atomic absorption measurements have indicated that most of the cell-bound Cd^{2+} is located in the cell envelope and polyphosphate granules, and that some Cd^{2+} is also bound to nucleic acid (Pawlett, 1983). The increase in surface area produced by blebbing of the outer membrane may increase the number of binding sites for Cd^{2+} during the lag phase. Polyphosphate granules

provide another important site for Cd^{2+} binding during the lag phase and binding of Cd^{2+} by both of these cell components may be critical for cell survival after inoculation.

Towards the end of the lag phase, the polyphosphate may be utilized as a source of inorganic phosphate for metabolism in the dividing cells. At this stage, the Cd^{2+} bound to polyphosphate is relocated, perhaps to cadmium-binding proteins (Higham *et al.*, 1984). The electron-dense aggregates observed during the late lag phase and exponential phase of Cd^{2+}-adapted cells may represent polyphosphate granules which have not been fully degraded.

During the lag phase, over 50% of the cellular cadmium content is located in the cell envelope (Pawlett, 1983). The high concentration of Cd^{2+} in the cell envelope may induce some of the observed changes in membrane structure (blebbing), loss of LPS and increased permeability. Similar effects have been observed in cells exposed to cyclic polypeptides such as polymyxin B (Storm *et al.*, 1977), EM49 (Rosenthal *et al.*, 1976) and PMBN (Vaara & Vaara, 1983), and also in LPS-defective ('rough') mutants (Irvin *et al.*, 1975). Gram-negative bacteria can also be rendered permeable to antibiotics by the action of EDTA (Eagon & Carson, 1965). However, the blebs of cadmium-adapted cells were much larger than those which have been observed on cells exposed to the latter agents. It is difficult to rule out the possibility that the blebs are artifacts produced during fixation of Cd^{2+}-weakened membranes, although polymyxin-induced blebs have been shown to be genuine in freeze-etched preparations (Schindler & Teuber, 1975). Small blebs occurring near the point of cell division, like those that we observed on control cells, have frequently been described (Burdett & Murray, 1974). Also we cannot rule out the possibility that the effects of Cd^{2+} are synergistic with those of Tris in the medium. Tris is known to permeabilize outer membranes, but does not itself cause blebbing (Hancock, 1984).

The release of LPS and protein from Cd^{2+}-adapted cells may represent the release of whole fragments of membrane (as blebs?), leaving lesions or holes in the membrane which could account for the increased permeability of the cells. The cell envelopes of these cells contained less LPS than those of control cells. In rough mutants, the degree of permeability has been correlated with the extent of the lesion in the LPS (Sanderson *et al.*, 1974; Meadow, 1975). Anomalously, cadmium-adapted *P. putida* cells were more resistant to ampicillin than control cells. Increased resistance towards penicillin and ampicillin has also been observed in 'rough' mutants of *Salmonella typhimurium* (Roantree *et al.*, 1969). Since the β-lactam sensitivity tests on cadmium-adapted cells were done in the absence of Cd^{2+}, it is possible that some of the increased sensitivity occurs during reversion to normal growth, which takes place within a few generations (Higham *et al.*, 1985).

Loss of LPS and protein from cadmium-adapted cells was reduced by Ca^{2+} but not by Mg^{2+}. This suggests that, like EDTA, and cationic antibiotics (Schindler & Osborn, 1979), Cd^{2+} interferes with the binding of divalent metal ions in the outer membrane. Unlike these agents, Cd^{2+} may interfere specifically with Ca^{2+} binding. Leive (1974) has postulated that divalent cations (Ca^{2+}, Mg^{2+}) form ionic bridges between neighbouring LPS phosphate groups, stabilizing the outer membrane and establishing the principal permeability barrier. Cd^{2+} may disrupt the packing of membrane lipids by competitively displacing Ca^{2+} ions which normally function as such cationic bridges. Cd^{2+}-induced lesions in the LPS may induce the synthesis of new membrane. An over-production of membrane may give rise to blebs, loops of membrane and the structures which appear to be filled with membranous material. The tendency of Cd^{2+}-adapted cells to grow in chains may indicate an impairment of the separation mechanism. This may also be a reflection of an altered outer membrane structure induced by Cd^{2+}. Alternatively Cd^{2+} may inhibit autolysins involved in cell separation. It is interesting that no chains of cells were evident only 1 h after inoculation into fresh Cd^{2+}-containing medium.

In conclusion, cadmium resistance in *P. putida* involves both decreased cadmium transport (Higham *et al.*, 1985) and binding of cadmium by four major cell components: cell envelope, polyphosphate granules, nucleic acid, and three cadmium-binding proteins (pseudothioneins). It is not yet clear whether these characters are plasmid-mediated; this is being investigated. The high intracellular Cd^{2+} concentration does, however, appear to interfere with much of the normal cell metabolism, including the degradation of polyphosphate granules, the accumulation of poly-β-hydroxybutyrate and the completion of cell separation.

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