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Aluminum Tolerance of *Pseudomonas fluorescens* in a Phosphate-Deficient Medium

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Although aluminum is the most widely occurring metal in the environment, it is not known to participate in the normal functioning of living systems. On the contrary, most organisms appear to have somehow circumvented the necessity of utilizing this trivalent metal (Lewis 1989). Hence, the presence of aluminum in foods and pharmaceuticals and its increased bioavailability triggered by acid rain have made this metal a potential hazard to the ecosystem. Indeed most reports implicate this metal in diverse cellular malfunctioning (Exley and Birchall 1992).

As part of our ongoing investigation on the impact of metals on living systems, we have uncovered the ability of the soil bacterium *Pseudomonas fluorescens* to tolerate up to 50 mM aluminum (Appanna et al. 1994). This resistance to aluminum is mediated by the elaboration of a gelatinous lipid-rich residue where most of the trivalent metal is sequestered at stationary phase of growth. The metal and phosphate appear to be present in approximately a 1:1 ratio. Since phosphate is an important ligand involved in the insolubilization of the toxic metal, the obvious question to explore is the essentiality of phosphate in aluminum tolerance. We report here on the influence of phosphate on aluminum detoxification in *Pseudomonas fluorescens*. The reduced, but substantial ability of this microbe to tolerate up to 5 mM aluminum in a phosphate-limited medium and the involvement of a soluble aluminum metabolite(s) in aluminum homeostasis are discussed. This finding is in sharp contrast to that observed in a phosphate-rich medium where most of the trivalent metal was sequestered in a gelatinous lipid-rich residue (Appanna et al. 1994).

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MATERIALS AND METHODS

The bacterial strain *Pseudomonas fluorescens* ATCC 13525 was obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). It was grown in a phosphate-deficient, mineral medium as described in Appanna and Huang (1992). Trace elements were present in the following concentrations: $FeCl_3.6H_20$ (2μM) $MnCl_2.4H_20$ (lμM), $Zn(NO_3)_2.6H_20$ (0.5μM), $CaCl_2$(1μM), $CoSO_4.7H_2O$(0.25μM), $CuCl_2.H_2O$ (0.1μM), and $NaMoO_4.2H_2O$ (0.1μM). Aluminum chloride in concentrations ranging from I to 10 mM was complexed to the tricarboxylic acid prior to sterilization. The pH of the medium was adjusted to 6.8 with dilute NaOH. The media were dispensed in 200 mL amounts in 500 mL Erlenmeyer flasks and inoculated with I mL of stationary phase cells grown in a phosphate-rich medium unamended with the test metal (Al-Aoukaty et al. 1992). The cultures were aerated on a gyratory water bath shaker model G76 (New Brunswick Scientific) at 26ºC at 140 rpm. At various time intervals, microbial multiplication was measured by monitoring solubilized bacterial protein by the method of Lowry et al. (1951). The harvested cells were treated with 0.5 M NaOH and bovine serum albumin was used as standard. The pH of the spent broth, devoid of bacteria, was monitored with the aid of a Fisher pH meter model 610A.

At various time intervals, 20 mL aliquots of bacterial cultures were centrifuged at 10,000xg. The pellets were then sonicated to yield soluble fractions and cell debris. These fractions and the clear supernatant fluids were then analyzed for aluminum by the aluminon colorimetric assay (Hsu 1963). The concentrations of citrate in the spent fluids from aluminum supplemented and control cultures were monitored at different incubation times with the citrate assay kit from Boehringer (Moellering and Gruber 1966).

The carbohydrate contents of supernatants were monitored at timed growth intervals by the phenol-sulfuric acid method (Dubois et al. 1956). D-glucose was the standard. Spent fluids were also analyzed for protein content by the methods of Lowry (1951) and Bradford (1976). At various periods of incubation, the supernatants were dialyzed in membranes with molecular weight cut off (MWCO) of 100 Da and I kDa. The dialyzates were examined for aluminum by the colorimetric assay. Supernatants from stationary phases of growth were fractionated for aluminum-rich components by molecular weight exclusion chromatography (Biogel-P2). All experiments were performed twice and in duplicate.

RESULTS AND DISCUSSION

Pseudomonas fluorescens multiplied in a phosphate-deficient medium and attained a cell yield of 290 μg of protein mL^{-1} of culture. In a phosphate-rich medium, the cellular yield was 485 μg mL^{-1} of culture. Inclusion of I mM, 2 mM, 3 mM and 5 mM aluminum appeared to have only a slight negative impact on growth rate. There was approximately a 14% diminution in cell yield in media containing I mM, 2 mM and 3 mM aluminum. In cultures supplemented with 5 mM aluminum, the bacterium experienced a 23% reduction in cell mass at stationary phase of growth (Fig. 1). No bacterial growth was recorded in media with 10 mM aluminum. Citrate was rapidly utilized in all growing cultures. In media with 2 mM and 5 mM aluminum, all the citrate was consumed within 60 hr of incubation. In the control medium all the citrate was consumed within 38 hr (Fig. 2). Examination of aluminum at various growth intervals revealed that most of the trivalent metal was localized in soluble form(s) in the spent fluid at stationary phase of growth. The cell debris accounted for 10% of the aluminum, while the soluble cellular components contained only minute amounts of the trivalent metal (Figs. 3a, 3b). The exocellular carbohydrate and protein contents did not appear to vary significantly in metal-supplemented and control media. The carbohydrate level ranged from 35.3 to 43.4 μg of glucose equivalent mL^{-1} of culture, while the protein concentration was 98.1 to 124.8 μg mL^{-1} at stationary phase of growth. The pH of the spent fluid increased in all cultures, and at the cessation of cellular multiplication the pH varied between 8.2 - 8.9.

Chromatographic studies on the supernatant isolated at stationary phase of growth indicated that aluminum was associated with metabolite(s) with molecular weights in the range of 400-700 Da. This observation was further confirmed by dialysis experiments. Aluminum-rich dialyzate was retained in a membrane with MWCO of 100 Da even though no citrate was detected. No aluminum-rich dialyzate was obtained in a membrane with MWCO of 1 KDa (Fig. 4). The foregoing data demonstrate the reduced, but substantial ability of *Pseudomonas fluorescens* to survive in a phosphate-deficient medium supplemented with millimolar amounts of aluminum. In contrast to the phosphate-rich medium where the microbe was able to tolerate up to 50 mM aluminum (Appanna et al. 1994), the bacterium in the present study failed to grow in 10 mM aluminum. In the former medium, tolerance was mediated by the deposition of a gelatinous lipid-rich residue; in the current study, aluminum appeared to be mobilized in soluble component(s) in the spent fluid.

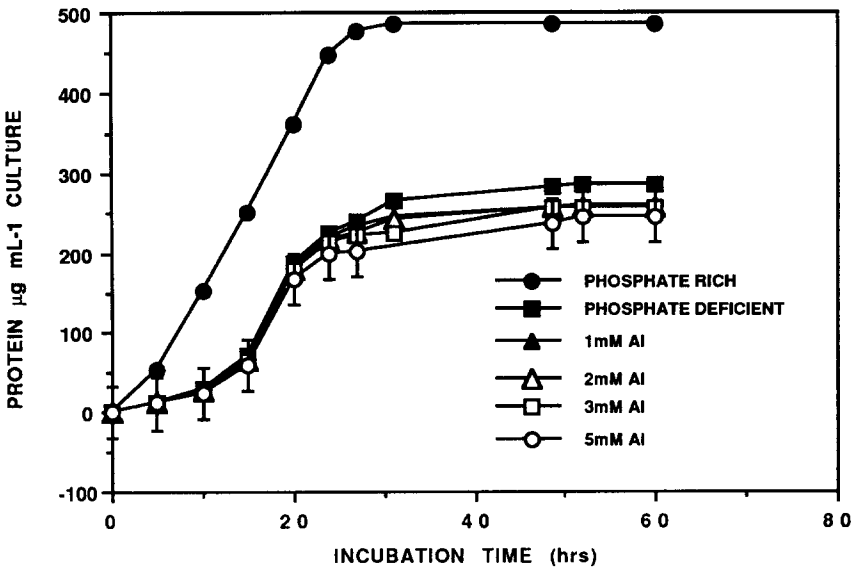

Figure 1. Growth profile of Pseudomonas fluorescens cultured in phosphate deficient medium with varying concentrations of aluminum.

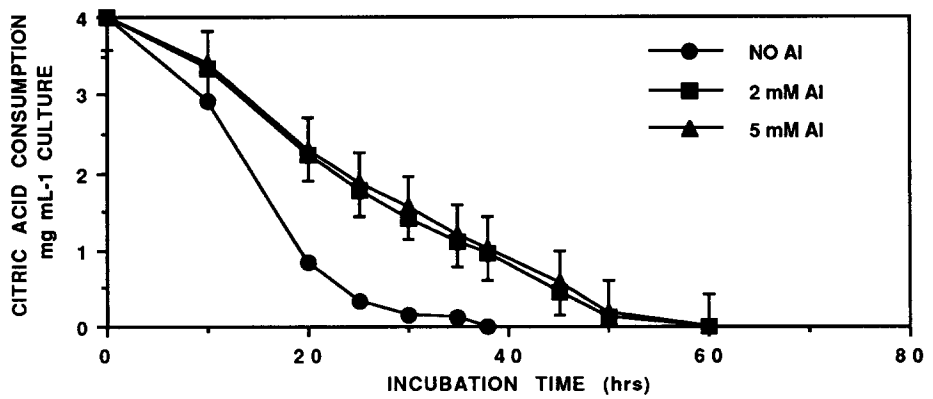

Figure 2. Citrate utilization by Pseudomonas fluorescens in medium supplemented with aluminum.

Aluminum is known to be toxic and has been reported to interact with nucleic acids (Johnson and Wood 1990); thus, its detoxification is essential for bacterial multiplication.

Insolubilization of metal is an important strategy initiated by this organism to circumvent the presence of abnormal levels of metals in its surroundings. Calcium and strontium evoke the biogenesis of crystalline carbonate derivatives (Anderson et al. 1992, Anderson

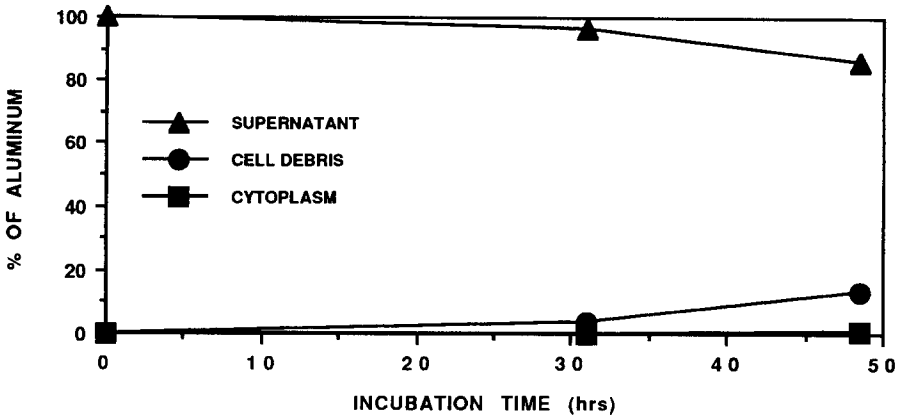

Figure 3a. Aluminum distribution in cultures with 2 mM aluminum.

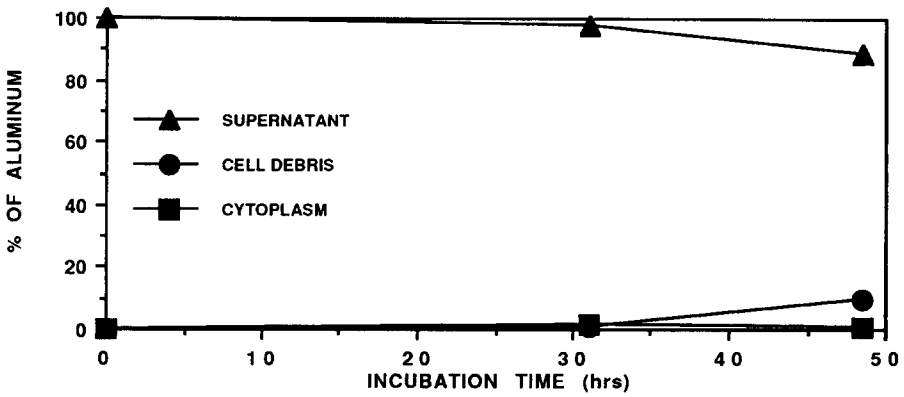

Figure 3b. Aluminum distribution in cultures with 5 mM aluminum.

and Appanna 1994). Lead, yttrium and indium are deposited as phosphorus-containing complexes in phosphate-rich cultures (Al-Aoukaty et al. 1991, 1992; Anderson and Appanna 1993). However, in phosphate-deficient media, lead and yttrium are associated within the bacterial cells (Appanna and Huang 1992) and the presence of 1 mM indium results in no microbial growth (Anderson and Appanna 1993).

Gallium, a metal that shares the same charge as aluminum and is known to mimic iron in vivo, arrested microbial multiplication when present in I mM amounts. However, inclusion of 25 μM iron in the medium reversed the toxic influence of gallium and it was immobilized in soluble form(s) in the spent fluid (Al-Aoukaty et al. 1992). The detoxification of iron in the same phosphate-deficient medium is attained by its precipitation as oxide derivative(s) associated with lipids (Appanna and Finn 1995). As the citrate was completely utilized and the pH increased during growth, it is

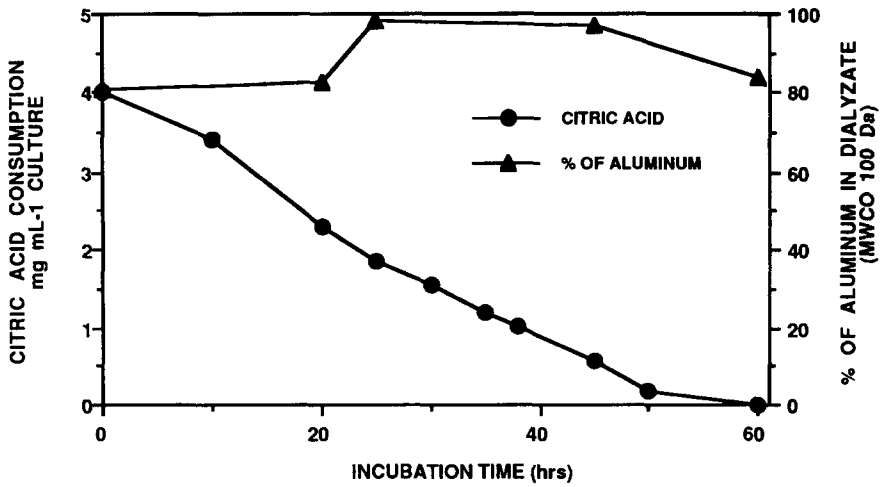

Figure 4. Biotransformation of the trivalent metal in cultures with 5 mM aluminum.

unlikely that aluminum is freed from the ligand exocellularly. Citrate and citrate-metal complexes are known to be transported in microbial systems (Harding and Royt 1990); thus it is quite conceivable that following its liberation from citrate via degradation or acidification, aluminum, a metal of no known biological function, is processed for its elimination as a soluble product(s) in the spent fluid. Furthermore, it is unlikely that aluminum would stay in solution at the pH of the spent fluid in the absence of a stabilizing molecule(s). Indeed, the dialysis and chromatographic data do argue for the presence of low molecular-weight, aluminum-binding metabolite(s). The physicochemical analyses of these moieties are in progress.

This report shows that the soil microbe *Pseudomonas fluorescens* can cope with abnormal, but limited, levels of aluminum even in media where phosphate is a limiting nutrient. However, the dearth of phosphate evoked a detoxification mechanism quite disparate from that observed in a phosphate-rich medium. Furthermore, this finding also illustrates: (a) the ability of this microbe to adapt to diverse environmental stresses, and (b) the susceptibility of aluminum-citrate complexes to microbial degradation even in phosphate-deficient surroundings.

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