ENUMERATION OF IRON BACTERIA

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Prepared for:

Microbiology Section
Public Health Engineering Division
Canada Center for Inland Waters
P.O. Box 5050
Burlington, Ontario
L7R 4A6

Prepared by:

Division of Applied Biology
B.C. Research
3650 Wesbrook Mall
Vancouver, B.C.
V6S 2L2

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SUMMARY

The literature was reviewed for publications concerning the iron bacteria subsequent to the review articles published in the early 1960's. An examination of this literature revealed that the iron bacteria could be placed in four major groups: the Sphaerotilus-Leptothrix group; Gallionella (stalked bacteria); the budding bacteria and the gram-negative chemolithotrophs.

The organisms of most interest to the microbiologists at the Canada Center for Inland Waters were the budding bacteria and the Siderocapsaceae a Family (of dubious validity) classed among the chemolithotrophic bacteria. These organisms appear to be organotrophic for the most part. They metabolize the organic portion of naturally occurring iron-organic complexes and are thus iron precipitators rather than iron oxidizers. They appear to have little else in common.

Attempts to develop a simple, reliable enumeration technique were unsuccessful due to lack of knowledge concerning any unique substrates or metabolic products produced by the various genera of bacteria capable of precipitating iron. Attempts to develop a technique capable of enumerating bacteria (if they even exist) which oxidize the iron portion of the complexes were also unsuccessful.

Preliminary studies were undertaken to evaluate existing procedures for growing Gallionella and to determine if a reliable counting technique could be developed. Studies were discontinued prior to completion when the focus of the project changed to the budding bacteria.

A simple reliable technique for enumerating the iron bacteria of interest cannot be developed based on present knowledge. The only procedure of any value is collection on membrane filters, selective staining and direct microscopic counting as practiced by workers in the Soviet Union.

INTRODUCTION

OBJECTIVE

To develop simple, reliable techniques for the enumeration of heterotrophic and autotrophic iron bacteria present in lake and river waters and sediments.

BACKGROUND

The staff of the microbiology laboratory of the Canada Center for Inland Waters is studying the role and the origin of transient and endemic populations in the sediments and overlying waters of fresh water lakes. As part of their studies they are interested in determining the density and distribution of various physiological groups of bacteria. In particular, they wish to establish the relationship between the bacterial populations and land use practice in the environs, as affecting the receiving waters in which the bacteria are found.

One physiological group which is of interest is the iron-oxidizing and iron-precipitating organisms. The existence of bacteria capable of oxidizing iron was recognized as early as 1836, and by 1964 at least three distinct groups of iron bacteria were recognized (1), i.e. the sheath- forming bacteria (the Sphaerotilus-Leptothrix group); the stalked iron bacteria (Gallionella); and the unicellular forms (which include the budding bacteria).

Recently two other genera of iron-oxidizing bacteria have been reported. The Brierleys (2) have isolated an autotrophic, acidophilic thermophile which they felt was similar to Sulfolobus (3), and Walsh and Mitchell (4) isolated acid tolerant, filamentous Metallogenium strains. This latter organism is morphologically similar to Gallionella, but it lacks a conventional cell body and is acid tolerant.

The staff of the microbiology section at the Canada Center for Inland Waters recognized that the extensive literature on the Sphaerotilus-Leptothrix group (1, 5, 6, 7, 8, 9, 10, 11, 12, 13) documents that these heterotrophic organisms are primarily associated with polluted waters. Their interest is primarily in the bacterial populations occurring in lakes and streams, particularly those organisms living at the air-water interface or the mud-water interface in relatively normal aquatic environments; i.e. they are not interested at this time in organisms such as Sulfolobus types which grow under thermophilic conditions or in Thiobacillus ferrooxidans which grows in an extremely acidic environment. The stalked organisms such as the Gallionella were deemed only of minor interest and so the study was planned to deal with iron-oxidizing unicellular organisms, the budding bacteria and any bacterial population whose growth is stimulated by iron in the aquatic environment.

EXPERIMENTAL METHODS

LITERATURE REVIEW

Background literature concerned with iron-oxidizing bacteria was surveyed by reference to recent review articles in this area such as the ones by Pringsheim (5, 6) and Mulder and his co-workers (1, 10, 11, 12) and in particular the work by Hirsch (14) on the budding bacteria. The more recent literature was surveyed by reference to Chemical Abstracts, Biological Abstracts and by means of the CAN/OLE computerized literature searching service.

LABORATORY STUDIES

Source of Inocula

Several samples of drainage water were collected from the University golf course in the environs of our laboratories, from an area where plugging of drain tile by iron bacteria had occurred in the past. Samples of unchlorinated water from the balancing water reservoir of the Greater Nanaimo Water District, water from the drainage tunnel of the Mica Dam on the Columbia River and seepage from the downstream side of the tailings dam on the Anvil Mine property at Faro, Yukon Territory were also obtained. A five-gallon sample of Hamilton harbour water and a 2-lb bag of mud was supplied by A. Kwan of the Canada Centre for Inland Waters.

Media

The standard medium was that of Kucera and Wolfe (15), either as modified by Wolfe (16) or by Nunley and Krieg (17). The medium of Winogradski (18) which contains ferric ammonium citrate was also used as a test medium. Variations of and modifications to these media were tested and details are listed in the text.

Incubation was at 20-22^{o}C, with or without carbon dioxide-enriched atmospheres, for up to 30 days.

LITERATURE SURVEY

SPHAEROTILUS-LEPTOTHRIX GROUP

The literature concerning the Sphaerotilus-Lepthothrix group of bacteria has been very contradictory, especially with regard to the ability of these organisms to oxidize iron. Mulder and his co-workers (1, 11, 12) concluded that Sphaerotilus was a heterotroph (chemoorganotroph) and that it probably did not oxidize iron although ferric iron would accumulate on its sheath under certain conditions. Leptothrix probably does not oxidize iron either but it does oxidize manganese (1, 11). In his 1968 review, Phaup (19), concluded that Sphaerotilus spp. grew heterotrophically and that the possibility of autotrophic growth remained to be proven. The 8th Edition of Bergey's Manual (20) states that Sphaerotilus and Leptothrix are chemoorganotrophs.

Rogers and Anderson (21) concluded that the available literature demonstrates that Sphaerotilus spp. are not autotrophic but are iron precipitators. In their studies, S. discophorus exhibited a characteristic temporal pattern of iron deposition, which was delayed until the latter portion of the exponential or the onset of the stationary growth phase. The growth rate was independent of the iron concentration in the medium and there was no correlation between iron concentration and final cell yield. Sessile populations derived no physiological advantage over free living cells and iron deposition occurred at the same rate in both populations. These authors felt iron deposition was mediated by certain constituents of the organisms' sheath.

Mac Rae and Celo (22) studied the influence of colloidal iron on the respiration of various "non-iron bacteria" and concluded that when they were growing in a high iron environment, iron precipitated on the cells and lowered the endogenous respiration rate of iron encrusted cells by 32 to 70% as compared to unencrusted cells.

In a subsequent study Rogers and Anderson (23) examined the following hypothesis concerning deposition of iron by S. discophorus:

- That the organism's capacity to remove iron might starve other competing organisms with high iron requirements.
- That iron deposition is useful in fulfilling the organism's physiological iron requirement under conditions of low exogenous iron.
- That iron deposition serves a protective function which might facilitate resistance to toxic concentrations of iron, manganese or other chemically similar trace elements.

They were able to show that none of these hypotheses were valid, but they could not come up with any specific function for the iron deposition phenomenon.

No satisfactory procedure exists for enumerating Sphaerotilus due in part to the filamentous style of growth. Mulder and van Veen (11) described procedures for isolating pure cultures and Armbruster (24) published an improved technique based on a medium of very low nutrient composition. It could possibly be used as the basis for an MPN technique. Grainge and Lund (25) developed a quick procedure for culturing iron bacteria, particularly those causing problems in water supplies, which was based on the use of a soft steel washer as an iron source. It would be cumbersome as an MPN technique.

In recent studies on the occurrence of Leptothrix ochracea in a fresh-water lake Jones (13) enumerated the organisms microscopically after passing known volumes of water through a 0.22 micron pore size, membrane filter. He found a relationship between filament numbers and iron concentration and the highest populations occurred in zones of very low oxygen concentration. Leptothrix isolated from de-oxygenated water had iron-free sheaths.

GALLIONELLA

Gallionella are chemolithotrophic; deriving their energy from the oxidation of ferrous iron, and fixing carbon dioxide. The organisms are microaerophilic and the daughter cells are motile (swarmers) before they become attached and form characteristic stalks.

The taxonomy of Gallionella has been reviewed by Balashova (26) who proposed three species. However, the 8th Edition of Bergey's which groups the Gallionella with the budding and/or appendaged bacteria, only recognizes two species: G. ferruginea and G. filamenta (20).

The stalk of Gallionella is coated with ferric hydroxide (27) and Mardanyan and Balashova (28) have shown that there are strong chemical bonds formed between the iron atoms and the organic structures of Gallionella fibres. They consider that the iron is actively bound.

Wolfe (16) has studied the Gallionella and has developed a medium for growing the organism in pure culture. The original medium as described by Kucera and Wolfe (15) consisted of 0.1% ammonium chloride, 0.05% dipotassium hydrogen phosphate, 0.02% magnesium sulphate, with precipitated ferrous sulfide as an energy source. Subsequently (16) calcium (0.01% calcium chloride) was found to be necessary for growth of Gallionella in this medium and that better results were obtained if the ferrous sulfide was incorporated in 3% agar slants. Nunley and Krieg (17) improved the medium by incorporating formalin to inhibit other organisms. However, no attempt was made by either group of investigators to quantitatively determine the numbers of organisms present.

In her extensive studies of the Gallionella, Balashova (29) either used undefined media in flow through equipment or the medium of Kucera and Wolfe (15). The major purpose of her studies was to obtain growth of pure cultures; not to estimate numbers. During this study she concluded that the iron coated stalks contained living material.

Recently Hanert has published a series of papers on the rate of growth of Gallionella under natural and laboratory conditions (30, 31, 32, 33). However, again no attempt was made to quantitatively determine the numbers present.

Balashova (34) has noted a similarity between Gallionella and a parasitic group of organisms named Mycoplasma. However she could not relate the Mycoplasma and the manganese-oxidizing organism Zavarzin (35) found in iron-rich waters. Zavarsin (35) described a method of growing Metallogenium but his studies did not indicate that this genus oxidized iron. Walsh and Mitchell (4) isolated in pure culture an acid-tolerant, iron-oxidizing organism which they described as an iron-oxidizing strain of Metallogenium. Their simple medium contained ferrous sulphate as the form of reduced iron and KH phthalate as a buffer. Formalin was added to inhibit Thiobacillus ferrooxidans, an autotrophic iron oxidizer.

THE BUDDING BACTERIA

The budding bacteria as a group contain a number of genera which precipitate and may oxidize iron. This group of organisms was the subject of a review published by Hirsch in 1974 (14). Hirsch reports that among the many genera which make up the budding bacteria the following either oxidize or precipitate iron:

Pedomicrobium - found in lake waters (usually eutrophic lakes); the stalk is usually encrusted with iron deposits.

Planctomyces - stalked bacteria in the shape of a drumstick; occur as planktonic organisms in eutrophic lakes; P. bekefii stalks are usually covered with iron deposits.

Blastobacter - encapsulated rod-shaped organsims which grow in iron-rich waters in forest brooks and ponds; probably heterotrophic.

Naumanniella - encapsulated rod-shaped organisms, found in brooks; appear golden yellow due to precipitation of iron compounds. Iron is not oxidized by this heterotrophic organism.

Siderococcus - very small coccoid organisms found in iron-rich waters and in mud. Older colonies are covered with iron oxides (usually only Fe_2O_3) but single cells are free of iron deposits.

Seliberia - rod-shaped, spiral organisms which form rosettes; found in soil and aquatic environments especially those rich in humic complexes: chemoorganotrophic; iron released by oxidation of humic complexes stains the cells.

Acholeplasma - a chemoorganotrophic organism that is able to oxidize iron during periods of active growth. The oxidized iron precipitates on the cells but it was concluded (34) that the oxidation was a secondary process of A. laidlawii's metabolic processes. Bergey's Manual (20) does not consider Acholeplasma to be budding bacteria but includes them under the Mycoplasma.

Caulococcus - a rarely found, poorly defined coccoid-shaped organism. Colonies are heavily encrusted with manganese and iron.

The budding organisms are usually studied by slide culture using natural environments. No attempts appear to have been made to enumerate the numbers of organisms with the exception of Dubinina (36) who estimated, by a capillary microscopy technique, that a brook water sample contained 12.4×10^3 cells of Naumanniella per ml. The adoption of any other technique is difficult because budding bacteria in general either form aggregates or attach themselves to a common holdfast.

GRAM-NEGATIVE CHEMOLITHOTROPHIC BACTERIA

This group includes the acidophilic organism, Thiobacillus ferrooxidans which is an inhabitant of all acid mine drainage streams but is not found in non-acidic lake waters. It is a true chemolithotroph which will grow on ferrous iron as well as on reduced sulphur compounds. It is normally counted by the MPN technique using the medium of Silverman and Lundgren (37) although Tuovinen and Kelly have published a membrane based procedure using ferrous iron agar (38). Recently Manning (39) has reported a technique for isolating the organism on a solid medium, which may be adaptable for enumeration.

Bergey's Manual (20) includes four genera of iron- and manganese-depositing organisms (the family Siderocapsaeae) among the chemolithotrophs primarily because they have nowhere else to put them. The organisms are mostly organotrophs and the cause of iron precipitation is the oxidation of the organic component of the organo-iron complexes frequently found in natural waters.

Siderocapsa are spherical to ovoid cells imbedded in a common capsule which is partially encrusted with iron and/or manganese compounds. Dubinina and Zadanov (40) have studied a pure culture of this genus and maintain it should be classified in the genus Arthrobacter of the Coryneform group of bacteria.

Other members of the Siderocapsaeae listed by Bergey include the Naumanniella which Hirsch (14) lists under the budding bacteria, Ochrobium, which are ellipsoidal to rod-shaped with a horseshoe shaped torus (marginal thickening) which is heavily encrusted with iron salts (Bergey notes this organism may be an algae), and the Siderococcus, which is a small motile coccus which again Hirsch has included in the budding bacteria.

Hardman and Henrici (41) studied the distribution of Siderocapsa in lakes and streams using the immersed slide technique and Drabkova (42) estimated the numbers in some lakes in the Soviet Union.

Dubinina et al (43) studied the vertical distribution of various micro-organisms in Lake Gek-Gel' and found massive development of Metallogenium and Siderocapsa in the chemocline zone where the oxygen disappears. The organisms were counted microscopically after being collected on membrane filters. The same technique, augmented by direct slide immersion, was used in a more recent study on Lasnaya Lamba, Karelia (44), to count Ochrobium, Siderococcus and Siderocapsa. They found that each type of bacteria, i.e. iron, sulfur, etc., occupied its own ecological niche. Maximum development of iron bacteria was independent of the depth of light penetration. It was influenced by the presence of oxygen and availability of reduced iron and manganese.

LABORATORY STUDIES

MEDIUM OF KUCERA AND WOLFE

Using this medium the presence of iron bacteria were confirmed in samples from the University golf course, and in the drainage samples from the Mica Dam. The cultures, which had the morphological appearance of Gallionella, were maintained as impure stock cultures of that organism.

The results with the Nanaimo Reservoir and Anvil Mines samples were ambiguous although the Anvil samples did contain filamentous organisms, probably Sphaerotilus. Motile rod-shaped organisms did appear in the medium and they could be maintained on repeated transfer but it could not be established clearly that they were Gallionella.

WINOGRADSKI'S MEDIUM

Winogradski's medium was made up according to the procedure of Rodina (18) and used as per Dutka et al (45) to enumerate the bacteria present in the Hamilton harbour water and mud. Samples from other sources were inoculated into this medium but no attempt was made to actually enumerate the number of organisms present.

Large rod-shaped motile bacteria were isolated from all water samples examined. These organisms grew very quickly, i.e. within 48-72 hr, and caused the precipitation of ferric iron. The Hamilton harbour water gave a count of 7,900 per 100 ml and the drained mud a count of 350,000 per 100 g wet weight by the MPN technique.

Since the iron in Winogradski's medium is in the ferric form, the bacteria involved were not iron oxidizers but iron precipitators. The bacteria destroyed the ferric citrate complex, which maintained the iron in solution, by metabolizing the citrate component.

When ammonium citrate was substituted for the ferric ammonium citrate in Winogradksi's medium, the isolated bacteria, as well fresh isolates from the original samples, grew as well as on the original Winogradski's medium. That is, the organisms were growing on the citrate and the iron precipitated only because the citrate had been metabolized.

MODIFICATIONS TO THE MEDIUM OF WOLFE

The medium of Wolfe was inconvenient to prepare and had a short shelf life due to the sensitivity to oxidation of the ferrous sulfide component. To try and overcome this disadvantage various other forms of iron were

substituted, i.e. pea-sized granules of ferrous sulfide; pyrite from Noranda Mines; washed iron washers; ferrous phosphate; ferrous carbonate. The pyrite was used as received or as ball-milled material (-400 mesh). The sources of iron were both slanted in agar and added to the bottom of the test tube under the liquid medium.

When the various media were examined microscopically, a mixed population was always encountered; primarily rods, many of which were motile. The rods were of various shapes and some coccoid forms were always present although they may just have been short rods.

Although the level of soluble iron varied between the various media no effect could be observed on the type or nature of the organisms present in the medium. Attempts to monitor the disappearance of ferrous iron with o-phenanthroline were unsuccessful. Some residual ferrous iron was always present, vitiating the use of a simple color change test.

After repeated transfer in the medium of Wolfe (without formalin) and various modifications of same, the population of Gallionella forms plus motile rods gave profuse growth on plates containing plate count agar indicating a heterotrophic population was being maintained without the addition of an energy source.

The most likely source of traces of organic matter was the agar and so Ionagar 2 was used to slant ferrous sulfide, ferrous phosphate or ferrous carbonate as the iron source with Wolfe's medium. Typical Gallionella types were observed and the populations of other organisms appeared to be reduced.

It was concluded from these studies that the medium of Wolfe, particularly when used with formalin, as suggested by Nunely and Krieg, was an effective way to determine the presence of Gallionella. A variety of iron sources could be used. However it appeared that large inocula were needed to achieve growth; visible growth seldom occurred at dilutions beyond 1-100 whereas the presence of the bacteria could be observed microscopically. The procedure did not seem to be applicable for enumerating Gallionella by the MPN technique.

OXIDATION OF CHELATED IRON

By popular usage the iron bacteria include true chemolithotrophs as well as those organotrophs which become encrusted with iron for any reason, known or unknown. In an attempt to determine if such chemolithotrophs, other than Gallionella, were present in the water samples available, several experiments were carried out with a variety of ferrous iron compounds and chelating agents. The objective was to try and grow the organisms on soluble ferrous iron at pH levels where iron is not significantly soluble unless complexed or chelated.

Ferrous iron was chelated with EDTA, nitrilotriacetic acid (NTA), potassium hydrogen phthalate, citrate and peat and used directly as the ferrocyanide salt.

Iron chelated with EDTA or NTA plus the mineral salts used by Wolfe supported a population of thick rods and cocci. However when iron was left out of the various media, similar populations appeared, indicating that the organisms were living on the organic component. Similar results occurred when the iron was chelated by citrate.

The phthalate plus iron medium was unsuitable for growth tests because of the extensive iron precipitation that resulted during autoclaving. The ferrocyanide complex gave indeterminate results in that populations could not be maintained on repeated transfer. Peat was not a satisfactory chelating agent inasmuch as it did not keep the iron from precipitating in uninoculated media.

DISCUSSION

The literature survey carried out as a major portion of this study has shown that there are four main groupings of iron-oxidizing bacteria, two of which, the Gallionella and the gram negative chemolithotrophs, are mostly chemolithotrophic, i.e. they get their energy for growth from the oxidation of ferrous iron. The other two groups, the Sphaerotilus-Leptothrix and the budding bacteria appear to be organotrophs. As the project advanced it became evident that at that time the organisms of most interest to the microbiologists at the Canada Center for Inland Waters were the budding bacteria and the Siderocapsaceae, with the Gallionella of lesser interest and the Sphaerotilus-Leptothrix group and the acidophilic chemolithotrophs of no interest.

Relatively specific media for Gallionella exist at the present time, based on the ability of the organism to oxidize ferrous iron. The actual source of the iron has little effect on the growth of the organism and a variety of insoluble iron sources can be used. The use of formalin, as suggested by Nunley and Krieg (17), reduced the level of extraneous organisms and caused growth to occur deeper in the tube. A microaerophilic environment is obviously necessary.

Since the presence of oxygen-utilizing contaminants helped maintain a microaerophilic environment most studies were done without adding formalin. Nevertheless our attempts to develop a statistically reliable MPN procedure for counting Gallionella were without success. The organism did not appear to develop when the field samples were diluted more than 100 fold. Planned studies using the laboratory cultures with and without the addition of formalin were not carried out when the emphasis of the study was shifted away from the Gallionella.

The available literature suggests that most, if not all, of the organisms of specific interest are organotrophs which precipitate iron by oxidizing the natural organic components that maintain iron in solution. As shown by Mac Rae (22, 46, 47) organisms capable of doing this are not always the so-called iron bacteria. In their studies on water supplies (46) they found that strains of Pseudomonas, Moraxella, Alcaligenes, Acinetobacter and Vibrio precipitated iron when grown on ferric ammonium citrate agar but only Pseudomonas and Moraxella caused iron to precipitate in a liquid medium. The others modified the gallate complex in some way but iron did not precipitate.

This characteristic of these iron bacteria, to cause iron precipitation by utilizing the organic component of an iron-organic complex, makes the development of a specific medium very difficult. Such a medium would be keyed to the ability of bacteria to oxidize some organic substrate, i.e. gallate, citrate, humic acids, etc. This approach seemed of little inherent value and lacking alternative approaches, work was stopped.

The available evidence implies that iron precipitation is caused by bacterial metabolism of the organic portion of natural chelates. However it is unclear whether the associated iron is oxidized chemically or biologically. One approach that seemed to be worth following was to determine if bacteria do exist in lakes, streams, drainage and sediments that grow by oxidizing the ferrous iron component of naturally occurring iron-organic complexes. Perhaps these organisms could be identified if the iron in solution were complexed with a compound which was resistant to bacterial attack.

The literature was consulted with regard to chelating agents for iron and four were selected for examination. EDTA, NTA, 8-hydroxy -5 quinoline sulfonic acid and cyanide. EDTA and NTA did not prove to be satisfactory since they were utilized by the bacteria when iron was absent. A sample of the sulfonic acid could not be obtained soon enough for inclusion in the study.

Experiments using the tightly bound ferrocyanide complex were initially inconclusive. Growth occurred (as determined by microscopic observation) following inoculation from the various test waters but it could not be maintained on repeated transfer. It was finally concluded that the initial growth was attributable to compounds carried over with the inoculum and so experimental work was terminated. This problem of growth due to carry over of organic material may complicate any procedure developed for enumerating iron-oxidizing organisms, assuming that they do exist.

Many of the iron bacteria appear to develop in zones of low oxygen tension which suggests that the presence of reduced iron is physiologically important. However no information appears to exist as to what that role might be.

The only established technique for collecting and enumerating the iron bacteria of interest appears to be collection on membrane filters followed by selective staining and counting microscopically (42, 43, 44). This technique is valid only for free swimming organisms since to measure sessile forms they must be dislodged from their stationary attachments. The immersion of slides and electron microscope grids is another technique which may have some application in indicating if certain morphologically recognizable types of bacteria are present (36). However it is tedious and does not provide a ready means of determining numbers.

The objective of this investigation - to develop simple reliable techniques of enumeration - has not been achieved. A technique based on the oxidation of iron does not seem to be the answer. The organisms of interest metabolize organic matter, frequently organic matter which chelates iron, and thus any simple technique must be based on a single unique substrate or the production of a unique product. There is insufficient knowledge available at the present time concerning these organisms to suggest what such substrates or products should be.

D.W. Duncan
Division of Applied Biology

C.C. Walden
Head, Division of Applied Biology

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