A SELECTIVE ENRICHMENT METHOD FOR GALLIONELLA FERRUGINEA

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The genus Gallionella is characterized by a beanshaped bacterial cell which elongates during growth and then divides in typical bacterial fashion (Cholodny, 1924, 1926), but which possesses the unusual ability of secreting a twisted, lifeless stalk that gives a strong Prussian blue reaction with ferrocyanide, indicating the presence of ferric ions. The organisms are found in nature under conditions where water with ferrous ion content is exposed to sufficient oxygen to satisfy growth requirements. These properties have contributed to the classification of the gallionella as true iron bacteria, especially since the precipitation of iron takes place in a morphologically defined form (Pringsheim, 1949).

For almost 50 years attempts have been made to cultivate these organisms, but in the light of present knowledge it is doubtful if they have ever been obtained in pure culture. An enrichment method developed by Lieske (1911) employed a liquid medium containing the following compounds (values in g/100 ml): KCl, 0.05; MgSO₄, 0.005; K₂HPO₄, 0.005; (NH₄)₂SO₄, 0.15. Iron filings or iron wire were added to this medium. Best growth was obtained when flasks containing a shallow layer of the medium were incubated at 4 to 10°C in an atmosphere containing 1 per cent CO₂. Unfortunately Lieske never observed the actual cells of the organism and saw only the fragmented stalks. Cholodny (1924) succeeded in getting gallionella to grow attached to glass coverslips which he suspended in iron-bearing waters and discovered the true morphology of the cell and stalk. Although the thesis of Teichmann (1935) is not readily available, Pringsheim (1949) reports that this worker obtained primary enrichments of gallionella by allowing tap water to run over iron filings. The ochre-colored growth obtained by this method was then transferred to a mineral salts medium containing small amounts of nitrate, phosphate, magnesium sulfate, and ferrous sulfate at pH 6.

In agreement with Lieske (1911) poor growth was obtained above 10°C. More recently, Sartory et al. (1950) have reported isolation techniques for Gallionella ferruginea. A primary enrichment was obtained using iron filings in a liquid medium similar to that of Lieske, but 2 per cent agar was added to this medium in subsequent isolation steps. Another medium reported by these authors consisted of 0.01 per cent manganous carbonate, tap water, and 1.5 per cent agar at pH 7.8.

The present study was begun in the laboratory of Dr. C. B. van Niel at the Hopkins Marine Station of Stanford University in an attempt to isolate G. ferruginea on solid media using the techniques of Sartory et al. (1950). In the hands of the authors these methods failed to yield cultures of the organism. Previous observations by Dr. van Niel had indicated that ferrous sulfide might be satisfactory as a source of reduced iron. This proved to be an excellent source of iron, and the present report concerns a selective enrichment technique for G. ferruginea based on the use of ferrous sulfide. A brief account of some of these results (Vatter and Wolfe, 1955) as well as a study of the morphology of G. ferruginea as revealed by the electron microscope (Vatter and Wolfe, 1956) have appeared.

MATERIALS AND METHODS

Inoculum. Sediments from iron-bearing water were obtained from seepsages along the beaches at Pacific Grove, California; from springs near Harrisville, New Jersey (kindly supplied by Dr. D. J. O’Kane); from the aeration manifold of the Northern Illinois Water Corporation, Urbana; and from Rocky Hollow, Turkey Run State Park, Indiana. Although Sphaerotilus, (Leptothrix), was frequently the dominant organism in these natural samples, this organism was unable to grow under the experimental conditions. The deposits at Turkey Run provided excellent collections of G. ferruginea. An illustration of these iron bacterial deposits is presented in figure 1. An area of vertical rock wall (approx 0.5 by 0.9 m) is shown possessing a seepage crack

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Figure 1. A natural deposit of iron bacteria formed below a seepage area on a vertical rock wall, Turkey Run, Indiana. The white line indicates 0.5 m.

Figures 2, 3, and 4. Test tube cultures showing colonies of Gallionella ferruginea 4 days old attached to the sides of the test tubes. X 2.2.

Figure 5. Colonies 60 hr old illustrating their cottony nature. X 12.

Figure 6. A mature colony exhibiting satellite growth. X 8.

Figure 7. The edge of a mature colony photographed by transmitted light on the upper inside surface of a Carrel flask. X 900.

Figure 8. A colony about 20 hr old photographed in a manner similar to figure 7. X 900.
below which the iron organisms have grown forming masses of stalks and sheaths 2 to 3 cm thick.

Source of iron. Ferrous sulfide was prepared by reacting equal molar quantities of ferrous ammonium sulfate and sodium sulfide in boiling distilled water. The resulting precipitate was allowed to settle from the hot solution in a completely filled and stoppered bottle. The supernatant was then decanted and was replaced with boiling distilled water. In this manner the ferrous sulfide precipitate was washed 4 times and then stored in a completely filled glass stoppered bottle. For preparation of the culture medium the ferrous sulfide was previously sterilized in 10 ml amounts in cotton plugged 16 by 150 mm test tubes which could be stored for a week or so with only negligible surface oxidation of the ferrous sulfide.

Culture vessels. Carrel flasks were chosen for the initial experiments because they provided a low surface/volume ratio of the liquid medium when completely filled and because a microscopic examination of the flask contents could be made conveniently. Growth of the organism was obtained in a variety of ampules, vials, test tubes and flasks, provided the surface/volume ratio of the liquid contents was sufficiently low to maintain reduced ferrous sulfide for at least a week. Test tubes were finally adopted as the most convenient vessel, the experiments described here being carried out in 1 by 7.5 cm pyrex tubes.

Preparation of the medium. Constituents of the basal medium were sterilized separately as 10-fold concentrated solutions and were added to sterile distilled water in appropriate amounts to give the following per cent composition: NH₄Cl, 0.1; K₂HPO₄, 0.05; MgSO₄, 0.02. The medium was dispensed into cotton plugged test tubes using standard aseptic techniques so that each tube was approximately half full. Cotton filtered carbon dioxide was then bubbled through each tube for about 5 sec using a capillary pipette. The ferrous sulfide precipitate was next added slowly from a pipette and allowed to settle gently to the bottom so that it did not become suspended. It was observed that if the addition of ferrous sulfide was performed carefully the precipitate tended to cling together and settle to the bottom in the form of a moving column. The tubes were then inoculated and stoppered.

Prussian blue reaction. Microscopic observation of the organism was facilitated by the Prussian blue reaction. For the observation of intact colonies slides were suspended in large test tubes of the medium until attached colonies were observed. The slides were then carefully removed and allowed to dry. A drop of 0.2 ml hydrochloric acid was added to the dried colonies followed by a drop of a 1 per cent solution of potassium ferrocyanide. After a reaction time of approximately one minute the slide was washed and dried. A conventional stain such as crystal violet could then be added to the same preparation to stain the cells.

RESULTS

Colony morphology. Macroscopically the colonies appeared white and cottony by reflected light as illustrated in figures 5 and 6, but with age the colonies gradually turned a rust color. The organism was sessile, always being attached to a solid surface such as the side of a test tube (figures 2, 3, and 4). Rarely did individual colonies exceed a diameter of 1 mm, but under optimum conditions secondary growth was observed around colonies which were several days old. This satellite growth which is shown in figure 6 frequently spread between the colonies forming a continuous mass of stalks around the test tube (figure 9). Under low magnification the individual stalks of a colony appeared to radiate from a central point, but at higher magnifications this pattern was masked by the masses of interwoven, branching stalks. Figures 7 and 8 represent growing colonies of G. ferruginea photographed by transmitted light on the upper inside surface of a Carrel flask. The former shows the edge of a mature colony whereas the latter shows a very young microcolony. The third dimensional growth of the organism is apparent in these figures as well as in figure 10. Figures 7, 8, and 11 to 14 reveal the type of precipitate which was deposited over the stalks and glass surfaces during incubation. Old colonies became encrusted with this precipitate and the definite shape of the stalks gradually disappeared so that colonies a few weeks old no longer exhibited the characteristic stalks of the organism. This precipitate was a ferric salt as indicated by a Prussian blue reaction with ferrocyanide and probably resulted from a purely chemical oxidation of ferrous ions.

Ferrous sulfide. The requirement for reduced iron was absolute, but the amount of ferrous sul-
Figure 9. Secondary growth between colonies forming a continuous mass of stalks. X 20.

Figure 10. Metal shadowed portion of a colony on a glass slide showing third dimensional nature of stalks. X 2000.

Figures 11 to 14. Dried preparations exposed to dilute hydrochloric acid and a 1 per cent solution of potassium ferrocyanide. Cells stained with crystal violet. The type of precipitate which frequently was deposited on the stalks is apparent. The line in each figure represents 2 µ. X 2000.

Figure 15. Tubes illustrating the technique for culturing Gallionella ferruginea. A, Colonies one week old, ferrous sulfide completely oxidized. B, Colonies 4 days old, illustrating the relation of growth to oxygen and ferrous sulfide. X 1.2.
fide added to each tube was found not to be critical. In routine culturing about 10 per cent of the volume of liquid medium was ferrous sulfide, although good results were obtained also at a volume of 5 per cent. If ferrous sulfide was added in lesser amounts, it was completely oxidized and colonies formed all the way to the bottom of the tube (figure 15A). With a limited amount of ferrous sulfide, growth was inconsistent.

Carbon dioxide. During first attempts to isolate the organism, initiation of growth was very erratic; sometimes colonies appeared in a few days, sometimes not until after 10 days or more. The addition of a small amount of carbon dioxide to the medium by bubbling from a capillary pipette prior to the addition of ferrous sulfide greatly stimulated growth. However, entirely consistent results were not obtained until a cork stopper was added to each tube in addition to the cotton plug to prevent loss of carbon dioxide. This technique is illustrated in figure 15.

Oxygen. No growth was obtained in the medium under anaerobic conditions in a nitrogen atmosphere containing 1 per cent carbon dioxide; in addition, potassium nitrate did not serve as an electron acceptor for anaerobic growth. In the culture method described here G. ferruginea exhibited the characteristics of a microaerophile. Test tube colonies first appeared in a localized region forming a narrow ring of growth in the upper 1/4 of the tube. From this initial area the formation of colonies progressed downward until at a definite region just above the ferrous sulfide precipitate growth ceased. The growth habit is illustrated in figures 2, 3, 4, and 15. In the stoppered test tubes best growth was obtained when the volume of air was about twice that of the liquid volume. Uninoculated tubes produced an amorphous chemical precipitate of ferric salts which was deposited in a manner similar to the growth habit of G. ferruginea.

Nitrogen. Only negligible growth was obtained in the absence of ammonium ions, the minute colonies quickly being oxidized to a rust color. Nitrate was not utilized as a nitrogen source. In view of these results it is probable that G. ferruginea is unable to fix nitrogen, but precise studies on this point as well as on the ability to use organic nitrogen must wait for a pure culture of the organism.

Temperature. At 25 C colonies of G. ferruginea appeared in 18 to 36 hr. Growth was only slightly slower at 20 C but was very slow at 12 C. No growth was obtained above 30 C.

pH. Since the medium used in these studies was poorly buffered the pH was dependent upon the amount of carbon dioxide added. Best growth of the organism was always obtained in tubes where the medium possessed a pH of 6.3 to 6.6. This pH range was also confirmed using a concentration of 0.1 M acetate or phosphate buffer in the medium at pH values from 3.3 to 7.5.

Phosphate. The addition of K2HPO4 in 0.05 per cent concentration greatly stimulated growth. This concentration was sufficient to produce a slight buffering effect and to satisfy the phosphate requirement of the organism; higher concentrations of phosphate produced an excessive amount of precipitate in the medium.

Water. Continuous serial transfer of the organism was not possible when only distilled water was used in the preparation of the medium, colonies in each successive transfer becoming smaller until after 3 or 4 such transfers no growth was detected. Continuous subcultures were possible upon the addition of sterile tap water or water from a natural source of the organism at a concentration of 20 per cent of the total volume of water used in the preparation of the medium.

Transfers. In subculturing, best results were obtained with weekly transfers; cultures older than 2 or 3 weeks frequently were nonviable. Although the cells of G. ferruginea were easily dislodged from the stalks, they did not exhibit other fragile properties and responded to standard dilution techniques without loss of viability.

DISCUSSION

The colonies of G. ferruginea obtained in this study differ from the ochre-colored masses described by Teichmann (1935), and from the shiny, brown growth on solid media illustrated by Sartory et al. (1950). Newly formed colonies were colorless, appearing white due to light diffraction.

The manner in which the organism grows in test tubes indicates that there is initially a narrow zone in which it is possible to compete with the auto-oxidation of ferrous ions. If an upward diffusion of ferrous ions and a downward diffusion of oxygen molecules is pictured, then it is possible for G. ferruginea to initiate growth only at a position where an adequate supply of both ferrous ions and oxygen are available. The initial ring of
colonies around the test tube seems logically explained on this basis.

This enrichment method has made possible the continuous subculturings of *G. ferruginea* at 25 °C but has not yielded pure cultures. Although cultures have been obtained which possessed only one contaminant that would grow on nutrient agar, if glass slides were suspended in larger tubes of the medium using the technique of Cholodny (1924), several morphologically different types of bacteria were observed attached to the slides. These contaminants persisted under the minimal nutrition of the method. Since a completely synthetic medium has not been developed, the added tap water or iron-bearing water from nature provided a source of various nutrients, allowing the persistence of the contaminants. A study of the nutrition of *G. ferruginea* is being continued.

Several species of gallonella have been described by Cholodny (1924), but these species were defined solely on the basis of morphological features exhibited by organisms collected from nature. It is probable that stalk as well as cell size may be related to a nutritional condition such as abundance of ferrous iron. A lack of precise culture studies has led Pringsheim (1949) to suggest that perhaps the genus consists of only a single species. It is significant that the widely distributed sources of inocula used in these studies all yielded cultures which were indistinguishable from each other.

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SUMMARY

A liquid enrichment medium for *Gallionella ferruginea* has been developed which permits continuous subculture of the organism at 25 °C. The sessile colonies are white and cottony. Constituents of the medium include ferrous sulfide, ammonium chloride, potassium phosphate, and carbon dioxide. A small percentage of tap water or iron-bearing water from nature is required in the medium for continued growth.

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