Volutin Granules in Zoogloea ramigera

FRANK A. ROINESTAD AND IRVING YALL

Department of Microbiology and Medical Technology, The University of Arizona, Tucson, Arizona 85721

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Zoogloea ramigera, a gram-negative bacterium found in activated sludge, formed volutin granules when excess orthophosphate was added to a phosphate-starved culture. These volutin granules were stainable by hydrogen sulfide after lead acetate treatment and extractable by N-perchloric acid but were not adsorbed by activated charcoal. They appeared to consist of inorganic polyphosphate. Optimum granule formation in the arginine broth required 10 g of glucose, 3 mg of phosphate, and 1 to 20 mg of magnesium per liter of medium. At an Mg²⁺ concentration of 1 mg/liter, very large granules appeared which often appeared to fill the cell. An excess of glucose, orthophosphate, or magnesium reduced granule formation. In the absence of sulfate, moderate granulation occurred in arginine broth before the addition of excess orthophosphate; granulation did not increase after the addition of phosphate.

Increased use of fertilizers and detergents has resulted in large amounts of phosphorus being present in our wastewaters. The presence of this element in amounts exceeding 0.5 mg/liter results in bursts of algal growth in lakes or streams receiving wastewater effluent from a treatment plant (11). Activated sludge, a mixture of many microorganisms, provides an effective means of ridding wastewater of its organic matter but, under the usual conditions of treatment, does little to relieve it of its phosphorus content. However, work in this laboratory has shown that biological phosphorus uptake from sewage by sludge can be significantly increased (Yall et al., Bacteriol. Proc., p. 8, 1970).

Volutin is a granular inclusion found in some bacteria that is thought to be associated with phosphorus uptake. These granules seem to contain inorganic polyphosphate although other compounds may also be present (8). Some organisms such as Aerobacter aerogenes rapidly accumulate large amounts of inorganic polyphosphate when supplemented with orthophosphate after phosphate starvation; this phenomenon is called polyphosphate overplus (7). During overplus, A. aerogenes forms volutin granules; overplus has not been found in mutants that do not form volutin granules (9).

As a part of a study concerned with the ability of the bacterial population of sludge to remove phosphorus from wastewaters, we examined the conditions necessary to promote optimal volutin formation in a microorganism, Zoogloea ramigera, which has been reported to be present in the flocs of activated sludge (Berger's Manual).

MATERIALS AND METHODS

Microorganism. Z. ramigera ATCC 19623 was employed in this work. It was maintained on Trypticase soy (BBL) agar slants and supplemented with 0.25% glucose. Other media used included: Brain Heart Infusion (BHI; Difco) agar slants; Hall's synthetic sewage medium (M. W. Hall, Ph.D. Thesis, Univ. of Illinois, Urbana, 1968); phosphate-deficient Hall's medium; Crabtree and McCoy's arginine broth (2); inoculating broth, a modification of Crabtree and McCoy's arginine broth containing 0.2 g of K₂HPO₄ and 0.1 g of KH₂PO₄ per liter of medium; and arginine broth, which was a modification of Crabtree and McCoy's broth containing 4 mg of KH₂PO₄ per liter.

Inoculum. The standard inoculum for liquid media consisted of 0.01 ml of stationary-phase Z. ramigera.

Growth conditions. Z. ramigera was grown in 100 ml of medium in 500-ml Erlenmeyer flasks continuously shaken at 200 rev/min on a New Brunswick rotary shaker (model C.S.) at 24 C.

Measurements of mass. The transmittance of uniform culture suspensions was measured at 540 nm with a Bausch & Lomb Spectronic-20 colorimeter. The dry weights were determined by collecting the cells on preweighed membrane filters (0.45 μm pore size; Millipore Corp., Bedford, Mass.) and drying at 70 C overnight.

Staining procedures. Neisser's stain was used to stain volutin granules. The solution of methylene blue
plus gentian violet stains the granules deep blue, and the chrysoidin solution stains the cells yellow (1).

Tandler's inorganic phosphate stain was used to show the presence of inorganic phosphate in volutin granules. (14). The cells were counter-stained red with safranin.

**Granule counting.** Smears stained for volutin were observed at 970 X by using bright-field microscopy. Photographs of some smears were taken with a Leitz Orthomat Microscope Camera. An area near the top of the slide was chosen for granule counting where the yellow counterstain had thoroughly drained off. The number of volutin granules in each of 30 cells was recorded. The number of granules per cell approximated a Poisson distribution and was treated as such in computing confidence limits of means.

**Radioactive assays.** Carrier free $^{32}$P$_2$PO$_4$, in 0.02 N HCl, supplied by the New England Nuclear Corp., Boston, Mass., was added to a 120-hr culture of *Z. ramigera* in arginine broth. After 30 min, 0.2 g of K$_2$HPO$_4$ and 0.1 g of KH$_2$PO$_4$ were added to dilute out the activity and provide chromatographic markers. After 24 hr, approximately 0.1 ml of packed cells from the culture was washed in distilled water and extracted by the procedure of Ogur and Rosen (12), modified by using 70% ethanol to remove the soluble pool, and by using 0.1 N KOH to extract the final residue. Normal perchloric acid (PCA) fractions were adsorbed with Norit A (3). The disappearance of nucleic acids was measured at 260 nm with a Beckman DU Spectrophotometer. The cells were centrifuged at 20,200 × g for 20 min at 0 C. Duplicate 0.1-ml samples of each extract were added to 10 ml of scintillation counting fluid, consisting of, per liter, 4 g of 2,5-bis-[2-(5-tert-butylbenzoxazolyl)]-thiophene, 80 g of naphthalene, 400 ml of methyl cellosolve, and 600 ml of toluene. The radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer (model 314 EX-2).

**Chromatography.** Samples and standards (H$_2$P$_2$PO$_4$ and Na$_2$P$_2$O$_7$) were applied to Whatman no. 4 chromatography paper cut to 9 by 9 inches (23 by 23 cm), developed in a mixture of isopropanol, concentrated HCl, and water [170:41:39, v/v (15)], and sprayed with a mixture of 60% PCA, N-HCl, 4% ammonium molybdate, and water [5:10:25:60, v/v (5)]. The paper was sprayed, air-dried, and exposed to ultraviolet light at 260 nm for 10 min, whereupon the inorganic phosphate appeared as blue spots.

**RESULTS**

**Growth characteristics.** *Z. ramigera* reached stationary phase in both arginine broth and inoculating broth in approximately 72 to 120 hr (Fig. 1). Its doubling time was 5.5 hr in Trypticase soy broth, 6 hr in arginine broth, 7 hr in Crabtree and McCoy's arginine broth, and 8 hr in inoculating broth. The total cell mass in arginine broth was usually 17 mg (dry weight) in 120 hr. If the glucose concentration of arginine broth was raised from 10 to 30 g/liter, there was no change in total cell mass, but, if glucose was deleted from the medium, cell mass was reduced to 4 mg (dry weight). If the initial phosphate concentration of arginine broth was increased to 6 mg/liter, there was no change in cell mass. No growth occurred in the absence of added phosphate. If the magnesium concentration was decreased from 20 to 2 mg/liter, there was no change in cell mass. If no magnesium salt was added to the medium, the cell mass was reduced to 2 mg (dry weight). If 1.8 g of phosphate per liter was added to a 120-hr culture in arginine broth, the cell mass increased to 46 mg in 24 hr.

**Granule formation.** Moderate granulation (two granules per cell) occurred in Trypticase soy broth, on Trypticase soy agar, and on BHI agar. Since arginine broth contained only 3 mg of phosphate per liter, *Z. ramigera* normally gave a phosphate-starved culture. On the average, 2.5 granules per cell were rapidly formed after the addition of 0.18% phosphate (0.2 g of K$_2$HPO$_4$ plus 0.1 g of KH$_2$PO$_4$) to a 120-hr culture growing in arginine broth (Fig. 2A). No granules were observed in Hall's medium, but, on the average, 2.5 granules per cell were formed when 0.18% phosphate was added to a 120-hr culture grown in phosphate-deficient Hall's medium. When the organism was grown in coarsely filtered, autoclaved, activated sludge supplemented with 2 g of glucose/liter, an average of one granule per cell was formed. No granules were detected when glucose was omitted or present at a concentration of 0.01%.

![Fig. 1. Growth of Zoogloe ramigera in arginine broth and inoculating broth. Growth was at 24 C on a rotary shaker. Symbols: O optical density (at 540 nm) of growth in arginine broth; O, dry weight of growth in arginine broth; •, OD of growth in inoculating broth. Ordinate scale is linear in optical density.](image-url)
FIG. 2. Photomicrographs of volutin granules in Zoogloea ramigera. Smears, prepared 24 hr after adding 1.8 g of orthophosphate per liter to phosphate-starved cultures of Z. ramigera grown for 120 hr in arginine broth, were stained by Neisser’s procedure. ×1,250. (A) Unmodified arginine broth; (B) arginine broth with 1 mg of magnesium ion per liter; (C) arginine broth with 80 mg of magnesium ion per liter.
The pH of arginine broth during granule formation depended on the amount of orthophosphate added. When 0.18% phosphate was added to a 120-hr culture in arginine broth, the initial pH of 8.0 decreased to a pH of 7.0, where it remained. If the added phosphate was reduced from 1.8 to 0.18 g/liter or to 0.018 g/liter, the buffering capacity was decreased; however, the average granule yield was still 2.5 granules per cell.

To maintain maximum buffering capability in the medium, 0.18% phosphate was routinely used as excess orthophosphate, under which condition the number of granules per cell varied from none to seven. If only one granule was present in a cell, it was always polar; if two granules were present, they were usually both polar; if three or more granules were present, two were usually polar with the others randomly distributed between them.

These granules apparently contained inorganic phosphate since they stained with Tandler's inorganic phosphate stain. However, they were very soluble in ammonium acetate and dissolved in that reagent despite the presence of lead acetate and the previous Formalin treatment. If the addition of excess orthophosphate was withheld from a culture in arginine broth, granulation never occurred.

**Extractions.** When cells of *Z. ramigera*, labeled by adding carrier-free H$_5$PO$_4$ to a phosphate-starved culture in arginine broth, were extracted by the modified method of Ogur and Rosen (12), 11% of the activity was eluted with ethanol, 4% with ethanol-ether, 3% with 0.1 n PCA, 51% with cold N-PCA, 30% with hot N-PCA, and 1% with KOH. Adsorption of the N-PCA fractions by Norit A removed the nucleic acids and left most of the activity with the unadsorbed inorganic phosphate. Chromatograms revealed that most of the radioactivity resided in the N-PCA fractions as orthophosphate and pyrophosphate rather than as nucleotides. Microscopically the granules remained distinct but completely disappeared when treated with cold N-PCA.

**Time variation.** Volutin granules began to form immediately upon addition of 0.18% orthophosphate to a phosphate-starved culture in arginine broth; the maximum number of 2.5 granules per cell was reached in 4 hr. The ability to form granules continued with succeeding generations, maintaining the maximum number (2.5/cell) for 3 days, during which time the dry weight of the cell mass increased from 17 to 77 mg; then the number of granules gradually decreased until there were only 0.3 granules per cell in a 2-week-old culture. The age of the culture at the time of phosphate addition could be anywhere from 4 to 8 days without changing the number of granules formed in 24 hr.

**Glucose variation.** Only a few granules were
formed in arginine broth in the absence of glucose. The presence of 0.1 g of glucose per liter gave abundant granules that were too faint to count. The presence of 2 g of glucose per liter gave dark granules averaging about 1.5/cell. A further increase in glucose concentration gave darker and more numerous granules; the optimum of 2.5 granules per cell was reached at the level of 10 g of glucose per liter. Increased amounts of carbohydrate, up to 70 g/liter, caused gradual decreases in granule counts (Fig. 3).

The ability of cells to remove $^{32}$P radioactivity from arginine broth supplemented with 0 or 10 g of glucose per liter was studied. Carrier-free $\text{H}_3\text{PO}_4$ was added to 120-hr cultures of Z. ramigera. The cells in the presence of glucose removed 4,825 counts per min per mg (dry weight) in 30 min. The cells in the absence of glucose removed 860 counts per min per mg (dry weight) in that length of time.

**Initial phosphate variation.** With only 0.6 mg of initial phosphate per liter in arginine broth, the granule yield was low; however, the yield rapidly increased to abundant granulation (2.5 granules/cell) with 3 mg/liter and then rapidly decreased to a low level with 12 mg/liter (Fig. 4).

**Magnesium variation.** No granules were found in the small amount of cells resulting from growth in arginine broth without added magnesium salt; however, 1 mg of magnesium per liter gave large, dark, abundant granules which often filled the whole cell (Fig. 2B). The maximum average number of 2.5 granules per cell occurred when magnesium was present in a range of from 1 to 20 mg/liter, but at 20 mg the extra-large granules associated with the 1-mg amount were absent. Further increases in magnesium concentration gave decreased yields (Fig. 5). At a magnesium level of 80 mg/liter, the granules were faint (Fig. 2C). The magnesium concentration was varied by changing the concentration of magnesium sulfate. However, the same results were obtained when the sulfate level was held constant.

**Interactions.** A factorial design was used to find interactions between glucose (2 and 10 g/liter), initial phosphate (3 and 18 mg/liter), and magnesium (2 and 20 mg/liter) in arginine broth.

Z. ramigera was grown in arginine broth with the eight possible combinations of the above three nutrients. As usual, 1.8 g of phosphate per liter was added at 120 hr, and the smears were made 24 hr later.

The results were illustrated with three-dimensional coordinates by drawing two planes, one representing 2 g of glucose per liter and the other representing 10 g of glucose per liter (Fig. 6). No significant difference was found between the two planes at three of the corners, but one corner of the 2 g/liter glucose plane was irregularly elevated to 1.4 granules per cell at 18 mg of initial phosphate per liter and 20 mg of magnesium per liter. This represented increased granule

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**Fig. 5.** Granulation in Zoogloea ramigera at different magnesium concentrations. Granule counts were made 24 hr after adding 1.8 g of orthophosphate per liter to phosphate-starved cultures in arginine broth. The brackets indicate the 95% confidence limits for each mean.

**Fig. 6.** Granulation in Zoogloea ramigera at different concentrations of glucose, initial phosphate, and magnesium. Granule counts were made 24 hr after adding 1.8 g of orthophosphate per liter to phosphate-starved cultures in arginine broth. The brackets indicate the 95% confidence limits for each mean.
production resulting from an interaction between low glucose, high phosphate, and high magnesium concentrations.

Other variations. The granule count was not affected by varying the amount of arginine (0.05 to 1 g/liter), vitamin B₁₂ and biotin (0.0005 to 0.025 mg/liter), K⁺ (1 to 63 mg/liter), or Ca⁺ (0 to 50 mg/liter). When magnesium chloride was substituted for magnesium sulfate in arginine broth so that no sulfate was added, 1.2 granules per cell were formed before the addition of excess orthophosphate. The number of granules did not increase after the addition of phosphate and remained lower than if the broth contained sulfate.

DISCUSSION

The volutin granules in Z. ramigera appeared to be composed of long-chain polyphosphate. They were formed immediately after adding excess orthophosphate to a phosphate-starved culture and were metachromatic, a characteristic of long-chain polyphosphate but not of orthophosphate, pyrophosphate, metaphosphate, or polyphosphate of less than eight phosphate units in length (4). They stained with Tandler's technique which is specific for inorganic phosphate; however, they dissolved in ammonium acetate, the reagent used to remove the sulfates and oxalates present, perhaps because of the greater solubility of polyphosphate than of orthophosphate in ammonium acetate (14). Most of the radioactivity of phosphate ³²P taken up by phosphate-starved cells was extracted with the nucleic acids but was not subsequently adsorbed by activated charcoal, indicating insoluble polyphosphate, the polyphosphates of long-chain length (8). Chromatographically this label was mostly in the orthophosphate and pyrophosphate position, probably due to considerable degradation during extraction.

The apparent accumulation of polyphosphate by Z. ramigera when excess orthophosphate was added to a phosphate-starved culture was similar to the polyphosphate overplus phenomenon in A. aerogenes (7). An enzyme was isolated from Escherichia coli which takes the terminal phosphate from adenosine triphosphate (ATP) and builds the polyphosphate polymer (10). In A. aerogenes a similar enzyme, polyphosphate kinase, mediates the only route of polyphosphate biosynthesis (7). It is possible that a similar system is responsible for the uptake of phosphorus into the volutin granules of Z. ramigera.

The optimum concentrations for granulation in arginine broth were found to be 10 g of glucose, 3 mg of initial phosphate, and 1 to 20 mg of magnesium per liter of medium. Quantities of less than 10 g of glucose per liter in the medium apparently caused a shortage of ATP, the intracellular phosphatase source for polyphosphate biosynthesis; more than 10 g of the hexose per liter caused typical catabolic repression. More than 3 mg of initial phosphate per liter apparently caused enzyme repression similar to that reported for polyphosphate kinase in A. aerogenes. More than 20 mg of magnesium per liter caused typical cationic inhibition.

Sulfur starvation resulted in the formation of 1.2 granules per cell prior to the addition of excess orthophosphate. Sulfur starvation stops nucleic acid synthesis which is in competition with polyphosphate synthesis for the available ATP. When nucleic acid synthesis in A. aerogenes was stopped by sulfur starvation, polyphosphate accumulated until exogenous sulfate was added (6). Similarly, the sulfur-starved Z. ramigera formed granules even though the culture was also phosphate-starved because, in the absence of nucleic acid synthesis, a phosphate concentration of 3 mg/liter is apparently an excess.

Granulation occurred in activated sludge in the presence of 2 g of glucose per liter. Granulation in arginine broth showed an unexpected increase in the factorial design (1.4/cell) with the combination of 18 mg of initial phosphate, 20 mg of magnesium, and 2 g of glucose per liter of medium. Laboratory analysis showed that Tucson raw sewage contains about 34 mg of orthophosphate per liter and 19 mg of magnesium ion per liter. The carbon level of whole sewage in an English study was about 44 mg/liter; most of this was glucose and sucrose (13). Thus, the phosphate and magnesium levels used in the factorial design were close to sewage levels; however, the glucose concentration was 18 times that reported for sewage.

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