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Utilization of Iron Gallate and Other Organic Iron Complexes by Bacteria from Water Supplies

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The degradation of four soluble organic iron compounds by bacteria isolated from surface waters and the precipitation of iron from these complexes by the isolates was studied. All eight isolates brought about the precipitation of iron when grown on ferric ammonium citrate agar. Three isolates were able to degrade ferric malonate, and three others degraded ferric malate with iron precipitation. Only three isolates, two strains of *Pseudomonas* and one of *Moraxella*, were able to degrade gallic acid when this was supplied as the sole carbon source. One strain of *Pseudomonas* was found to be active in degrading ferric gallate. Electron microscopy of cells of this bacterium after growth in ferric gallate as the sole carbon source yielded results indicating uniform deposition of the iron on or in the bacterial cells. Seven of the isolates could degrade the iron gallate complex if supplied with additional carbon in the form of yeast extract.

Iron problems in water supplies basically involve two reactions: (i) mobilization or solubilization of iron that can then be transported by water, and (ii) precipitation of the iron at some distance from the site of mobilization.

One way in which iron can be mobilized in nature is by the formation of organic-metal complexes which result from the combination of iron with the products of organic matter decomposition in the uppermost soil layer (1). Polyphenols of plants are also important in dissolving and complexing iron (3). The type of complexing agent depends largely upon the type of vegetation. For example, the solution of iron oxides by extracts from eucalypts seems to depend largely upon the appreciable amounts of free gallic acid that are present in these plants (5). Once formed, the relatively soluble organic iron complexes are susceptible to leaching in the soil profile or to lateral movement with water runoff. In either circumstance there is a good chance that these complexes will accumulate in surface water supplies such as reservoirs. Iron precipitation problems could arise at any stage of water treatment and supply in which microflora able to degrade the complexing agent can be supported.

The present investigation was aimed at determining the ability of bacteria isolated from water supplies to bring about the precipitation of iron from a number of organic iron complexes. Because eucalypts make up a large percentage of the vegetation in and around water catchment areas of Australia, the ability of water bacteria to degrade gallic acid and the iron-gallic acid complex was examined in more detail.

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MATERIALS AND METHODS

Media. The following media were modifications of a basal mineral salts solution (BMS) and were composed of 0.1 g of K_2HPO_4, 0.5 g of $(NH_4)_2HPO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.01 g of $Ca(NO_3)_2 \cdot 2H_2O$ per liter of distilled water. Ferric ammonium citrate medium was prepared as follows. Five grams of ferric ammonium citrate and 0.2 g of yeast extract (Difco) were added to 1 liter of the basal medium, pH was adjusted to 7.0, and the medium was sterilized by autoclaving at 120 C for 20 min. When a solid medium was required, 14.0 g of agar (Difco) was added. Ferric malonate medium was prepared in halves (parts A and B), which were mixed aseptically just before inoculation. Part A consisted of 0.04 g of yeast extract added to 100 ml of the BMS and sterilized as described above. Part B was prepared by dissolving 4.18 g of malonic acid and 0.75 g of $FeCl_3 \cdot 6H_2O$ in 100 ml of BMS. The pH was adjusted to 5.5 with NH_4OH and the medium was sterilized by filtration through a membrane filter (0.22 μm mean pore size; Millipore Corp., Bedford, Mass.). Ferric malate medium was prepared in the same way as ferric malonate medium, except that part B consisted of 2.70 g of malic acid and 0.75 g of $FeCl_3 \cdot 6H_2O$ dissolved in 100 ml of BMS with a pH of 6.5. Ferric gallate medium was prepared by dissolving 0.2 g of yeast extract (Difco), 0.024 g of

$FeCl_3 \cdot 6H_2O$, and 0.045 g of gallic acid in 1 liter of BMS. The pH was adjusted to 7.0 and the medium was sterilized by filtration. Yeast extract-mineral salts medium consisted of 0.2 g of yeast extract in 1 liter of BMS.

Cultures. Isolates were obtained from ferric ammonium citrate agar plates that had been inoculated with water samples and incubated at 30 C for 48 h. Dark-brown colonies showing the deposition of iron were selected for further purification and indentification. Isolates were identified by using the scheme of Cowan and Steel (4). The type of flagellation was determined by electron microscopy.

Electron microscopy. The bacteria were grown in either ferric gallate medium or yeast extract-mineral salts medium at 30 C for 48 h. The cells were harvested and washed by centrifugation in distilled water and then resuspended in this medium. The suspensions were put onto 200-mesh copper grids of carbon-coated nitrocellulose film and examined by using a Phillips EM300 electron microscope. Staining of the bacteria before electron microscopy was not performed except to determine the type of flagellation. In the latter case the bacteria were stained for 15 s with a solution containing 1% uranyl acetate and 0.4% sucrose.

Determination of ferric gallate and gallic acid. Determination of the violet-colored iron gallate complex in ferric gallate medium was carried out by the method of Sommer (7), in which the absorbance at 430 nm was measured by spectrophotometry. The determination of gallic acid in iron-free media was carried out in either of two ways. In the first method, samples of the cultures were centrifuged to remove the bacterial cells, and 5 ml of the clear supernatant fluid was added to 10 ml of a solution of ferrous ammonium sulfate containing 100 μg/ml iron. The absorbance at 430 nm was then read. In the second method, the absorption of samples of the culture supernatant fluids was measured with a recording spectrophotometer at 185 to 360 nm.

RESULTS

Of the eight isolates chosen for their rapid deposition of iron on ferric ammonium citrate agar, three were identified as strains of the genus *Pseudomonas*, two as *Moraxella*, one as *Alcaligenes*, one as *Acinetobacter*, and one as *Vibrio*. All of these strains produced dark-brown colonies on ferric ammonium citrate agar, indicating the utilization of citrate. However, utilization of malate and malonate was not so wide-spread (Table 1).

Growth of the isolates in liquid ferric ammonium citrate medium produced results different from those obtained with the agar medium. All cultures grew equally well in the liquid medium, but this growth was not measured quantitatively.

At the time of inoculation with six of the isolates, the medium was a clear, yellow liquid with a pH of 7.0. During incubation, changes in the appearance of the cultures were observed (Table 2). After 12 days of inoculation, *Moraxella* strain 2 and *Pseudomonas* strain 3 produced a heavy, orange-red precipitate in the medium, leaving a water-clear supernatant fluid, whereas the other isolates had caused no precipitation of the iron even after 34 days of incubation. All isolates changed the color of the medium to orange-red. The pH of all the culture media after incubation was 8.2. The pH and color of uninoculated samples of the medium remained unchanged during the incubation period.

When all of the eight isolates were inoculated into iron-free medium with gallic acid (100 μg/ml) as the sole added carbon source, only three of the isolates showed any appreciable utilization of gallic acid. Because absorption due to gallic acid interfered with the spectrophotometry determination of growth at 540 nm, growth was assessed visually and by measuring the absorbance at 540 nm of the cultures before and after centrifugation and comparing the difference between these two figures. The difference for uninoculated medium was zero, whereas the differences for *Pseudomonas* strains 1 and 3 and *Moraxella* strain 2 were 0.07, 0.05, and 0.07, respectively. Table 3 shows the absorbance due to the iron gallate complex formed when ferric ammonium sulfate was reacted with residual gallic acid in the culture supernatant fluids after incubation for 72 h at 30 C.

To find the optimal concentration of gallic

TABLE 1. *Deposition of iron by water bacteria growing on organic iron agar media*[a]

Organism	Medium		
	Ferric ammonium citrate	Ferric malonate	Ferric malate
Alcaligenes sp.	+	−	+
Acinetobacter sp.	+	−	−
Vibrio sp.	+	+	−
Pseudomonas strain 1	+	+	−
Pseudomonas strain 2	+	−	−
Pseudomonas strain 3	+	+	−
Moraxella strain 1	+	−	+
Moraxella strain 2	+	−	+

[a] Incubation period was 5 days.

TABLE 2. *Reactions of isolates in liquid ferric ammonium citrate medium*

Organism	Appearance of medium at time of incubation (days)[a]			
	0	6	12	34
Moraxella strain 1	Clear yellow	NC	Orange-red	Orange-red
Moraxella strain 2	Clear yellow	Orange-red	Orange-red precipitate	Orange-red precipitate
Pseudomonas strain 1	Clear yellow	NC	Orange-red	Orange-red
Pseudomonas strain 2	Clear yellow	NC	Orange-red	Orange-red
Pseudomonas strain 3	Clear yellow	Orange-red	Orange-red precipitate	Orange-red precipitate
Alcaligenes sp.	Clear yellow	NC	Orange-red	Orange-red

[a] NC, no change.

TABLE 3. *Utilization of gallic acid by water bacteria*

Organism	Absorbance of iron gallate complex at 430 nm[a]
Uninoculated control	0.313
Pseudomonas strain 1	0.088
Pseudomonas strain 3	0.068
Moraxella strain 2	0.097

[a] After incubation for 72 h.

acid for the growth of *Pseudomonas* strain 3, the organism was inoculated into gallic acid-mineral salts medium containing gallic acid at concentrations of 100, 200, 300, 400, and 500 μg/ml, and incubated for 72 h at 30 C. By measuring the difference in absorption of cultures at 540 nm before and after centrifugation, growth was found to be best at a gallic acid concentration of 200 μg/ml, where the difference was 0.26. No growth was obtained at the 400- or 500-μg/ml levels.

Degradation of gallic acid by *Pseudomonas* strain 3 with disruption of the aromatic nucleus was demonstrated by spectrophotometry by following the loss of absorption due to the aromatic ring structure in cultures of the bacterium growing in gallic acid medium. Gallic acid concentration in the medium at the time of inoculation was 200 μg/ml. Both peaks (210 and 260 nm) completely disappeared.

Freshly prepared ferric gallate medium containing no yeast extract was violet in color because of the iron-gallic acid complex. After inoculation with *Pseudomonas* strain 3 and incubation for 48 h at 30 C, the violet color had disappeared and growth of the bacterium was visible. Addition of gallic acid at 100 μg/ml did not restore the color. When separate cultures were acidified to pH 1.0 with HCl, the violet color could be restored by adding gallic acid and readjusting the pH to 8.0. However, when the cultures were filtered through a membrane filter (0.05 μm mean pore size; Millipore Corp., Bedford, Mass.) before the acidification and addition of gallic acid, no color developed.

Cells of *Pseudomonas* strain 3 grown in ferric gallate medium without yeast extract showed an even distribution of electron-dense material when viewed by electron microscopy (Fig. 1A). Even a single polar flagellum could be seen without the usual negative staining. In contrast, cells of the bacterium grown in yeast extract-mineral salts medium were less electron dense (Fig. 1B). Figure 1C shows cells of the same bacterium after negative staining.

The ability of seven of the isolates to degrade the iron gallate complex was tested. The organisms were inoculated into ferric gallate medium and incubated for 72 h at 30 C. After incubation, the cultures were centrifuged to remove the cells, and the absorbance of the supernatant fluids at 430 nm was determined (Table 4).

DISCUSSION

The results demonstrate that a variety of bacteria from surface water supplies can precipitate iron from certain organic salts and iron complexes that could exist in surface waters. The precipitation occurs after the release of iron, when the bacteria utilize the organic part of the complex (1). Some of the isolates can cause formation of a heavy red precipitate of iron when grown in liquid ferric ammonium citrate medium. The precipitate is probably a mixture of bacterial cells and ferric oxide hydrate. Other isolates were unable to cause the precipitation of iron but changed the color of the medium to orange-red. The color change in the medium without iron precipitation could mean either that the ferric iron was in the form of a stabilized colloid or that the organism had not utilized the citrate but, instead, caused a rise in pH of the medium which in turn affected the species of iron complex.

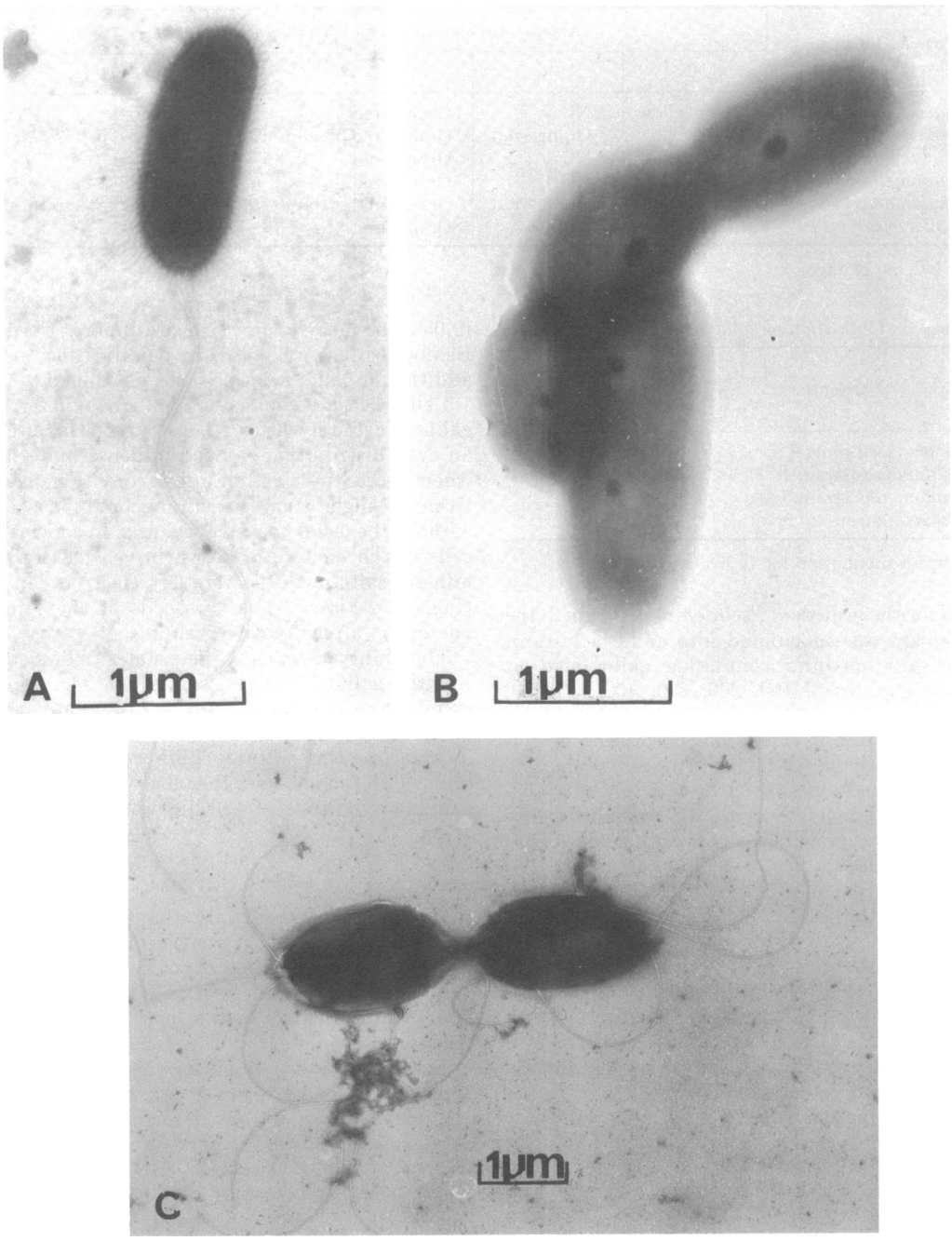

FIG. 1. *Electron micrographs of Pseudomonas strain 3. A, Cell from iron gallate culture, unstained; B, cells from yeast extract-mineral salts culture, unstained; and C, cells from yeast extract-mineral salts culture, stained with uranyl acetate.*

Only three of the isolates could utilize gallic acid as a sole source of carbon for growth. Beveridge and Hugo (2) isolated the bacterium *Pseudomonas convexa* X.I, which could use gallic acid as a sole carbon source. Unlike *P. convexa* X.I, the strains of *Pseudomonas* iso-

TABLE 4. *Degradation of iron gallate complex by water bacteria*

Organism	Absorbance of iron gallate complex at 430 nm[a]
Uninoculated control	0.520
Alcaligenes sp.	0.175
Vibrio sp.	0.340
Acinetobacter sp.	0.192
Pseudomonas strain 1	0.115
Pseudomonas strain 2	0.167
Pseudomonas strain 3	0.165
Moraxella strain 2	0.195

[a] After incubation for 72 h.

lated in this study did not produce pigments. No growth was obtained with any of the isolates at a gallic acid concentration greater than 300 μg/ml, and this was possibly due to toxic effects.

The results show that *Pseudomonas* strain 3 can destroy the colored iron galate complex by degrading the gallate part. The iron was liberated in a form that cannot be complexed with additional gallic acid unless the pH is lowered to 1.0. Because the liberated iron was retained on a membrane filter (0.05 μm mean pore size), it is highly likely that it is either closely associated with the bacterial cell or is in a colloidal form. Examination of cells of *Pseudomonas* strain 3 by electron microscopy showed that iron gallate-grown cells were uniformly electron dense, presumably because of deposition of iron on the cells during degradation of the gallate part of the complex. Seven of the isolates could degrade the iron gallate complex in the presence of an additional carbon source in the form of yeast extract. A comparable finding has been made for species of *Arthrobacter* (6).

The degree to which bacteria such as the isolates in this study participate in iron-accumulation problems in water supplies has not been determined; but, considering their ability to precipitate iron from the various organic iron complexes tested, it seems likely that these bacteria could play a significant role.

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