Bull. Environ. Contam. Toxicol. (1996) 57:875-880

Bulletin of Environmental Contamination and Toxicology

Mechanism of Chromium Detoxification in *Pseudomonas fluorescens* Is Dependent on Iron

V. D. Appanna,[1] L. G. Gazsó,[2] J. Huang,[1] M. St. Pierre[1]

[1]Department of Chemistry and Biochemistry, Laurentian University, Sudbury, Ontario P3A 2C6, Canada
[2]Department of Radiobiology, Joliot-Curie National Research Institute for Radiobiology and Radiohygiene, Anna Str. S., P.O. Box 101, H-1221 Budapest, Hungary

Received: 4 March 1996/Accepted: 26 August 1996

Biotechnology may provide an efficient and environmentally friendly route to the management of industrial wastes. Microbes in particular, owing to their ability to proliferate in most ecological niches, can be engineered for the immobilization of metal pollutants. The utilization of chromium in steel production, wood preservation, leather tanning, paints and pigments has led to a sharp increase of this metal in the environment where it occurs primarily in trivalent or hexavalent forms (Frausto da Silva and Williams 1993). In trace amounts chromium is considered an essential nutrient for numerous organisms; in elevated concentrations it is toxic and mutagenic (Shen and Wang 1993).

As part of our study to evaluate the biochemical adaptation to abiotic-environmental stress (Appanna and Finn 1995; Appanna and St. Pierre 1994), we investigated the interaction of chromium (III) on the soil bacterium *Pseudomonas fluorescens*. This microbe, known for its nutritional versatility, was challenged by millimolar concentrations of the trivalent element complexed to citrate, a natural tricarboxylic acid, as the sole carbon source. The influence of iron (III) on the interaction between chromium (III) and the microbe was also examined in an effort to assess its suitability in metal waste management.

MATERIALS AND METHODS

Pseudomonas fluorescens ATCC 13525 was obtained from the American Type Culture Collection and was maintained at 4°C by monthly subculture on a mineral citrate medium solidified by the inclusion of 2% agar. The liquid culture contained Na_2HPO_4 (0.6g), KH_2PO_4 (0.3g), NH_4Cl (0.09g), $MgSO_4 \cdot H_2O$ (0.2g) and citric acid monohydrate (4.0g) per liter of deionized double distilled H_2O. Trace elements were added in concentrations as described in (Anderson et al. 1992). Iron(III) and chromium(III) were included in the test cultures as chlorides and were complexed to citrate prior to sterilization. The medium without added test metals served as a control. The pH of the media was adjusted to 6.8 with dilute NaOH.

Correspondence to: V. D. Appanna

The media were dispensed in 200 mL amounts in 500-mL Erlenmeyer flasks and inoculations were made with 1 mL of stationary-phase cells grown in the control medium. The cultures were aerated on a gyratory waterbath shaker model G76 (New Brunswick Scientific) at 26°C at 140 rev. min^{-1}. At timed intervals, cells were harvested by centrifugation at 10,000xg for 20 min at 4°C . Following the disruption of the cells with 0.5 *M* NaOH in boiling water, microbial growth was monitored by measuring soluble protein by the methods of Lowry et al. (1951) and Bradford (1976). Bovine serum albumin was used as the standard. The spent fluid devoid of bacterial cells was analyzed for its protein and carbohydrate contents, respectively (Bradford 1976; Dubois et al. 1956). The pH was recorded with the aid of a Fisher pH meter model 610A. Citrate utilization was assayed with citrate assay kit (Moellering and Gruber 1966).

At various periods of growth, the fate of chromium and iron was monitored by x-ray fluorescence analysis (XRF) on a Philips PW1404 automatic sequential spectrometer as described in Al-Aoukaty et al. 1992. The bacterial cells harvested at 10,000xg and the supernatant were lyophilized and examined by XRF. The pellet that appeared in the iron cultures following ultracentrifugation at 159,000xg for 3.5 hr was freeze-dried and analyzed. A methane/argon gas mixture was used in the flow proportional counter and a rhodium x-ray tube was employed throughout this investigation. The analyzing crystals were LiF 200 and PX_1. Intensities are given in kilocounts per second (kcps) and peak shifts in two theta degrees (20°). Lipids from the pellet were extracted with a mixture of CH_3OH, $CHCl_3$ and H_2O (2:1:0.8). The lipids were then placed as spots on thin-layer silica-gel plates (Whatman, Germany) and resolved by ascending chromatography using chloroform-methanol-acetic acid- water (25:15:4:2 vol/vol). The lipids were visualized with I, vapor, ninhydrin and molybdate reagents (Kates 1988).

At various timed intervals the supernatant obtained at 10,000xg was further spun at 159,000xg for 3.5 hr. The pellet obtained was then analyzed for its metal and lipid contents by the same methods as described before.

RESULTS AND DISCUSSION

Pseudomonas fluorescens was found to experience a 35% diminution in cell yield, as compared to the control, when cultured in a medium with 1 m*M* chromium; inclusion of iron did not appear to have a beneficial influence on growth rate and cellular yield (Fig. 1). Citrate, the sole carbon source to which the test metals were bound, was completely utilized albeit at different times. In the chromium-iron rich cultures, most of the tricarboxylic acid was consumed by 50 hr of growth (Fig. 2). The pH of the spent fluid increased and ranged in values from 8.1-8.9 in these experiments. There did not appear to be any marked variation in exocellular protein and carbohydrates in these cultures. The protein

concentration ranged from 42 to 58 µg mL^{-1} while the carbohydrate content varied between 55 and 75 µg mL^{-1}.

Even though most of the chromium (III) was located in soluble form(s) in the supernatant fluid at the stationary phase of growth, at 30 hr of growth 2.0 to 3.0% of this trivalent metal was observed in the soluble cellular component. The cell debris accounted for 2% of the chromium (data not shown). In the medium where the microbe was challenged with both chromium and iron, a lesser amount of chromium was localized within the bacterial cells at 30 hr of incubation. As growth progressed, the iron and chromium were immobilized in a gelatinous residue (Fig. 3). This precipitate contained peaks at 14.96 20°, 69.4 20° and 57.6 20° that are attributable to phosphorus (not shown), chromium and iron, respectively (White and Johnson 1970). The residue was found to contain a $CHCl_3/CH_3OH$- extractable component. Analysis of this extract by thin layer chromatography revealed a spot with a R_f value of 0.86 that comigrated with phosphatidylethanolamine (PE).

The presence of 1 m*M* chromium inhibited microbial multiplication and the trivalent metal was localized predominantly in soluble form(s) in the supernatant fluid. The inclusion of iron in the growth medium elicited a disparate detoxification mechanism. In this instance an insoluble pellet, isolated by ultracentrifugation, appeared to contain both of the trivalent metals. Since citrate, the sole source of carbon, was rapidly utilized, it is quite conceivable that citrate-metal complex(es) interacted directly with the bacterial cells. The increase of pH and the absence of a citrate lyase-like activity in the spent fluid would support such a possibility. Furthermore, the association of the test-metals within the bacterial cells and their eventual elimination also would indicate a direct interaction between the metals and the microbe.

Chromium (III) is known to be toxic and is usually excluded from entering the cells (Shen and Wang 1993). In this study, the chromium uptake may have arisen owing to the necessity of the microbe to acquire its carbon source. Indeed, Fe(III)-citrate complexes have been shown to be transported in *Pseudomonas* (Harding and Royt 1990). Although chromium accumulated predominantly in the spent fluid in the culture with iron (III), 30% of the chromium was immobilized as an insoluble residue. Most of the iron was insolubilized. It appears that the test metals were associated with PE from early stages of growth. The possible involvement of phospholipid in the mineralization of metals has been shown both in vivo and in vitro (Gorby et al. 1988; Archibald and Mann 1993). The detoxification of aluminum also appears to involve the participation of PE (Appanna et al. 1995). However, when the phosphate concentration is decreased by 100 fold, the aluminum is primarily sequestered in a soluble metabolite (Appanna and St. Pierre 1994). It is important to note that calcium and

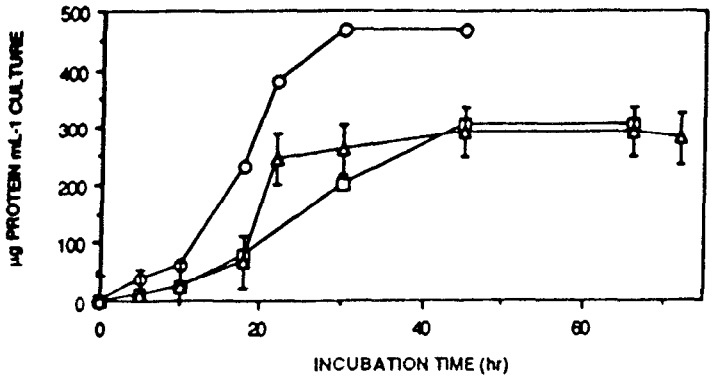

Figure 1. Growth profile of *Pseudomonas fluorescens* in control (○), chromium (□), and chromium and iron (Δ) cultures.

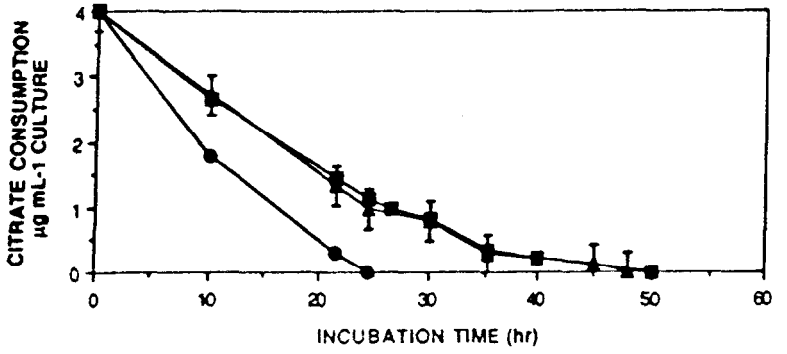

Figure 2. Citrate consumption by *Pseudomonas fluorescens* in control (●), chromium (■) and chromium and iron (Δ) cultures.

strontium evoke the biogenesis of crystalline carbonates (Anderson et al. 1992; Anderson and Appanna 1994).

This study shows that the response of *Pseudomonas fluorescens* is quite disparate when chromium (III) is the sole metallic stress and when it is present together with iron (III). Although further studies are necessary, this model may have potential application in the immobilization of chromium.

Acknowledgments. This research was supported by grants from the Ontario Ministry of Northern Development and Mines and a NATO Collaborative research grant (CRG No. 94.07.49).

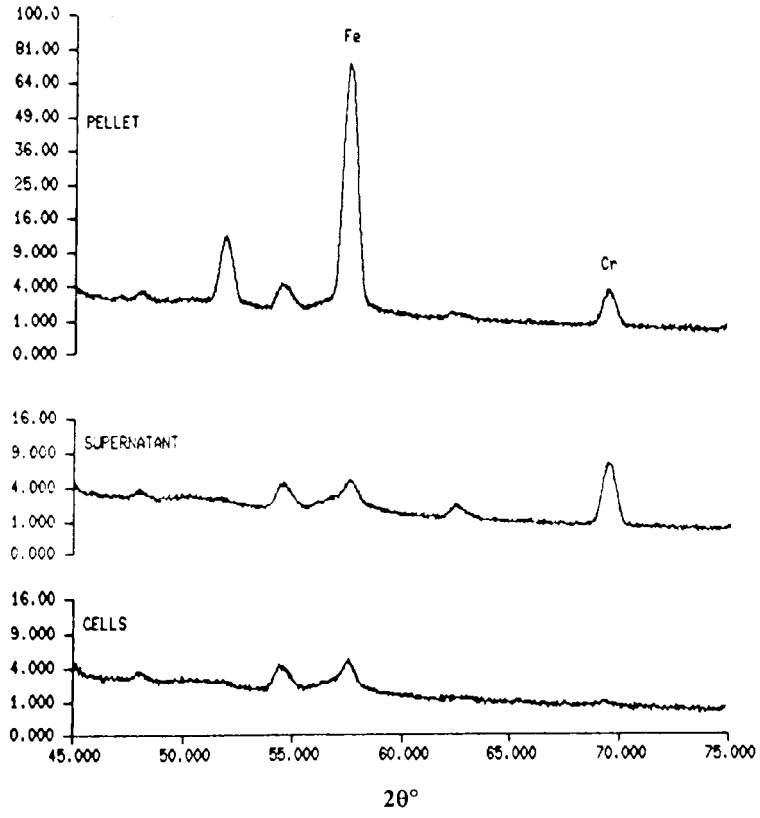

Figure 3. XRF analysis of pellet, supernatant and cells at stationary phase of growth in cultures with iron and chromium. Fifty ml of culture were centrifuged and analyzed in the three components.

REFERENCES

Al Aoukaty A, Appanna VD, Falter H (1992) Gallium toxicity and adaptation in *Pseudomonas fluorescens.* FEMS Microbiol Letts 92:265-272

Anderson S, Appanna VD (1994) Microbial formation of crystalline strontium carbonate. FEMS Microbiol Letts 116:43-48

Anderson S, Appanna VD, Huang J, Viswanatha TA (1992) A novel role for calcite in calcium homeostasis. FEBS Letts 308:94-96

Appanna VD, Finn H (1995) Microbial adaptation to iron: A possible role of phosphatidylethanolamine in iron mineral deposition. Biometals 8:142-148

Appanna VD, Huang J, Prusak-Sochaczewski E, St.Pierre M (1995) Microbial adaptation to aluminum. Biotechnol Prog 11:159-163

Appanna VD, St.Pierre M (1994) Influence of phosphate on aluminum tolerance in *Pseudomonas fluorescens.* FEMS Microbiol Letts 124:327-332

Archibald DD, Mann S (1993) Template mineralization of self-assembled anisotropic lipid microstructures. Nature 364:430-433

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein, utilizing the principle of protein-dye binding. Analyt Biochem 72:248-254

Dubois M, Gilles KA, Hamilton JK, Robers PA, Smith F (1956) Calorimetric method for the determination of sugar and related substances. Analytical Chem 28:350-356

Frausto da Silva JJR, Williams RJP (1993) The biological chemistry of the elements. Clarendon Press, Oxford

Gorby YA, Beveridge TJ, Blakemore R.P. (1988) Characterization of the bacterial magnetosome membrane. J Bacteriol 154:708-718

Harding RA, Royt RW (1990) Acquisition of iron from citrate by *Pseudomonas aeroginosa.* J Gen Microbiol 36:1959-1967

Kates M (1988) Techniques of lipidology, isolation, analysis and identification of lipids, 2nd ed. Elsevier Publication, New York

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275

Moellering H, Gruber W (1966) Determination of citrate with citrate lyase. Anal Biochem 17:369-376

Shen H, Wang Y-T (1993) Characterization of enzymatic reduction of hexavalent chromium by *Escherichia coli* ATCC 33456. Appl and Env Microbiol 59 11:3771-3777

White FW, Johnson GG (1970) X-ray and absorption wavelength and two theta tables, 2nd ed. American Society for testing and materials, Philadelphia