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A novel role for calcite in calcium homeostasis

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Calcium carbonate ($CaCO_3$) minerals are known to be deposited in a wide array of different organisms, ranging from microbes to vertebrates [(1989) On Biomineralization, Oxford University Press, New York]. Calcite, aragonite and vaterite are the major crystalline structural polymorphs of $CaCO_3$ associated with living systems, and participate in a variety of biological functions [(1989) Biomineralization: Chemical and Biochemical Perspectives, VCH Publishers, Weinham, Germany; (1991) Advances in Inorganic Chemistry 36, 137–200]. Here we report on the ability of a soil bacterium to synthesize calcite in a calcium-stressed environment. The elaboration of this exocellular crystalline residue enables the organism to regulate its calcium content. The attainment of calcium homeostasis via the exocellular deposition of bacterial calcite with unique crystal habits is a novel biological phenomenon.

Prokaryote; Calcium; Homeostasis; Calcite

1. INTRODUCTION

Calcium plays a pivotal role in numerous biological processes in both prokaryotes and eukaryotes [4,5]. The variation in cellular concentration of this divalent element provides the basis for calcium to act as a mediator for a vast array of biological activities. The appearance of the metal in the cytoplasm triggers biochemical and physiological reactions, and elevated levels of free calcium are known to cause irreversible damage to the cells [6,7]. Thus the maintenance of low intracellular concentrations of calcium is essential both for the survival of an organism and for this divalent metal to function as a secondary messenger. In mammalian cells cytoplasmic calcium is maintained to a level that is 10,000-fold lower than that of the extracellular environment. A wide array of mechanisms participate in attaining such a homeostatic condition. Ligand-gated channels, Ca^{2+}/ATP-ase and Na^+/Ca^{2+} antiporters are among some of strategies involved in calcium regulation. Intracellular liberation of this divalent metal via secondary messengers has also been reported [5,8]. Microorganisms have also evolved intricate extrusive mechanisms in an effort to preserve a constant low level of intracellular calcium. The cytosolic calcium in bacteria is kept as low as 90 nM, and ATP-driven calcium efflux appears to play a pivotal role in microbial calcium regulation [4,7]. Here we report that *Pseudomonas fluorescens* ATCC 13525 produces exocellular calcite to combat the presence of elevated amounts of calcium in its surroundings. The maintenance of innocuous levels of cytosolic calcium by the extracellular deposition of crystalline $CaCO_3$ is an uncommon occurrence.

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2. MATERIALS AND METHODS

The bacterial strain, *Pseudomonas fluorescens* ATCC 13535, was from the American Type Culture Collection, Rockville, MA, USA. The medium in which the microbe was grown at 26°C contained Na_2HPO_4 (0.06 g · l^{-1}), KH_2PO_4 (0.03 g · l^{-1}), $(NH_4)_2SO_4$ (1.0 g · l^{-1}), $MgSO_4.7H_2O$ (0.2 g · l^{-1}) and trace elements. Citrate (4 g · l^{-1}) was the sole source of carbon, and calcium (10 mM) was provided as its citrate salt. The pH was adjusted to 6.8 with NaOH. Medium without added calcium served as the control. Cultures were aerated on a gyratory water bath shaker, model G76 (New Brunswick Scientific), at 140 rev · min^{-1}. Bacterial growth was monitored at different time intervals by measuring the increase in total culture protein using the method of Lowry et al. [9]. pH of the spent fluid was recorded with the aid of a Fisher pH meter, model 610A.

2.1. *Calcium and citrate measurements*

At various incubation intervals, the growth medium was spun at 800 rpm to isolate any insoluble residues. The bacterial cells obtained following centrifugation at 10,000 rpm were sonicated to afford two fractions, the cell debris and the soluble fraction. The calcium content was monitored with the aid of a Perkin-Elmer atomic absorption spectrometer Model 703. Rate of citrate consumption was monitored enzymatically with the citrate assay kit from Boehringer [10].

2.2. *Isolation and identification of exocellular precipitate*

At stationary phase of growth the precipitate was isolated by centrifuging the spent fluid at 800 rpm, and purified by solvent extraction to remove water-, ethanol- and chloroform-soluble impurities and by dialysis (MWCO 12–14 kDa). This residue was examined by Fourier transformation infra-red spectroscopy using a KBr disc on a Perkin-Elmer 983 spectrometer. X-ray fluorescence analyses were performed on a Philips PW/404 Automatic sequential spectrometer according to standard procedures [11]. LiF200 was used as the analyzing crystal. The X-ray powder photograph of the bioprecipitate was taken with a 114.6 mm Straumanis-type camera on a Philips PW-1010 generator

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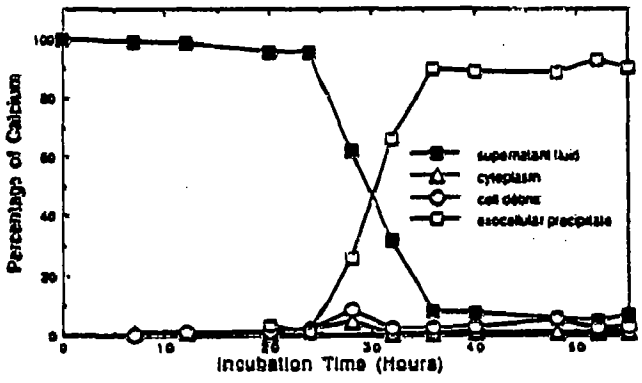

Fig. 1. Calcium metabolism in *Pseudomonas fluorescens* ATCC 13525. Distribution of the divalent metal in different cellular fractions, the supernatant fluid and the exocellular precipitate as measured by a Perkin-Elmer atomic absorption spectrometer Model 703.

(Ni-filtered CuKα radiation). The residue was examined in a Cambridge Stereoscan 120 scanning electron microscope after coating with gold or carbon.

3. RESULTS

Inclusion of calcium in the culture medium did not appear to have an inhibitory influence on cellular multiplication. As growth progressed no significant increase in cytoplasmic calcium was recorded. Only after 16 h of growth was exocellular deposition of a calcium containing residue observed which continued through to stationary phase of growth. Most of the calcium was sequestered in this precipitate (Fig. 1). At cessation of bacterial multiplication, the pH of the spent broth from both control and calcium-supplemented media showed an increment to 8.23–8.35. No exocellular residue was evident if the culture medium was not inoculated and the pH raised up to 10.0. Solutions of calcium (10 mM) devoid of the tricarboxylic acid and with a pH ranging from 8.5 to 10.5 did not precipitate upon incubation for two weeks. These results indicated that the metabolism of *Pseudomonas fluorescens* ATCC 13525 was necessary for the formation of the white precipitate. After 24 h of incubation no citrate was detected in the spent fluid.

X-ray fluorescence spectroscopic analysis of the insoluble residue revealed peaks attributable to calcium only (Fig. 2). The infra-red absorption spectrum of a sample of the bacterial crystals was similar to that of calcite with major bands characteristic of carbonate at 1,446, 873 and 705 cm^{-1}. The precipitate dissolved in dilute acids with effervescence. X-ray diffraction patterns exposed lines that are identical to calcite (Fig. 3). Examination of the white residue with a scanning electron microscope revealed structures with hexagonal crystal habits (Fig. 4).

4. DISCUSSION

These results demonstrate that *Pseudomonas fluorescens* ATCC 13525 promotes the formation of calcite in a calcium-rich environment. This crystalline $CaCO_3$ allows the organism to survive in the calcium-stressed surrounding by contributing to the maintenance of a low intracellular concentration of the divalent element. The involvement of Ca^{2+}/ATPase pumps and Ca^{2+}/proton antiporters in regulation of cytoplasmic calcium in bacterial systems have been suggested [5]. In this study, during the exponential phase of growth, the cytoplasmic calcium content appears to be regulated by mechanisms(s) other than biotransformation of the metal as an insoluble residue. As the deposition of calcite occurs predominantly at the stationary phase of growth, it is not unlikely that bioprecipitation of the divalent element may ensure a more efficient utilisation of energy. In numerous organisms an excess or a dearth of metallic elements is known to trigger the exocellular deposition of the metals sulphides and phosphates [11–13], the production of modified exopolysaccharides [14], and the elaboration of siderophores [15]. The detoxification of calcium, in this instance, is mediated by the elaboration of $CaCO_3$ with unique crystal habits. In higher organisms crystalline polymorphs of $CaCO_3$ provide skeletal structures, mediate acid–base control, store ions, detect

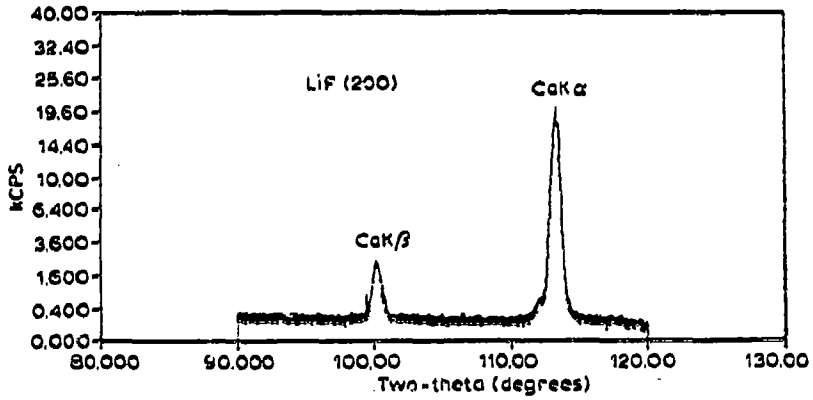

Fig. 2. X-ray fluorescence spectrum of the exocellular precipitate recorded on a Philips PW1404 automatic sequential spectrometer using an Rh X-ray tube. LiF200 served as the analyzing crystal.

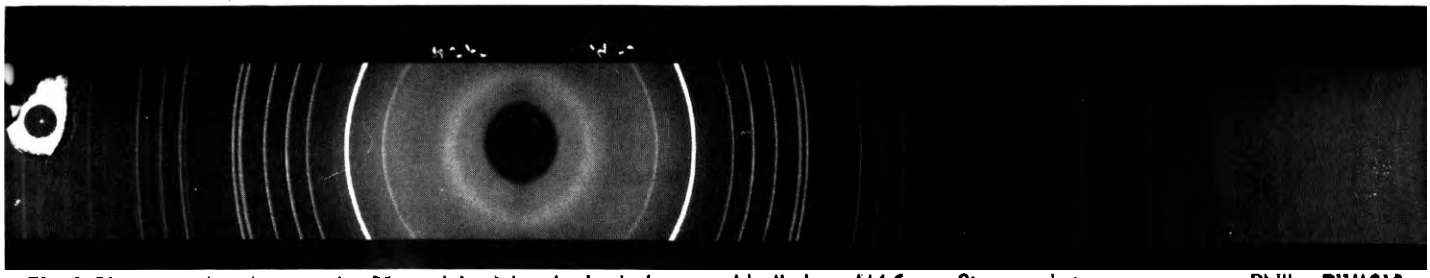

Fig. 3. X-ray powder photograph of bacterial calcite obtained photographically by a 114.6 mm Straumanis-type camera on a Philips PW1010 generator, (Ni-filtered Cukα radiation).

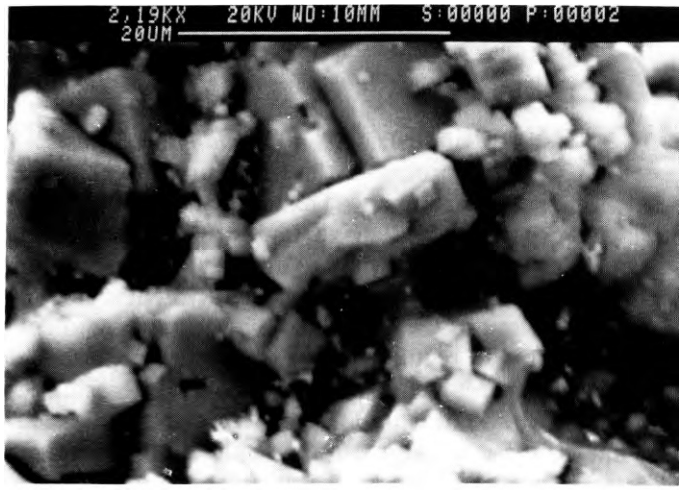

Fig. 4. Scanning electron micrograph of exocellular calcite with hexagonal crystal habit elaborated by *Pseudomonas* in a calcium-rich environment was observed with a Cambridge Stereoscan 120.

changes in linear acceleration and act as gravity sensors, and their production appears to be either biologically induced or controlled [16,17]. Nucleation and growth of $CaCO_3$ crystal would necessitate the maintenance of an environment supersaturated in carbonate and calcium ions. Carbonic anhydrase and pH modifications are known to provide a constant supply of inorganic carbon [18]. Whether the formation of calcite with hexagonal crystal habits is a result of adventitious precipitation or biologically regulated processes, it is evident that this microbe is instrumental in the deposition of the calcareous mineral.

In conclusion, our results indicate that production of $CaCO_3$ with crystal habits of calcite enables *Pseudomonas fluorescens* ATCC 13525 to regulate its cytosolic calcium. The involvement of crystalline $CaCO_3$ in calcium homeostasis is a novel phenomenon. This microbial model of calcium insolubilization may also have a potential application in the immobilization of excess of calcium in the environment. And the biogenesis of the calcium mineral in a prokaryote indeed provides a simple system to delineate biocrystallization processes.

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