Aluminum Elicits Exocellular Phosphatidylethanolamine Production in *Pseudomonas fluorescens*

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Pseudomonas fluorescens ATCC 13525 was found to grow in a minimal mineral medium supplemented with millimolar amounts of aluminum, a known environmental toxicant. During the stationary phase of growth, the trivalent metal was localized in a phosphatidylethanolamine (PE)-containing residue. The concentration of PE in pellets ranged from 1.7 to 13.9 mg ml of culture⁻¹ in media supplemented with 1 to 30 mM aluminum. Although the gelatinous residue was observed during the stationary phase of growth, ultracentrifugation and dialysis experiments revealed that PE was produced from earlier stages of incubation and was associated with aluminum. A sharp diminution in the levels of PE and aluminum in the spent fluid was concomitant with the formation of the insoluble deposit. The aluminum content of the soluble cellular fraction increased during growth and reached an optimum of 1.85 mM of test metal at 45 h in cultures with 15 mM aluminum. Further incubation, however, led to a marked decrease in the cellular aluminum content, and during the stationary phase of growth, only trace amounts of the trivalent metal were detected in this fraction. When 45-h cells were incubated in fresh citrate medium, most of the intracellular aluminum was secreted in the spent fluid and citrate was rapidly consumed. Aluminum efflux was also observed in cultures in which D-glucose was substituted for citrate. However, no efflux of this trivalent metal was evident in media devoid of either citrate or D-glucose. Scanning electron microscopic studies and X-ray energy-dispersive analyses of the dialyzed supernatant aided in the visualization of nodule-like aluminum- and phosphorus-rich bodies associated with thread-like carbon-, oxygen-, and phosphorus-containing structures. Transmission electron microscopic and electron energy loss spectroscopic analyses revealed the presence of aluminum within bacteria after 45 h of incubation. Cells harvested after aluminum insolubilization did not show aluminum inclusions. This aluminum-tolerant microbe may have potential application in bioremediation processes.

Aluminum is the most abundant metal and constitutes approximately 8% of the earth's crust. It occurs predominantly as insoluble deposits of silicon, the second most common element. This chemical association sharply reduces the availability of this trivalent element throughout the biological world. However, acid rain and anthropogenic activities have led to a sharp increase in the solubilization of aluminum. This phenomenon has been further compounded by the introduction of aluminum into food products, pharmaceuticals, and water treatment (11, 13). Heightened exposure to aluminum has become a major cause of concern, as this trivalent metal is known to be toxic to numerous organisms. A considerable body of evidence implicates aluminum in a variety of complications, such as abnormal development in fish, retardation of root growth, defective eggshell formation, and some neurological disorders (13, 19).

Although prokaryotes may be affected by fluxes in environmental factors, they are known to harbor adaptive features that enable them to circumvent many of these challenges. The mechanisms underlying the resistance of microbes to a variety of metals have been studied, but few reports of the strategies evoked in response to aluminum stress exist (15). As part of our studies to evaluate the impact of metals on living systems, we have identified a minimal mineral medium, with citrate as the sole source of carbon, in which the soil bacterium *Pseudomonas fluorescens* readily multiplies. This culture has afforded a unique system to probe metal-microbe interactions. Re-

cently, we reported the ability of this microbe to tolerate up to 50 mM aluminum (8). In this study, we demonstrate that this tolerance to aluminum is mediated by the deposition of a gelatinous phosphatidylethanolamine (PE)-containing residue in which most of this trivalent metal is sequestered. The role of intracellular aluminum, its secretion, and its eventual deposition are also discussed.

MATERIALS AND METHODS

All chemicals were reagent grade. Folin Ciocalteu phenol reagent, serum albumin, Victoria Blue reagent, and PE were from Sigma Chemical Co. The

FIG. 1. Distribution of aluminum at various growth intervals in cultures with 15 mM aluminum. (Standard deviations ranged from 0.06 to 1.56.)

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FIG. 2. (A) PE and aluminum concentrations in pellets isolated by centrifugation ($159,000 \times g$ for 3.5 h) of spent fluid at various incubation times (cultures with 15 mM aluminum). (Standard deviations ranged from 1.10 to 2.93.) (B) PE and aluminum concentrations in dialysates (MWCO of 3.5 kDa) from spent fluid at different growth intervals. (Standard deviations ranged from 1.16 to 3.42.)

Bradford assay kit and citrate determination kit used were from Bio-Rad and Boehringer, respectively.

Microbial culture conditions and growth measurement. The bacterial strain *P. fluorescens* ATCC 13525, an organism known for its nutritional versatility and biotechnological potential (24), was obtained from the American Type Culture Collection (Rockville, Md.). It was maintained and grown in a mineral medium that contained Na_2HPO_4 (6.0 g), KH_2PO_4 (3.0 g), NH_4Cl (0.8 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g), and citric acid (4.0 g/liter of deionized distilled water). Trace elements were present as previously described (4). Aluminum chloride in concentrations ranging from 0.1 to 30 mM was complexed to the tricarboxylic acid prior to sterilization. The pH of the medium was adjusted to 6.8 with dilute NaOH. Media were dispensed in 200-ml amounts in 500-ml Erlenmeyer flasks and inoculated with 1 ml of stationary-phase cells grown in a medium unamended with the test metal. Cultures were aerated on a gyratory water bath shaker, model G76 (New Brunswick Scientific), at 26°C at 140 rpm. At various growth intervals, microbial multiplication was measured by monitoring solubilized bacterial protein by the method of Lowry et al. (20). Harvested cells were treated with 0.5 M NaOH, and bovine serum albumin was used as the standard. The pH of the spent broth devoid of bacteria was monitored with a model 610A pH meter (Fisher). Citrate was assayed enzymatically (22).

Aluminum analyses. At various growth intervals, 100 ml of bacterial culture was centrifuged at $10,000 \times g$ at 4°C for 20 min to afford a pellet and a supernatant. The bacterial pellet was sonicated in order to yield a soluble fraction and cell debris. The aluminum in all three components was then analyzed by aluminum assay (14). The gelatinous residue that appeared during the later stages of growth was isolated by spinning the culture broth at $100 \times g$ at 4°C for 30 min. This insoluble deposit was suspended in distilled water and spun at $100 \times g$ in order to ensure a microbe free pellet. This process was repeated twice. Light microscopy and protein analyses confirmed a bacterium-free aluminum deposit. After hydrolysis in 6 M HCl, the aluminum and phosphate contents were measured colorimetrically (12, 14). The presence of aluminum and phosphorus in the freeze-dried residue was further analyzed by X-ray fluorescence spectroscopy on a Philips PW1404 automatic sequential spectrometer as previously described (3), with PX₁ crystal used as the analyzing crystal (25). The supernatants obtained at different incubation intervals were centrifuged at $159,000 \times g$ for 3.5 h and/or dialyzed extensively against distilled water in a membrane with a molecular mass

cutoff (MWCO) of 3.5 kDa. After acidic hydrolysis, the aluminum contents in the pellet and dialysate were monitored colorimetrically. The gelatinous pellet isolated at 75 h of growth in media with various concentrations of aluminum was also hydrolyzed and analyzed for aluminum and phosphate with colorimetric reagents.

Lipid analyses. As growth progressed, aliquots of 20 ml of spent fluid were centrifuged at $159,000 \times g$ for 3.5 h. The pelletized product was extracted with a mixture of $\text{CH}_3\text{OH}-\text{CHCl}_3-\text{H}_2\text{O}$ (2:1:0.8). Lipids were placed as spots on thin-layer silica gel plates (Whatman, Kent, England) and resolved by ascending chromatography using a $\text{CHCl}_3-\text{CH}_3\text{OH}-\text{NH}_4\text{OH}$ (65:25:5 [vol/vol]) mixture. Lipids were visualized with I_2 vapor, ninhydrin, and molybdate reagents (17). Phospholipids in these samples were quantitated with the dye Victoria Blue R (10). PE was used as the standard. Similar experiments were performed with the dialysate from the membrane with an MWCO of 3.5 kDa. The PE from the pellet isolated at 75 h in cultures with various concentrations of aluminum was also extracted and analyzed.

Aluminum efflux assays. Cells grown in minimal mineral medium supplemented with 15 mM aluminum for 45 h were centrifuged ($10,000 \times g$ for 10 min) and washed twice with 0.85% NaCl. Cells amounting to 16 mg of solubilized protein were inoculated in a fresh citrate medium devoid of aluminum for up to 8 h, and the aluminum in the spent fluid was analyzed colorimetrically at timed intervals. Citrate utilization was monitored by enzyme-coupled assay (22). Similar bacterial inoculations were performed in fresh medium without citrate and in a medium in which D-glucose was used in lieu of citrate.

Electron microscopic studies. The supernatants isolated during the late logarithmic phase of growth from both test and control cultures were dialyzed in membranes each with an MWCO of 3.5 kDa. After lyophilization or sequential drying with ethanol, these samples were analyzed with a model 6400 scanning electron microscope (JEOL) equipped with a TN5500 energy-dispersive X-ray microanalytical system. Similar experiments were performed with control cultures. Bacteria harvested at various incubation periods were washed twice with 0.85% NaCl solution and were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2). Postfixation was achieved in 1% osmium tetroxide dissolved in 0.1 M phosphate buffer. After being washed twice with double-distilled water, samples were further fixed, stained in 2% uranyl acetate, and embedded in 2% agar. The solidified agar was sliced into small blocks, dehydrated in a series of alcohol, embedded in Epon 812 resin, and cured at 60°C for 36 h. Thin sections were examined with or without further lead citrate staining by electron energy loss spectroscopy in a Zeiss 902A transmission electron microscope. All experiments were performed three times and in duplicate.

RESULTS

P. fluorescens cells multiplied in minimal mineral medium supplemented with millimolar amounts of aluminum. In most of the cultures examined, aluminum was deposited as an insoluble gelatinous mass during the later stages of growth. However, during the initial growth period, most of the aluminum was localized in the supernatant, even though citrate was rapidly consumed. In the 15 mM culture, the insoluble aluminum deposit was initially reported at 35 h of bacterial growth. Further incubation led to more insolubilization of this metal, and in the 80-h culture, 65.1% of the soluble aluminum was sequestered as a gelatinous residue. The concentrations of aluminum in bacterial cells after sonication were also analyzed. No significant amounts of this trivalent element were observed

TABLE 1. Aluminum stress and exocellular PE production in *P. fluorescens*^a

Concn (mM) of Al^{3+} in medium	Pellet wt (mg/ml of culture)	Total PE (mg) ^b	Aluminum (μmol/mg [wet wt] of pellet)
0.0	Nil	Nil	Nil
0.1	Nil	Nil	Nil
1.0	5.67	0.34	0.07
3.0	8.81	1.32	0.14
7.5	14.9	2.11	0.24
15.0	32.9	2.38	0.30
30.0	73.8	2.78	0.33

^a Data are the means of three experiments; standard deviations ranged from 0.7 to 2.12.

^b In pellet from 200-ml culture.

FIG. 3. Scanning electron micrograph of dialysate of spent fluid at 35 h from cultures with 15 mM aluminum. (Note the rounded bodies rich in aluminum and the thread-like structures rich in carbon.) Bar, 10 μ m.

in either the cell debris or the soluble cellular fraction during the early stages of incubation. However, as growth progressed, a marked increment in aluminum in the soluble cellular component was recorded. After 45 h of incubation, 12% of the aluminum found in the medium was localized in the soluble cellular fraction. Further incubation was characterized by a diminution in the cellular aluminum level, and at 75 h of growth, less than 1% of the aluminum was detected in the soluble cellular fraction (Fig. 1).

The fate of the soluble aluminum in the culture medium was analyzed by ultracentrifugation and dialysis. At various growth intervals, the spent fluid was examined for phospholipid and aluminum after centrifugation ($159,000 \times g$ for 3.5 h) and/or dialysis (membrane with an MWCO of 3.5 kDa). In the spent fluid isolated at 18 h of growth, the pellet harvested by ultracentrifugation was found to contain both aluminum and phospholipid. The amounts of aluminum and phospholipid increased up to 45 h of incubation when concomitant decreases in these moieties were seen in the pellet isolated by centrifugation at $159,000 \times g$. This diminution was characterized by the formation of the gelatinous residue isolated by centrifugation at $100 \times g$. The lipid extract of the pellet was examined by thin-layer chromatography. A major band with an R_f value of 0.42 that responded positively to ninhydrin and ammonium molybdate sprays was observed. This spot was found to comigrate with the standard PE. Similar aluminum and PE profiles were obtained in the dialysates of spent fluid harvested after various growth periods, indicating an association between PE and this trivalent metal as growth progressed (Fig. 2).

The nature of the gelatinous residue in cultures with various concentrations of aluminum isolated at 75 h of incubation was also examined. The weight of the precipitate ranged from 5.9

to 70.3 mg ml of culture⁻¹ in media with 1 to 30 mM aluminum, respectively. No precipitate was observed in the medium with 100 μ M aluminum. The percentage of aluminum immobilized in the pellet ranged from 38 to 80% in cultures with 1 to 30 mM aluminum, while the amount of extractable PE was found to vary from 1.7 to 13.9 μ g ml of culture⁻¹ in media with 1 to 30 mM aluminum (Table 1). When preconditioned cells, i.e., bacteria already exposed to 15 mM aluminum, were inoculated in fresh medium, improved rates of growth and aluminum insolubilization were observed (data not shown). The hydrolyzable phosphate and aluminum from the lipid-free pellet in cultures with 1 to 30 mM aluminum occurred approximately in a 1:1 ratio.

The soluble aluminum retained in the dialyzing membrane with an MWCO of 3.5 kDa was subjected to scanning electron microscopic studies. Carbon-, oxygen-, and phosphorus-rich filament-like structures were found associated with irregularly shaped bodies consisting of aluminum, phosphorus, and oxygen (Fig. 3). These features were absent from control cultures. Bacterial cells at different incubation times were visualized with a transmission electron microscope. The 45-h cells contained inclusions of aluminum-rich bodies in the cytoplasm (Fig. 4). These cytoplasmic aluminum-containing moieties did not appear in 60-h bacterial cells. When aluminum-containing cells were inoculated in fresh citrate medium, all the test metal was extruded in the spent fluid and citrate was rapidly utilized. After 1 h of incubation, no aluminum was detected within bacterial cells. Aluminum was also secreted in media in which D-glucose was the only carbon source. However, in fresh medium without citrate or D-glucose, no extrusion of aluminum was observed.

FIG. 4. Transmission electron micrograph of *P. fluorescens* cells isolated in a culture with 15 mM aluminum at 45 h. (Note the aluminum inclusions.) The intracellular bodies were shown to be aluminum rich by electron energy loss spectroscopy. Bar, 0.33 μm .

DISCUSSION

The foregoing evidence suggests that when challenged with aluminum complexed to its sole source of carbon, *P. fluorescens* elaborates exocellular PE. This moiety, which is associated with this trivalent metal from the early stages of growth, is involved in the formation of a gelatinous precipitate in which aluminum is eventually immobilized. Although the toxicity of this trivalent element is well documented, reports of tolerance mechanisms to aluminum are rare (9, 18). Aluminum binds to membrane components of cell walls and is also known to interfere with iron and calcium metabolism. Its positively charged species may also interact with nucleic acids, thus distressing cellular functions (16). To our knowledge, this study is the first demonstration of the possible involvement of PE in aluminum detoxification. Biotransformation, modifications in transport systems, and volatilization are some of the other defense strategies evoked by microbes subjected to high levels of metallic elements in their ecological niches (23). *P. fluorescens* ATCC 13525 has been shown to attain calcium and strontium homeostases by the elaboration of crystalline carbonate derivatives (5, 6). Yttrium and lead are detoxified as phosphorus residues (2, 7). Indium and gallium, trivalent elements like aluminum, are eliminated as a phosphorus derivative and an organic conjugate, respectively (1, 4).

Since the aluminum associated with PE was isolated from the spent fluid, it is quite likely that as citrate was consumed, this trivalent metal was processed for its removal from the earlier stages of growth. Only after the concentration of this trivalent metal or its organic complex associated with PE had

reached a certain critical point did spontaneous precipitation of these moieties occur. Indeed, the pH increment of the culture during growth and the presence of aluminum in the soluble component of cells indicate that the citrate complex was metabolized intracellularly. The phospholipid moieties of the inner membrane or the inner leaflet of the outer membrane may provide the nucleation sites of the metal or the metal-organic conjugate. As the concentration of the PE-containing component increased, precipitation occurred. The transmission electron micrograph depicting the aluminum inclusions in bacterial cells and their eventual disappearance on further incubation support such a postulation. The absence of lipopolysaccharide in the residue and spent fluid indicates that aluminum is not bound fortuitously but is indeed aided by PE in its immobilization. The involvement of synthetic lipid vesicles in metal deposition has previously been demonstrated (21). Furthermore, preconditioned cells harvested at the stationary phase of growth were found to grow in fresh aluminum-containing medium. In this instance, the trivalent element was also sequestered in a PE residue. All the observations in this study imply that PE is intricately involved in aluminum detoxification. In addition, the amounts of PE observed in the spent fluid increased with increases in the aluminum contents of media. The fact that cells containing aluminum readily secreted the test metal in fresh citrate or D-glucose medium but not in a medium devoid of citrate implies that this process is energy dependent.

Although further studies are needed to unravel the mechanisms of aluminum tolerance, it is tempting to propose that

after the uptake of aluminum, this trivalent metal or its organic conjugate is associated with PE. As the concentration of this aluminum-organic complex reaches a critical point, there is a phase change that leads to the insolubilization of the metal. Further studies aimed at the delineation of aluminum detoxification mechanism(s) are in progress in our laboratory.

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