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# Siderocapsa Major-- Fact or Artifact?

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SIDEROCAPSA MAJOR-- FACT OR ARTIFACT?

(TITLE)

BY

Ray W. Swanson

**THESIS**

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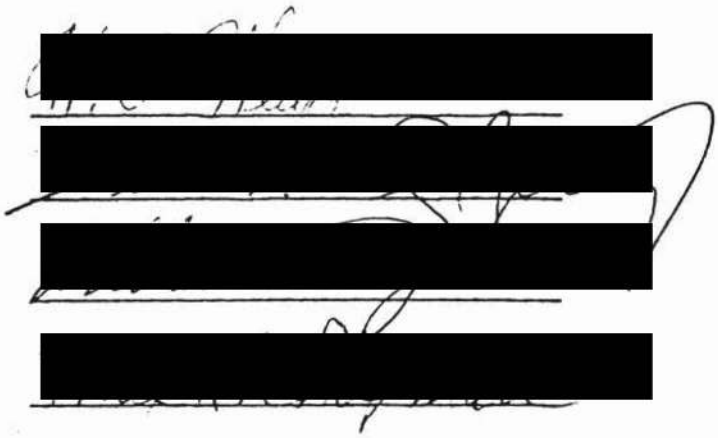
*Siderocapsa Major*-- Fact or Artifact?

Presented by

Ray W. Swanson

A Candidate for the Degree of Master of Science

And Hereby Certify That, In Their Opinion, It Is Acceptable



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## ABSTRACT

Swanson, Ray W. M.S., Eastern Illinois University. August 1979. *Siderocapsa major*-- Fact or Artifact? Major Professor: Dr. William A. Weiler.

The validity of the genus *Siderocapsa*, a group of unicellular iron-depositing bacteria, has been a subject of debate for many years. Lack of photomicroscopic and cultural studies has kept this group in taxonomic obscurity. Bacteria were collected from the Campus pond, Eastern Illinois University, Charleston, Illinois, by a submerged slide/coverslip technique. These bacteria were identified as *Siderocapsa major* according to the description of Molisch (1909). Scanning electron microscopy of *Siderocapsa major* microcolonies has shown cells (0.5 by 1.15 µm), in a clear central well (2-6 µm), surrounded by an amorphous material which accumulates with the age of the colony. Most authors have failed to explain how they have concluded that the material sequestered by *Siderocapsa* microcolonies is iron and/or manganese. Preliminary work employing the Prussian blue Reaction (and modifications) failed to demonstrate the presence of iron in the capsules around these bacteria. X-ray microanalysis also failed to demonstrate the presence of iron. However significant amounts of manganese were found to be present in the extra-cellular material.

Some investigators have suggested that *Siderocapsa* microcolonies are artifacts and the result of rod-shaped bacteria that have become entrapped in the well of a *Sphaerotilus natans* holdfast whose filament

has become dislodged. Scanning electron microscopy has shown that the size and morphology of the holdfast of Sphaerotilus-Leptothrix members does not correspond to the size of the deposit around the Siderocapsa microcapsule. In addition, statistical analysis has shown a definite developmental pattern relating the total number of cells in the microcapsule to the total amount of material deposited.

The author concludes, from material observed here, that the Siderocapsa microcolonies are discretely different entities from members of the Sphaerotilus-Leptothrix group but may be related to other heterotrophic bacteria.

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# INTRODUCTION

Iron is present in natural waters in solution and in particulate flocs. The universal occurrence of iron deposits in water system pipes and on the surfaces of leaves of aquatic plants suggests the presence of bacteria which have the ability to sequester iron.

The iron bacteria are capable of depositing ferric or manganic oxides without regard to the physiological significance of this function to the bacteria themselves. Because of this the term iron bacteria refers not to a taxonomic group but to an ecological group. This is exemplified by the diversity in habitat seen among the members of this group. Chemoautotrophic species exist in acid mine waters. Heterotrophic iron bacteria are found in neutral to alkaline waters. These two types have essentially nothing in common except the ability to accumulate iron and/or manganese.

Much confusion has evolved from attempts to taxonomically identify the heterotrophic members, specifically the Sphaerotilus-Leptothrix group and the Siderocapsaceae. Ambiguous results and conflicting reports from cultural studies have not reduced the confusion. The validity of the genus Siderocapsa, first described by Molisch in 1909, has been a subject of debate for many years. Lack of photomicroscopic and cultural studies has kept this group in taxonomic obscurity.

This study will attempt to answer several questions concerning the siderocapsas. First, what is the true morphology of these bacteria? This researcher finds the illustrations and transmission electron

micrographs of Siderocapsa now in the literature vague and easily misinterpreted. A second question concerns the validity of this genus. Are these bacteria artifacts as some have suggested? Photomicroscopy and cultural studies will be used to answer this question. Finally, do these bacteria indeed accumulate iron and manganese? Nowhere is there conclusive evidence of their ability to do so although it has always been assumed. Through microchemical and X-ray analyses a characterization of the material deposited around these bacteria will be made.

# HISTORICAL

## Types of Iron Bacteria

Transformations of iron by oxidation or reduction in natural waters occur either biologically or non-biologically. Non-biological or auto-oxidation -reduction of iron or ferrous compounds occurs where the redox gradient is characterized by a pronounced difference between reducing (low pH and Eh) and oxidizing (high Eh, high pH, and by free $O_2$) conditions. As a rule oxidizing conditions exist at a reduction intensity (rH) above 25, which means a redox potential of at least +390 mv, at a pH of 6.0 (Glathe and Ottow, 1972)..

Biological transformation of iron may occur in several ways. Heterotrophic microorganisms may be responsible for the oxidation or reduction of iron and iron-containing compounds. Such microorganisms may alter the environmental conditions making it favorable for iron to exist either in solution or as a precipitate, by lowering or raising the acid content of the water, changing the oxidation-reduction conditions, and/or varying the $CO_2$ content of the water. These microbes may also produce and decompose organic compounds of iron. Organic iron compounds which are slightly ionized tend to remain in solution, but decomposition of slightly ionized iron compounds will cause iron to precipitate (Starkey, 1945). The importance of these microorganisms in iron transformations must not be underestimated. Any bacterium, actinomycete or mold capable of utilizing the organic portion of the iron compound may be involved. Most of these organisms are nutritionally inexacting, and large portions of these flora remain

metabolically active in a wide pH range (Glathe and Ottow, 1972).

In addition to non-specific bacteria, the chemoautotrophic sulfur bacteria and sulfate-reducing bacteria also play a part in iron transformations in water. The sulfur bacteria, as a group, are characterized by their ability to oxidize sulfur or various sulfur compounds generally to sulfate, and to utilize these reactions for their energy requirement. These bacteria may bring iron into solution by lowering the pH or may precipitate it by producing insoluble iron-sulfates.

One common sulfur bacterium which may bring large amounts of iron into solution is Thiobacillus thiooxidans, an aerobic bacterium capable of tolerating a low pH and able to produce acid as well. Thiobacillus ferrooxidans (also known as Ferrobacillus ferrooxidans and Ferrobacillus sulfooxidans) was first cultivated and described by Temple and Colmer (1951). This bacterium has been observed to oxidize ferrous iron as well as sulfur (Temple and Colmer, 1951; Razzell and Trussell, 1963; Tuovinen and Kelley, 1972) and has been found in an acid environment of pH 1.6 (Temple and Colmer, 1951). Maintenance on a sulfur containing medium does not lead to loss of the ability of this organism to utilize ferrous iron nor does prolonged cultivation on an iron medium lead to loss of ability to oxidize sulfur compounds (Touvinen and Kelley, 1972).

Another method of iron transformation in waters is by oxidation of inorganic ferrous compounds by heterotrophic "iron organisms", as far as is known without any chemoautotrophic energy gain. It is with the members of this group that this investigation is primarily concerned. Bacteria of this type include the stalked bacterium Gallionella, some members of the sheathed bacteria, specifically members of the Sphaerotilus-Leptothrix group, and some unicellular iron bacteria of questionable taxonomy, members

of Siderocapsaceae (Bergey's Manual of Determinative Bacteriology, 8th edition).

Gallionella ferruginea is described as a kidney-shaped cell on the end of a long stalk with the long axis of the cell transverse to the stalk. Ellis (1919, as cited by Pringsheim, 1949a) described the stalks of Gallionella as living filaments reproducing by fragmentation and budding.

Cholodny (1926, as cited by Pringsheim, 1949a), by suspending cover-slips in its natural habitat, described the cells as loosely attached to the end of the stalks. These cells easily break away and therefore will not be seen in preparations made in the usual way.

Now it is generally assumed that the stalk is non-living and is produced by excretion of products from the cell itself (Bergey's Manual of Determinative Bacteriology, 8th edition). However, as late as 1968 Hanert proposed that the stalk was a living filament (as cited by Bergey's Manual of Determinative Bacteriology, 8th edition).

The stalks of Gallionella are formed of bundles of fibrils and are commonly ribbon-shaped and twisted. They may often be seen to branch. Characteristically ferric hydroxide may be precipitated on the stalk, but manganese compounds are not deposited by this organism. Electron microscope studies have revealed extreme polymorphism in stalk morphology of this bacterium (Bergey's Manual of Determinative Bacteriology, 8th edition).

Gallionella ferruginea is widely distributed in iron bearing waters. The stalk is a flat twisted band 0.4-1.0 μm in width, formed by numerous (up to 90) fibrils. The stalks often are dichotomously branched. On the other hand, Gallionella filamenta (Balashova, 1967) is rarely found in iron bearing waters. Morphology of this species is basically the same as that

for the genus. However the stalks are formed by usually less than twelve cylindrical fibrils having the appearance of spirally twisted hairpins (Bergey's Manual of Determinative Bacteriology, 8th edition).

The taxonomic position of Gallionella is uncertain. Zavarzin (1961) proposed that Gallionella may be related to Metallogenium, a manganese oxidizing organism whose taxonomy itself is questionable. Dubinina (1969), on the basis of cultural studies, proposed that Metallogenium should be included among the Mycoplasmatales.

## Taxonomy of the Heterotrophic Iron Bacteria

The Sphaerotilus-Leptothrix group of the sheathed bacteria is a small natural group of bacteria characterized by cells arranged in chains or filaments which are covered by a sheath of organic matter. In or on this sheath are deposited, under certain environmental conditions, precipitates of inorganic nature, usually ferric hydroxide, although manganese oxides are precipitated as well.

The first sheathed bacterium to be discovered was Sphaerotilus natans (Kutzing, 1833). It was found to occur in polluted running waters as greyish white tassels attached to woodwork and other substrata (as cited from Pringsheim, 1949a). In general Sphaerotilus natans Kutzing may be described as a thread-forming bacterium without spores. The rods are 1.0 µm wide, 2-6 µm long, Gram-negative and often with refractive granules (oil and volutin). Young filaments are naked (without a sheath) and difficult to observe. Around older filaments the sheath may be rigid and brittle, up to 1 µm thick, refractive and colorless to yellow. Although they are easily broken free, filaments are often attached to the substratum by a mucilaginous holdfast. Reproduction is by fragmentation of naked filaments or by the production of swarm cells with subpolar flagella. Swarmers are found

only when the sheath is soft and are liberated by its swelling and disintegrating (Pringsheim, 1949a).

In 1870 Cohn ( as cited from Pringsheim, 1949b) recorded observations on a filamentous iron bacterium which he called Cladothrix dichotoma. This organism was found in generally the same habitat as Sphaerotilus and was morphologically different from Sphaerotilus in that it was characteristically dichotomously branched. Cohn (1870, as cited from Pringsheim, 1949b) makes no inference as to the taxonomic position nor does he speculate on the origin of the iron compounds present.

Pringsheim (1949a), from evidence using pure cultures (which Cohn failed to obtain), states that Cladothrix is an interconvertible biotype with Sphaerotilus. He found that when the sheath was more rigid a single rod would slip sideways and grow into a filament which remained connected to the original thread by a continuation of the sheath. Thus false-branching could occur. Pringsheim (1949a) goes on to conclude that the S. natans form is characteristic of a rich supply of nutrients whereas the Cladothrix dichotoma form appears when the organism is grown on a medium poor in nutrients. Although iron was not visibly detected on the sheaths of these organisms its presence was demonstrated through use of the Prussian blue test.

Leptothrix ochracea, first described by Kutzing (1843), is also considered by Pringsheim (1949a) to be a form of S. natans. He states that the sheaths of older parts of the filaments become very brittle and are easily broken into bits which are readily perceivable as a bottom rust of red color containing a few cells. The L. ochracea type appeared after slow growth in hay infusion containing ferrous ammonium citrate (0.01-0.02%).

Due to the fact that Pringsheim was unable to isolate L. ochracea from natural habitats, some authors (van Beneden, 1951, Beger and Bringmann, 1953, Charlet and Schwartz, 1954, as cited from Mulder and van Veen, 1963; van Veen et al., 1978) have disputed Pringsheim's conclusion that L. ochracea is a variant of S. natans. Other investigators who claimed to have isolated this organism (Cataldi, 1939, Prave, 1957, as cited from Pringsheim, 1949a) actually dealt with another member of the Leptothrix genus.

Recent evidence (photomicrographs) presented by van Veen and co-workers (1978) suggests that S. natans and L. ochracea are indeed valid species. Using pure cultures of S. natans and crude cultures of L. ochracea they observed that the sheaths of older S. natans cultures were covered and sometimes encrusted with ferric hydroxide giving a granular surface, as contrasted with the smooth surface of L. ochracea sheaths. The latter were shorter than the S. natans sheaths and they were empty for more than 90% of their length, whereas the envelopes of S. natans were filled with cells to a much larger extent. Upon attempting to isolate S. natans from L. ochracea cultures, they were unable to do so. This is in direct conflict with Pringsheim (1949a, 1949b) who states that by changing the medium content he could produce cells of either S. natans type or L. ochracea type.

The iron bacterium Leptothrix discophora may be described as rods 1-4 μm in length (shorter than other members of the Sphaerotilus-Leptothrix group) and motile cells which may be either surrounded by a sheath or free swimming. The naked sheath diameter is approximately 3 μm, comparable to that of Sphaerotilus. This bacterium has the ability to oxidize manganese as well as iron. The sheath becomes covered with a thick, dark-brown, fluffy layer of ferric and manganic oxides which, at this stage, increase the total

sheath thickness to a diameter of 20-25 μm (van Veen et al., 1978). Under these conditions the sheaths may taper towards their tips (Pringsheim, 1949a; van Veen et al., 1978). Whether false-branching occurs in Leptothrix discophora is not quite clear. Cholodny (1926, as cited by van Veen et al., 1978) states that false-branching may occur in natural habitats while others (Pringsheim, 1949a; van Veen et al., 1978) tend to be skeptical.

According to Pringsheim (1949a) this bacterium was erroneously studied by Zopf (1882) under the name of Cladothrix dichotoma. Pringsheim (1949a) also noted that it was necessary for Molisch (1910) and Lieske (1919) to add manganese to their media in order to cultivate this organism. Actually, according to Pringsheim (1949b), Cataldi (1937) was the first investigator to actually culture Leptothrix ochracea. Cholodny (1926, as cited by Pringsheim, 1949b) changed the name of this iron/manganese oxidizer from Megalothrix discophora (as proposed by Schwers, 1912) to Leptothrix crassa. This name lasted until 1934 when it was changed to Leptothrix discophora by Dorff. Pringsheim (1949a, 1949b) claims that the morphological differences between this organism and Sphaerotilus were not enough to warrant a separate genus so he proposed that it be changed to Sphaerotilus discophora. Present classification however, has retained the genus Leptothrix (Bergey's Manual of Determinative Bacteriology, 8th edition).

According to van Veen and his collegues (1978) the description of Sphaerotilus discophora given by Rouf and Stokes (1964) does not fit with the morphological features of Leptothrix discophora. They have stated that the organism described is more closely related to Leptothrix cholodnii.

Leptothrix cholodnii was first described by Mulder and van Veen (1963) and is found in non-polluted and polluted waters, as well as in activated sludge. The sheath of this bacterium when grown in the presence of

$Mn^{++}$ becomes irregularly encrusted with manganic oxides up to and exceeding 20 μm in diameter. The behavior of this organism in culture resembles that of S. natans as to growth rate, amount of cell material produced, and amounts of intracellular storage compounds formed (van Veen et al., 1978). However, researchers in the laboratory of van Veen (1978) found this organism to differ in cellular diameter as well as in sheath morphology, flagellation, manganese oxidizing ability and tendency to form sheathless mutants spontaneously. The authors suggested that, from Cataldi's (1939) description of Leptothrix winogradskii, she was actually dealing with this organism.

In the literature there occurs a considerable amount of disagreement concerning the validity of Clonothrix and Crenothrix as separate genera.

Roze (1896, as reported by Pringsheim, 1949a) described an organism which he named Clonothrix fusca. He observed the presence of a green pigment and thus classified it as a blue-green alga. No one since has been able to observe any green pigmentation although Kolk (1938) states: "The cells within the sheaths of Clonothrix fusca are colorless although an almost imperceptible greenish tinge was observed by me in one instance in the cells at the tip of a filament."

Schorler (1904, as cited by Pringsheim, 1949a) gave the same name to an organism morphologically similar to that described by Roze (1896). Yet he made no mention of this in his work. Bergey's Manual of Determinative Bacteriology ($6^{th}$ edition) supposes that the two forms seen by Roze (1896) and Schorler (1904) are identical and that the latter chose the same name by coincidence! Molisch (1910, as reported by Pringsheim, 1949a) gives an account of an organism he calls Clonothrix fusca although his description is not like any previous descriptions. According to Pringsheim (1949a) in

his monograph on the iron bacteria, Lieske's (1919) physiological work on iron organisms depicts a mixture of Crenothrix polyspora with the Cladothrix form of S. natans as Clonothrix fusca. Cholodny (1926, as reported by Kolk, 1938) suggests that his Leptothrix crassa (Leptothrix discophora) is the organism Naumann (1921) called Clonothrix fusca Roze.

Crenothrix polyspora, first observed by Cohn (1875) was considered by Pringsheim (1949a) to be a valid species. Much of the early work done on Crenothrix, according to Pringsheim (1949a), is supported with idealized drawings (Migula, 1900; Dorff, 1934). Adler (1904, as cited by Pringsheim, 1949a) tried to culture Crenothrix but was unable to obtain multiplication. Rossler (1895, as cited by Pringsheim, 1949a) claims to have cultured Crenothrix on bricks with traces of ferrous ammonium sulfate. Rulmann (1912, as cited by Pringsheim, 1949a) also claims to have cultured Crenothrix, using iron ammonium nitrite, which does not exist (as noted by Pringsheim). Other workers (DeVries, 1890, as cited by Pringsheim, 1949a; Lieske, 1922) show illustrations of Crenothrix although more than one type of organism appears to be present. Pringsheim (1949a) claims these to be modifications of Sphaerotilus.

Kolk (1938) claims to have differentiated between Clonothrix fusca and Crenothrix polyspora. She was unable to grow these organisms on culture media, but her observations on material from natural waters seem to show a clear differentiation between these two organisms. She describes Clonothrix fusca as being false-branched whereas Crenothrix polyspora is unbranched. Clonothrix filaments taper to the tip as they get older. She also states that the sheath itself gets thicker. In Crenothrix the sheath may be thicker at the apex or narrow depending upon spore arrangement. The sheath of Crenothrix invariably tapers at the base. The sheaths of Clonothrix are colorless

when young turning more yellow and becoming more brittle as they take on more ferric oxides. This is opposed to the Crenothrix sheaths which remain thin and colorless. Cells of Clonothrix are 2-10 μm long whereas the cells of Crenothrix are much smaller (2-5 μm). Finally, spore formation in Clonothrix is limited to short branches at younger portions of the filaments, whereas spore formation in Crenothrix may occur anywhere in the sheath with the spores being much smaller and more numerous than those of Clonothrix.

Pringsheim (1949a) evidently decided not to consider Kolk's (1938) description of these two organisms. Present classification (Bergey's Manual of Determinative Bacteriology, 8th edition) does accept both Clonothrix and Crenothrix as valid genera. Beger and Bringman (1953) compared Clonothrix fusca with Glaucothrix putealis (Kirchner, 1878) and on the basis of similar size and false-branching, concluded that Clonothrix fusca was a later synonym of Glaucothrix putealis. At present a decision has not been made on this matter pending more studies and until one, preferably both, has been pure cultured. Clonothrix fusca remains the type species.

Leptothrix lopholea (Dorff, 1934) is morphologically similar to S. natans with respect to (1) formation of holdfasts, (2) development of subpolar flagella and (3) formation of false-branches (van Veen et al., 1978). False-branching occurs in rich media, which is in contrast to S. natans where it occurs in poor media. Cells normally occur in a cluster of filaments radiating from a cluster of holdfasts. The accumulation of manganic and ferric oxides is usually more pronounced on the cluster of holdfasts than on the filaments. According to van Veen and co-workers (1978) these holdfasts show an identical morphology to those described for L. sideropous (Cholodny,

1926; Dorff, 1934; Cataldi, 1939). It is generally assumed that L. sideropous is probably synonymous with the accepted species (Bergey's Manual of Determinative Bacteriology, 8th edition).

Within this group of morphologically heterogeneous iron bacteria, the encapsulated unicellular forms have been included in the family Siderocapsaceae Naumann. Much controversy has surrounded the validity of the genera in this family. Molisch (1910, as reported by Buchanan, 1970) first observed and noted that these unicellular, encapsulated bacteria occurred as irregularly-shaped, more or less lobed brown deposits on leaves of various water weeds (Elodea, Nymphaea, Sagittaria, Salvinia, etc.) which were perforated at various points where coccoid bacteria settled. Molisch (1909) named this organism Siderocapsa treubii. He described these bacteria as unicellular, colorless, non-sporulating, spherical organisms 0.4-0.6 µm wide. One to six or more cells may occur at random in a primary capsule 5-20 µm wide. The clear central well, where the cells are located, is described as being 1.8-3.6 µm in diameter. Siderocapsa major, also described by Molisch (1909) is very similar morphologically except the bacteria are short rods 0.7-1.8 µm long. The diameter of iron deposition is described as being anywhere from 5-28 µm. The central well area, according to Molisch, may contain anywhere from 2-100 cells. The 8th edition of Bergey's Manual of Determinative Bacteriology lists six species in addition to the two previously mentioned. S. monoica (Naumann, 1921) has the same form as S. treubii except that only a single cell is found in the capsule. S. anulata (Kalbe et al., 1965) has a capsule that is moderately thick when embedded with iron compounds. The diameter of iron depostion is 1.2-1.9 µm with the central well having a diameter of 0.65-1.9 µm. The edge often has a cog wheel appearance. Normally 1-2 cells are present inside the capsule.

Siderocapsa geminata (Skuja, 1956) has been found in the hypolimnion of some Swedish lakes and in the epilimnion at the time of vernal and autumnal circulation. Roundish or oval cells are in pairs although single cells can be observed after division of a colony. The roundish capsule is 7-11 μm in diameter, homogeneous and colorless at first, later becoming stratified concentrically and brownish due to impregnation with iron and manganese compounds (Bergey's Manual of Determinative Bacteriology, 8th edition).

Siderocapsa coronata (Redinger, 1931) is described as containing 2-8 coccal cells in an irregularly spherical capsule which may attain a diameter of 24 μm. This organism is generally a neustic form of surface layers in alpine or subalpine lakes and pools. Up to 40 colonies aggregate in a zoogleal mass.

Siderocapsa albergensis (Wawrik, 1956), a planktonic form, commonly has one to four spherical cells per capsule, 0.4-1.0 μm each (Bergey's Manual of Determinative Bacteriology, 8th edition).

Siderocapsa eusphaera (Skuja, 1948) is another planktonic form. Here the older cells have been observed to contain a central gas vacuole. Cells may attain a diameter of 2 μm with up to 60 cells per capsule. Capsule diameter ranges from 10-50 μm and is usually stratified with two to three concentric brownish layers due to presence of iron compounds. The capsules generally occur singly and are free floating (Bergey's Manual of Determinative Bacteriology, 8th edition).

Bergey's Manual of Determinative Bacteriology (8th edition) currently lists three other genera of the Siderocapsaceae. The distinguishing characteristics among these genera are primarily morphological as opposed to physiological differences, due to the fact that most of these organisms have never been grown in culture media. Genus Naumanniella (Dorff, 1934)

is characterized as rod-shaped cells. In this genus the oxides form a delicate sheath (torus) which emphasizes the margin of the cell. In Naumanniella the torus completely surrounds the cell whereas in Ochrobium (Perfil'ev, 1921) the torus is open at one end, resembling a horseshoe. A fourth genus, Siderococcus (Dorff, 1934), is described as coccoid cells which deposit iron but not manganese oxides.

Other genera, Siderosphaera (Beger, 1949), Sideronema (Beger, 1941), Sideromonas (Cholodny, 1922) and Siderobacter (Naumann, 1921) have not been included in the 8th edition of Bergey's Manual presumably due to the overlap and variety of morphological characters not only among these genera but within each genus as well.

As was mentioned earlier, it can be seen that aside from morphological characteristics there has been no strict criterion for subdividing this family into genera as well as for subdividing each genus into separate species. Several workers have questioned the validity of these genera of unicellular, iron-oxidizing bacteria (Pringsheim, 1949a; Skerman, 1967; Hirsch and Pankratz, 1970; Dubinina and Zhdanov, 1975).

Earlier it was noted that most probably in neutral and alkaline waters the iron-depositing organisms release the iron by metabolizing the organic substance with which it is chelated. Skerman (1967) points out that almost any cell that metabolizes citrate will deposit iron around itself in a ferric ammonium citrate medium. Hence, he doubts the validity of Siderocapsa as well as the other genera previously mentioned.

Pringsheim (1949a) doubts the validity of Siderocapsa treubii Molisch, stating that Molisch (1909) probably observed the holdfasts of some other organism impregnated with iron compounds. Pringsheim (1949a) compares Molisch's (1909; 1910, p. 11) structures as being strikingly similar to

holdfasts depicted by Skuja (1948, pl. VI, Fig. 21) of Colacium sideropous, or to those of what Molisch (1910, p. 14) describes as Chlamydothrix sideropous.

Kolkwitz (1914, as cited by Pringsheim, 1949a) found structures similar to S. treubii Molisch on Elodea, Vaucheria and Cladophora and warns not to confuse them with holdfasts of filamentous algae. His illustration (p. 89, Fig. 11, as reported by Pringsheim, 1949a) is more similar to S. major than to that of S. treubii.

Hirsch and Pankratz (1970) upon obtaining transmission electron micrographs of what appear to be Siderocapsa colonies also expressed doubt as to the validity of the genus. The authors state (p. 595):

> In the presence of ferrous iron and at near neutral pH we have observed extensive depositions of ferric hydrate on the mucoid holdfasts. This is especially the case with Sphaerotilus filaments, which become separated from the well-shaped, mucoid structures at the attachment site. These wells often trapped rod- or coccal-shaped bacteria, thus resembling microcolonies of the long-known iron bacteria Siderocapsa or Siderococcus. In our case iron deposition onto the mucoid wells had occurred before these bacteria had arrived. There were no iron depositions on their cell surfaces, and these bacteria were thus identical to large numbers of cells present outside of the wells.

However, after careful observation and a comparison between the sizes of his Sphaerotilus filament with holdfast (p. 596) and with the Siderocapsa colony (p. 603) one can see that the diameter of the central well of Siderocapsa is much too large (4.0 µm) to accommodate the Sphaerotilus filament (0.5-0.75 µm diameter). Furthermore, this investigator questions how the authors determined that iron deposition on these "Siderocapsa-like" colonies occurred before the bacteria arrived. By virtue of electron microscopal methods alone this would be impossible to know; all material is fixed therefore the cells could not be observed entering or leaving the wells.

Dubinina and Zhdanov (1975) have also questioned the validity of Siderocapsa as a separate genus. They claim to have isolated two strains of Siderocapsa eusphaera Skuja, thus far the only members of this genus ever to have been grown in culture. They found the morphological variations of these strains, when grown in culture, to exceed the species' distinctions of the previously described members of the family Siderocapsaceae. Dubinina and Zhdanov (1975), after studying the physiological and developmental cycle of this bacterium, have concluded that it should be placed in the genus Arthrobacter of the family Corynebacteriaceae. They observed cells to change from rod to coccoid shape as the pure cultures aged. This is a characteristic of Arthrobacter species. Oval shaped cells and those showing false-branching or so called V-shaped Arthrobacter arrangements were found to occur as well.

These investigators have also shown that two species of Arthrobacter, A. citreus and A. simplex, are capable of oxidizing iron and manganese, thus forming morphological structures characteristic of Siderocapsaceae. Dubinina and Zhdanov (1975) have proposed that the family Siderocapsaceae be abolished as an independent taxonomic entity and that all members of the genus Siderocapsa be considered growth forms of Arthrobacter species developing under specific conditions of habitat. They have proposed the name Arthrobacter siderocapsulatus for the two strains of Siderocapsa eusphaera studied.

## Physiology

### Autotrophy vs. Heterotrophy

Oxidation of ferrous ions is often thought to be one of the most typical characteristics of bacteria of the Sphaerotilus-Leptothrix group and members of Siderocapsaceae. Several investigators have attempted to

prove that Leptothrix species could grow autotrophically or mixotrophically, utilizing the energy liberated by the oxidation of $Fe^{++}$ (Winogradsky, 1888, 1922; Lieske, 1919; Prave, 1957, as cited by van Veen et al., 1978).

Winogradsky's hypothesis favoring autotrophy of these bacteria has never been confirmed. The main difficulty in testing this hypothesis lies in the fact that these organisms grow at a pH of 6.0-8.0. At this pH, ferrous iron is readily auto-oxidized to ferric iron. It is difficult to decide whether these bacteria even contribute to the oxidation of iron, let alone determine whether they have the ability to grow autotrophically. In the case of Thiobacillus thiooxidans there has been no difficulty in demonstrating autotrophism as this organism grows at a pH less than 3.0, where auto-oxidation of ferrous ions does not occur (Colmer et al., 1950; Temple and Colmer, 1951; Razzell and Trussell, 1963).

The other way to exclude non-biological oxidation of iron is to grow the organism at a very low $pO_2$. This has not been used for the Sphaerotilus-Leptothrix group since they do not grow well under this condition. This method has been used to show autotrophic growth for Gallionella (Glathe and Ottow, 1972; Kucera and Wolfe, 1957). Wolfe (1958) grew Gallionella using ferrous sulfide as the sole energy source. It is possible that the energy obtained was derived from the ferrous iron but oxidative energy from the sulfur cannot be excluded.

Winogradsky (1888, as cited by Pringsheim, 1949a) did not attempt to prove that the organism he called Leptothrix ochracea could reproduce in the absence of organic matter, but rather that its growth in a medium containing ferrous bicarbonate and its failure to grow without this compound are in favor of utilization of energy from inorganic oxidation.

Lieske (1919) claimed to grow Leptothrix in a medium containing manganous

sulfate without organic matter. Pringsheim (1949a) suggests that Lieske's chemical calculations were faulty, and this is probably why he failed to draw clear conclusions.

Experiments by Prave (1957, as cited by Peterson, 1966) on L. ochracea are claimed to support autotrophy without actually proving it. A critical examination of his results (as cited by Peterson, 1966) shows that too little iron was oxidized for the amount of organic material produced.

Molisch (1892 and 1910, as cited by Pringsheim, 1949a) grew a species of Leptothrix in pure culture and found this organism to thrive without any addition of iron to the medium, and without depositing the characteristic sheaths. Upon dismissing Winogradsky's theory of iron autotrophs Molisch overlooked the possibility of this kind of nutrition occurring in an autotrophic species capable of growing heterotrophically under different conditions (Pringsheim, 1949a).

Cataldi (1937, 1939) and Kalinenko (1940) repeated Molisch's feeling that autotrophy is out of the question because Leptothrix could be grown in organic media like other heterotrophic bacteria (as cited by Pringsheim, 1949a). As was pointed out earlier this statement is not justified.

Cataldi (1939, as cited by Starkey, 1945) also made attempts to grow Leptothrix species on mineral media were $Fe^{++}$ and $Mn^{++}$ salts were the only sources of energy. In spite of numerous tests her results were always negative. Pringsheim (1949a) notes these results are not surprising in view of certain defects in her experiments. None of the media used contained calcium (a necessary sheath constituent), none was buffered, nor was the pH adjusted. Just as important, the possibility that both iron and manganese might be required was not envisaged. In addition, under the conditions prevailing in her culture vessels, ferrous compounds would have been

oxidized immediately by non-biological processes and thus rendered useless for the bacteria.

Van Veen and his collegues (1978) suggest that if autotrophy was to occur in any of the sheathed iron bacteria it would most likely occur in L. ochracea. In natural habitats one often sees many empty sheaths impregnated with iron (Cholodny, 1926; Charlet and Schwartz, 1954, as cited by van Veen et al., 1978; Mulder and van Veen, 1963; Mulder, 1964). However, microscopic observations show that cells, as well as newly synthesized material, were not impregnated with iron suggesting nonautotrophic propagation (Mulder and van Veen, 1965). From ratios of cell dry weight to ferric iron precipitated it can be seen that this ratio is much greater for T. ferrooxidans (1:200 to 500) as compared to Leptothrix species (1:4). Van Veen and co-workers (1978) suggest that in the case of Leptothrix species this is most probably due to the release of iron contained in organic compounds used for growth.

By growing S. discophorus (L. discophora) at differing concentrations of $FeCl_3$, Rogers and Anderson (1976a; 1976b) demonstrated that there is a direct relationship between the initial iron concentration of the medium and the final iron deposition. However, final cell yields were not affected by varying the iron concentration for Sphaerotilus (L. discophora) growing under conditions of limiting carbon and nitrogen. In other words, the growth rate was independent of the iron concentration. In addition, iron deposition was not seriously affected after blockage of protein synthesis (by chloramphenicol) after iron deposition had already been initiated. These workers have concluded that iron deposition is mediated by certain sheath constituents.

This data, as reported by van Veen and associates (1978), would seem

to indicate that the bacteria of the Sphaerotilus-Leptothrix group are probably unable to derive any energy from the oxidation of ferrous compounds. This possibly may be true for the members of Siderocapsaceae as well. It could therefore be argued that the description "iron bacteria" is incorrect for these organisms.

Although manganese-oxidizing, sheath-forming bacteria are unable to grow in media containing only inorganic compounds the possibility that the oxidation of manganous ions would contribute to the energy supply of these organisms should not be entirely excluded (van Veen et al., 1978).

Using an inorganic mineral salt medium supplemented with trace amounts of essential vitamins, thiamine, biotin and cyanocobalamin and with $Mn^{++}$ as the sole source of energy, Habib and Stokes (1971) claim to have observed autotrophic growth of Leptothrix species. Van Veen (1972) disagreed with these investigators and offers, as an explanation to these results, the suggestion that proteinaceous compounds released by the cells during growth adhered to the $MnO_2$. This in effect, may have contributed to the final cell yield.

Van Veen (1972) also states that a higher heterotrophic growth rate was seen with the addition of manganese and suggests that manganese acting as a cofactor may have stimulated this. Van Veen, however, does not preclude the possibility that autotrophy may have substantially increased cell growth.

Brantner (1970) using nine bacterial strains, including S. discophorus (L. discophora), showed that no energy was derived from the oxidation of manganese, but that the bacteria initially utilized the organic part of the manganous compound thereby releasing the manganous ion which was readily auto-oxidized.

In summary, the probability of the utilization of energy released

through the oxidation of $Mn^{++}$ is thought to be very low for the following reasons (van Veen et al., 1978): (1) The amount of energy released upon oxidation of $Mn^{++}$ to $Mn^{+++}$ or $Mn^{++++}$ is very small (Touvinen and Kelley, 1972). (2) The maximum concentration of $Mn^{++}$ tolerated by Leptothrix species is very low (Pringsheim, 1949a; Mulder, 1972, as cited by van Veen et al., 1978) compared with the high concentration of ferrous iron required for autotrophic growth of T. ferrooxidans (Colmer et al., 1950; Razzell and Trussell, 1963). In fact it is hardly higher than the minimum concentration of $Fe^{++}$ supporting growth of T. ferrooxidans (Temple and Colmer, 1951). (3) Oxidation of $Mn^{++}$ by Leptothrix species proceeds mainly outside the cells, sometimes outside the colonies. The enzyme catalyzing the oxidation of $Mn^{++}$ is often released by the cells and is present partly in the medium and partly on the sheaths. This means that only a small part of the energy released by manganese oxidation would be available to the ensheathed Leptothrix cells.

## Cultural Investigations

### Cultural Methods

Some of the most important contributions to the cultivation of iron bacteria were Winogradsky's (1888) imitation of natural conditions and his use of microcultures on slides, Busgen's (1894) pure culture of Cladothrix, and Molisch's (1910) pure culture of L. discophora under the name L. ochracea (Pringsheim, 1949a).

Pringsheim (1949b) attempted to improve culture methods for S. natans and Leptothrix strains. His technique for cultivation of S. natans involved the isolation of characteristic sheaths from natural material with a capillary pipet. The sheaths were transferred, after several washings in sterile fluid, to an agar plate containing either a low concentration of

beef extract (0.05%) or soil extract (0.002 to 0.005%). Manganous sulfate was added if L. discophora was expected to be present. After streaking with a loop, isolated colonies were then taken up with a capillary pipet and transferred to new plates until apparently pure cultures were obtained (Pringsheim, 1949b).

For maintaining cultures of S. natans on slopes, the agar concentration was reduced from an initial concentration of 2 percent to between 1.0 and 1.5 percent, that of beef or yeast extract raised to 0.2 to 0.3 percent, and the pH was adjusted to 7.0 to 7.5. This medium was also used without agar. For development of L. ochracea, Pringsheim added 0.02 percent ferrous ammonium sulfate and increased the manganous sulfate concentration to 0.01 percent.

As was noted earlier, the only members of the Siderocapsaceae to have been grown in culture are two strains of Siderocapsa eusphaera (Dubinina and Zhdanov, 1975). These bacteria were isolated with and cultured in Pringsheim medium with the following modifications: 0.002 percent $MnSO_4 \cdot 4H_2O$, 0.005 percent yeast extract (Difco) with or without 0.1 percent agar. For cultivation in liquid media in test tubes ferrous sulfate or ferrous oxalate was supplied to the test tubes (Dubinina and Zhdanov, 1975).

## Cultural Requirements

Carbon Sources. Members of the Sphaerotilus-Leptothrix group are able to utilize a great variety of carbon compounds. Glucose, fructose, galactose, maltose, lactose, sucrose, xylose, ribose, methanol, ethanol, butanol, glycerol, mannitol, acetate, propionate, butyrate, $\beta$-hydroxybutyrate, pyruvate, malonate, fumarate, succinate, malate, tartrate, gluconate, citrate, quinate and oxalate are reported to be utilized by one or more members of this group (Iackey and Wattie, 1940; Stokes, 1954; Hohnl, 1955,

as cited by van Veen et al., 1978; Mulder and van Veen, 1963; Dondero, 1975). In addition to these, S. natans is also able to utilize sorbital (Stokes, 1954), maltose (Linde, 1913, as reported by Stokes, 1954) and aspargine (Cataldi, 1939, as cited by Stokes, 1954) as carbon sources.

A number of investigators have failed to obtain growth of S. natans using cellobiose, starch or inulin (Linde, 1913, as cited by Stokes, 1954; Lackey and Wattie 1940, Hohnl, 1955, Scheuring, 1957, Dondero, 1975, as cited by van Veen et al., 1978), as well as dextrin and gum arabic (Linde, 1913, as cited by Stokes, 1954).

Studies on Sphaerotilus natans show better growth is obtained by increasing the glucose concentration from 0.1 percent to 0.2 percent. When the concentration of glucose is raised to 1 to 2 percent, it must be sterilized separately to avoid formation of inhibitory compounds. At this concentration growth is still maintained, although no better than that seen at the lower concentration of 0.2 percent (Stokes, 1954).

S. natans and L. cholodnii show a more pronounced response to an increase in the supply of organic nutrients than do the other members of the Sphaerotilus-Leptothrix group. This is especially evident with L. discophora which grows satisfactorily in running iron- and manganese-containing soil extract, but hardly responds to added nutrients. However, under prolonged culture in vitro, L. discophora responds better to an increased supply of organic nutrients (van Veen et al., 1978).

Initial isolates of S. natans form rough or R type colonies. When these isolates are cultivated on a medium rich in organic nutrients, the filamentous, R type colonies dissociate giving rise to smooth, shiny, S colonies in which the cells are mainly single and the sheaths few (Stokes,

1954). Hoflich (1901, as cited by Stokes, 1954) briefly mentions the occurrence of filamentous and smooth colonies in cultures of S. natans. Pringsheim (1949b) has found R to S dissociation in S. discophorus (Leptothrix discophora). S colonies may be formed spontaneously by L. discophora or induced by nutritional conditions. Temporary conversion to S colonies results when a 0.5 percent peptone-salts agar is supplemented with 0.2 to 1.0 percent glucose, galactose, mannitol, sucrose, or salicin (Dondero, 1975).

Nitrogen Sources. Much question has centered around the ability of the sheath-forming bacteria of the Sphaerotilus-Leptothrix group to utilize inorganic nitrogen compounds. Linde (1913, as cited by Stokes, 1954) reported obtaining good growth of S. natans with both ammonium and nitrate nitrogen in a mineral salts-sucrose medium. Similarly, Cataldi (1939, as cited by Stokes, 1954) reported good results using ammonium nitrogen in a mineral salts-glucose medium and in a mineral salts-citrate medium. Also Lackie and Wattie (1940) obtained fair growth with ammonium and nitrate salts in a mineral salts-glucose medium. Stokes (1954) found that ammonium nitrogen or nitrite was rapidly utilized when the source of carbon was glycerol, sucrose or succinate whereas utilization was poor when glucose was added as the carbon source.

Mulder and van Veen (1963) grew S. natans and L. cholodnii using $NH_4^+$ and $NO_3^-$ as sole nitrogen sources. However, growth was reported to be less luxuriant than it was with a mixture of aspartic and glutamic acids, peptone or other complex nitrogen compounds (Mulder and van Veen, 1963; Mulder and van Veen, 1965, as cited by van Veen et al., 1978).

Van Veen and co-workers (1978) suggest these inferior results may be due to a pronounced shift of pH after the uptake of $NH_4^+$ or $NO_3^-$. They

cite the work of Dias, Dondero and Finstein (1968) where more satisfactory results were obtained, using inorganic nitrogen, when S. natans was grown in continuous culture. Similar results were obtained by van Veen and his co-workers (1978) using S. natans grown in a pH controlled batch culture. In these experiments excellent growth of the organism was obtained with $NH_4^+$ or $NO_3^-$ as the nitrogen source and glucose as the carbon and energy source.

Another factor that may interfere with comparisons of various nitrogen sources in sheathed bacteria is the requirement for Vitamin $B_{12}$ (cyanocobalamin) when they are growing in media with $NH_4^+$, $NO_3^-$ or a mixture of aspartic and glutamic acids (van Veen et al., 1978). Vitamin $B_{12}$ is not required when the bacteria are growing with Casamino Acids, peptone, or other complex nitrogen compounds that contain methionine (Mulder and van Veen, 1963; Johnson and Stokes, 1965; Stokes and Johnson, 1965).

Stokes (1954) reported good growth of S. natans when L-glutamic acid, L-aspartic acid, L-aspargine, DL-alanine, L-leucine, L-cystine, DL-tryptophan, L-argine and L-proline are used as nitrogen sources. Little or no growth was obtained when urea or glycine was used. Iackey and Wattie (1940) recorded good growth when DL-alanine or L-aspargine was used, fair growth with urea, and poor growth when L-tyrosine, L-cystine, L-leucine, or L-glutamic acid was used. Stokes (1954) explains these descrepancies in results as being due to a difference in the strains used.

Growth of S. natans using individual amino acids is usually not as rapid nor as extensive as with the mixture of amino acids in the form of Casamino Acids (Stokes, 1954). In separate experiments it was shown that this effect depended on the occurrence in the synthetic mixture of a number of amino acids as DL-isomers instead of L-isomers occuring in Casamino Acids or peptone (Mulder and van Veen, 1963). A toxic effect of

certain DL-amino acids has been recorded by Wuhrmann (1946, 1949, as cited by Mulder, 1964).

The difference in the tolerance of amino acids by S. discophorus (presumably L. cholodnii, according to van Veen et al., 1978) is striking. Johnson and Stokes (1965) found their strains of S. discophorus (L. cholodnii) were completely inhibited by 1.0 percent Casamino Acids, by 0.54 percent simulated casein hydrolysate, and by a 0.2 percent mixture of 18 amino acids. In the range of 0.18 to 0.36 percent, L-arginine, L-glutamic acid, L-leucine, L-lysine, and L-proline were not inhibitory or only slightly so. A second group of amino acids, including L-aspartic and L-methionine, was moderately inhibitory. The most toxic amino acids, which suppressed growth partially or completely, were L-alanine, glycine, L-histidine, L-cystine, L-isoleucine, L-tyrosine, L-phenylalanine, L-tryptophan, DL-valine, DL-serine, and DL-threonine. DL-serine and DL-valine prevented growth at the lowest level of concentration. Van Veen and his collegues (1978) suggest that the toxicities of the individual amino acids may account for the toxicity of Casamino Acids.

S. discophorus (L. cholodnii) may tolerate peptone up to about 0.2 percent, but protease peptone, phytone, tryptone and tryptose were inhibitory at this concentration. This may have been due to a higher content of free amino acids in these complex nutrients (Johnson, 1966, Ph.D. thesis, as cited by van Veen et al., 1978).

Mineral Sources. Excellent growth of several isolated strains of S. natans and L. cholodnii has been obtained with a basal nutrient medium of the following composition (in milligrams per liter): $KH_2PO_4$, 27; $K_2HPO_4$, 40; $Na_2HPO_4 \cdot 2H_2O$, 40; $CaCl_2$, 50; $MgSO_4 \cdot 7H_2O$, 75; $FeCl_3 \cdot 6H_2O$, 10; $MnSO_4 \cdot H_2O$, 5; $ZnSO_4 \cdot 7H_2O$, 0.1; $CuSO_4 \cdot 5H_2O$, 0.1; $H_3BO_3$, 0.1; and $Na_2MoO_4 \cdot 2H_2O$,

0.05, and supplied with a carbon and energy source, an inorganic or organic nitrogen source and Vitamin $B_{12}$, 0.005 mg/l (van Veen et al., 1978). Most of the other strains grow moderately in this medium (Mulder and van Veen, 1963).

Van Veen and his collegues (1978) have noted that no detailed experiments have been carried out to determine the essentiality of the above mentioned elements. They have assumed that all of these elements are required for growth (except boron) when purified salts and mineral-free water are used for preparing nutrient media. Calcium, specifically, must be available in concentrations of at least 0.1 mM to both S. natans and L. discophora for formation of filaments and attachment to solid surfaces (Dias, Dondero and Finstein, 1968; Dias, Okrend, and Dondero, 1968). Neither substitution with strontium nor barium has been found to satisfy this requirement (Dias, Okrend and Dondero, 1968).

Growth Factors. S. natans and Leptothrix strains exhibit a requirement for cyanocobalamin when methionine is not present (van Veen et al., 1978). When methionine is provided, no Vitamin $B_{12}$ is needed for growth (Mulder and van Veen, 1963; Mulder, 1964; Okrend and Dondero, 1964; Johnson and Stokes, 1965; Stokes and Johnson, 1965; Mulder, 1972, as cited by van Veen et al., 1978). Furthermore, methionine may be supplied as an L- or D-isomer, however the latter is more toxic at higher levels (Johnson and Stokes, 1965).

Contrary evidence is seen concerning the requirements for biotin and thiamine. Several investigators have found these growth factors essential for S. discophorus (L. discophora) (Rouf and Stokes, 1964; Stokes and Johnson, 1965) whereas others (Mulder and van Veen, 1963) found them not necessary for growth.

pH. Although it is generally agreed that the sheathed iron bacteria as well as the unicellular forms grow rapidly at pH values between 6.5 to 8.1 (Stokes, 1954; Mulder and van Veen, 1963; Rouf and Stokes, 1964), with an optimum at approximately pH 7.5. Yet, there are some inconsistencies. Cataldi (1939, as cited by Stokes, 1954) reported obtaining growth of S. natans in the range of pH 5 to pH 9.8 and observed that the amount of growth increased with an increase in pH. Iackey and Wattie (1940, as cited by Stokes, 1954) obtained growth of S. natans in the range of pH 5.5 to 8.0. Experiments by Stokes (1954) suggest that growth of S. natans may be initiated at a pH as high as 9 or 10. There seems to be general agreement that the members of the Sphaerotilus-Leptothrix group cannot tolerate an acid environment and grow best in a neutral or slightly alkaline pH.

Temperature. Growth of the bacteria of the Sphaerotilus-Leptothrix group has generally been reported to occur at temperatures between 15 and 40C. Hoflich (1901, as cited by Stokes, 1954) reported growth for S. natans at 15 to 35C with an optimum temperature of 25 to 30C. Linde (1913, as cited by Stokes, 1954) reported no growth for S. natans below 10C nor above 40C with an optimum temperature anywhere from 30 to 35C. Zikes (1915, as cited by Stokes, 1954) reported no growth at 5C or above 39C with an optimum temperature of 25 to 29C (for S. natans). Other investigators have reported an optimum temperature at about 30C for S. natans and Leptothrix species (Stokes, 1954; Dondero, 1961; Mulder and van Veen, 1963). Cataldi (1939, as cited by Stokes, 1954) reported an optimum temperature of 39C for S. natans.

Salt Tolerance. The bacteria of the Sphaerotilus-Leptothrix group generally do not tolerate high salt concentrations. Concentrations of

inorganic salts between 0.3 and 2 g/liter may adversely affect growth of strains of Sphaerotilus natans (Iackey and Wattie, 1940; Stokes, 1954; Scheuring, 1957, as cited by van Veen et al., 1978). Despite this susceptibility to salts, sheath bacteria have been found to occur in salt water (Petitprez and Ieclerc, 1969, as cited by van Veen et al., 1978). Van Veen and co-workers (1978) suggest that suppression of growth by high amounts of complex organic nutrients (e.g. Casamino Acids, nutrient broth and Trypticase soy broth) may be partly due to the relatively high concentration of salts present in these nutrients.

Storage Products. As was mentioned earlier, the bacteria of the Sphaerotilus-Ieptothrix group are often characterized by formation of a sheath. This sheath is often surrounded by a slime layer which is closely connected with the iron-accumulating and manganese-oxidizing capacities of the organisms, which are characteristic of most of the Ieptothrix species (van Veen et al., 1978). In addition to these properties members of this group have the tendency to form internal globules of poly-$\beta$-hydroxybutyrate (PHB) as reserve material (Mulder et al., 1962; Rouf and Stokes, 1964; Mulder and van Veen, 1963; Mulder, 1964; Stokes and Powers, 1967; Stokes and Parsons, 1968; Dondero, 1975). These globules may account for as much as 30 to 40 percent of the dry cell weight when utilizable carbon compounds are plentiful and nitrogen is in relatively low concentration (Dondero, 1975). This sudanophilic substance can be used by cells as a reserve material (source of carbon and energy) to prolong its survival (Stokes and Parsons, 1968). Utilization of PHB by cells, as shown by an increased rate of endogenous oxygen consumption and disappearance of the polymer, was greatly stimulated by small amounts of $Mn^{++}$ and even more by $Mg^{++}$. It is not biochemically understood why this occurs (Stokes and Powers, 1967).

In addition to PHB, sheathed bacteria may store large amounts of glycogen. The general composition of cells of a S. natans culture grown for six days in a basal culture solution supplemented with 5 g glucose/liter and 1.5 g of peptone/liter was as follows: 38% polysaccharides, 29% PHB, 4% crude fat and 25% protein (Mulder et al., 1972, as cited by van Veen et al., 1978). Nitrogen and oxygen limitations are apparently the major factors in the accumulation of glycogen. After utilization of glucose in the nutrient medium, a rapid fall in the cell content of both PHB and glycogen occurs; the polysaccharide is degraded more readily than PHB (van Veen et al., 1978).

## Ecology and Economic Importance

The heterotrophic iron bacteria may be expected to occur where a low concentration of organic matter and a fair amount of oxygen are present. According to Pringsheim (1949a), iron and manganese, in lower oxidative states, are also necessary. Possibly this could be extended to include iron that is present as part of organic complexes when one is considering these heterotrophic bacteria.

Water currents may be negligible. Development, however, appears to be better when there is an appreciable flow because there seems to be more proliferation of these bacteria in water from springs and wells (Spencer et al., 1963, as cited by Dondero, 1975; Barbic et al., 1974), water pipes (Peterson, 1966; Hasselbarth and Ludemann, 1972; Brown, 1934, Olsen and Szybalski, 1949, Wilson, 1945, as cited by Dondero, 1975) and reservoirs (Clasen, 1969, as cited by Donder. 1975; Drabkowa and Stravinskaja, 1969; Dubinina, Gorlenko and Suleimanov, 1973; Zhdanov and Dubinina, 1975; Dubinina and Zhdanov, 1975; Dubinina, 1976).

Iron bacteria developing in water pipes may manifest themselves in several different ways. They may form hard deposits which fill the pipes

and subsequently reduce the water carrying capacity of the pipe. Slime and accumulations of filamentous bacteria may form on the walls of water tanks and pipes and scum may be produced on the water surface. These bacteria may cause turbidity and discoloration of the water and be responsible, either directly or indirectly, for the unpleasant tastes and odors which are produced as the dead bacterial cells are decomposed by other micro-organisms. Cases of very profuse developments of filamentous iron bacteria have been referred to as "water calamities" (Starkey, 1945).

The iron bacteria are fouling organisms and not agents of corrosion. It sometimes happens that the surface of the fouled metal shows signs of corrosion, but in such cases it is probable that the corrosion was caused indirectly by the bacteria (Starkey, 1945), as reducing conditions may be present at the surface of the metal due to the large amount of filamentous material present.

Members of the Sphaerotilus-Leptothrix group may attach not only to metallic pipes but to pipes coated internally with asphalt as well as concrete substrates (Starkey, 1945). This suggests that the bacteria obtain the iron they oxidize not from the pipes but from the water being delivered through the pipes. This is further substantiated by growth of numerous members of the Sphaerotilus-Leptothrix group and Siderocapsaceae on submerged glass slides for investigative purposes (Pringsheim, 1949a; Barbic et al., 1974; Dubinina and Zhdanov, 1975).

In the biological treatment of sewage and industrial wastes, it is necessary to separate the microbiological solids, or floc, from the treated effluent by sedimentation in order to discharge a clean effluent relatively free of organic matter. Activated sludges failing to settle because of insufficient density are "bulky" and frequently contain filamentous bacteria

usually identified as S. natans. Recently other genera have also been identified as originating from bulking sludge (van Veen, Geuze and van der Vlies, 1973). The extended filaments projecting from the flocs are thought to increase the effective diameter of the floc without a proportional increase in mass (Dondero, 1975). With attention focused upon the growth of Sphaerotilus, various factors, such as overloading with carbohydrates, insufficient aeration, and low levels of phosphates have been blamed for bulking (Dondero, 1961,1975).

# METHODS AND MATERIALS

## General

Glassware. All glassware was thoroughly washed in detergent (Liquinox) followed by several rinses in de-ionized water.

Sterilization. All media and glassware were sterilized by autoclaving in a horizontal Castle autoclave at 15 psi (121C) for 15 minutes. Pond water was sterilized using a Gelman 47 mm magnetic filter funnel, product #4200, on which a Gelman GN-6 0.45 μm pore size sterile membrane had been placed. Glass slides, used to show the transfer of bacteria in coplin jars, were sterilized by dipping in 95 percent ethanol and flaming.

Incubation. Cultures were incubated in either a Precision Scientific, model 805, BOD incubator at 26C or in a Thelco, Model 6, anhydric incubator at 35.5C.

Reagents. Reagents were prepared using reagent grade water obtained from a Millipore Milli-Q-Water Purification System, catalog number ZD 30 000 70.

Media Preparation. All media were prepared by mixing with de-ionized water, brought to a boil, sterilized by autoclaving and dispensed into culture tubes, petri dishes or 250 ml Erlenmeyer flasks. Pringsheim medium consisted of yeast extract, 0.5 g/l; $MnSO_4 \cdot H_2O$, 0.05 g/l; and agar (Difco), 20 g/l. Plate Count Agar (PCA) was purchased from Difco Laboratories and was prepared according to manufacturer's specifications. In addition to these media a slightly modified form of a medium for heterotrophic iron precipitating bacteria (Clark et al., 1967) was prepared using $(NH_4)_2SO_4$,

0.5 g/l; $NaNO_2$, 0.5 g/liter; $K_2HPO_4$, 0.5 g/l; $MgSO_4 \cdot 7H_2O$, 0.5 g/l; and ferric ammonium citrate, 10.0 g/l. Agar (15 g/l) was added to solidify the medium. To stimulate sheath production, calcium ($CaCl_2$, .05 g/l) was aseptically added to solidified medium in bottles, prior to melting and pouring. The hydrogen ion concentration of Pringsheim's and Clark's media was adjusted to pH 6.6-6.8 with NaOH or HCl, whichever was appropriate.

All media for physiological tests were purchased from Difco laboratories and were prepared according to manufacturer's specifications.

Sample Collection. Iron bacteria were collected from the Campus pond, Eastern Illinois University, Charleston, Illinois.

These bacteria were collected by taking water directly from the Campus pond, using sterile Erlenmeyer flasks, or by a submerged slide technique (Henrici and Johnson, 1935). In this method microscope slides or coverslips were cleaned with Bon Ami, rinsed in de-ionized water, and air dried. An apparatus used to accomodate the glass slides during submersion was prepared using a plastic reagent bottle for bouyancy, rubber stoppers with slits for slide or coverslip insertion (each stopper accomodating up to four slides), and nylon cord onto which all of the stoppers were strung. Slides or coverslips were inserted as appropriate and the entire apparatus was submerged in the Campus pond. It was often necessary to secure the apparatus to the bank with ordinary monofilament line in order to prevent loss of the device.

## Light Microscopy

Preparation. Upon removal from the Campus pond, glass slides were transported to the laboratory in coplin jars containing fresh pond water whereupon they were immediately air dried and heat fixed. Slides were then stained with crystal violet (Hucker, 1922), Sudan Black B (Cohn, 1953) or

by the Prussian blue Reaction (Appendix A).

Techniques. All light micrographs were taken on a Nikon Microflex Model AFM brightfield light microscope with an automatic photomicrographic attachment using Ektachrome 160, tungsten film. Prints were made by a local commercial firm, by a slide enlargement technique, on Kodak paper.

## Scanning Electron Microscopy

Preparation and Observation. For SEM, suspended coverslips were transported to the laboratory by submerging the coverslip apparatus in a one liter graduated cylinder containing fresh pond water. Once in the laboratory coverslips were fixed for 5 minutes in either osmium tetroxide or in Pardúcz's fixative (Appendix A). Following fixation, coverslips were rinsed twice in distilled water followed by graded (10, 20, 30, 40, 50, 60, 70, 80, 90, 95%) ethanols up to absolute alcohol. Coverslips were then critical point dried and sputtercoated first with aluminum followed by gold. Both the critical point dryer and the sputtercoater were constructed by research engineers at the University of Illinois. Bacteria were observed in a Japanese Electron Optics laboratory scanning electron microscope (model JSM U-3). Photographs were taken using Polaroid Black and White Positive/Negative type 55 film.

## X-ray Microanalysis

Preparation and Observation. Coverslips were collected and prepared in the same manner as for SEM. In this case coverslips were fixed in $OsO_4$. X-ray analysis was performed using the same electron microscope as for SEM but with an energy dispersive detector coupled to an ORTEC, Model 6200, multichannel analyzer. Photographs were taken using Polaroid Black and White Film.

## Cultural Techniques

Several different methods were employed in attempts to culture bacteria. Clean, sterile glass slides and slides that had been submerged in the Campus pond for varying periods of time were placed adjacent to each other in sterile coplin jars to which sterile pond water had been added. In some cases, reagent grade Milli-Q water was used instead of sterile pond water. In an attempt to further promote slide to slide transfer of these bacteria several drops of sterile aqueous solutions of 10% ferric ammonium citrate and/or 10% $MnSO_4 \cdot 7H_2O$ was added to both of these media.

In addition, slide to slide transfers were attempted in a clean 5 liter aquarium into which oxygen was continuously bubbled. The aquarium was half filled with pond water, inoculated with several slides that had previously been placed in the Campus pond for a period of 48-72 hours, and clean glass slides were placed inside.

In an attempt to grow these bacteria in culture media, slides from the Campus pond were pressed onto the surface of an agar (heterotrophic iron medium) plate. Characteristic colonies were then restreaked on this medium until pure cultures were obtained. These pure cultures were characterized on standard physiological test media.

## RESULTS AND DISCUSSION

Considerable confusion exists regarding the taxonomic position of Siderocapsa. Molisch (1909) first observed and illustrated members of the genus Siderocapsa on the leaves of aquatic plants. This genus was the first of the non-filamentous iron bacteria described. His use of drawings, as opposed to photomicroscopy, has not reduced the confusion in the literature. The bacteria studied here (Figs. 1 and 2) appear to be the same as those illustrated by Molisch.

Figures 1 and 2 show rod-shaped rather faintly stained cells surrounded by a clear central well (A). Enclosing this area is a deposition of golden material (B) which has been described in the literature as iron containing, hence the name iron bacteria. The clear central well measures 2.1-3.1 μm in diameter, but may range from 2-6 μm in diameter. The area of deposition varies considerably but most appear to be 9-17 μm in diameter. Cells within the central well measure 0.5 μm in diameter by 1.15 μm long (see also Fig. 3).

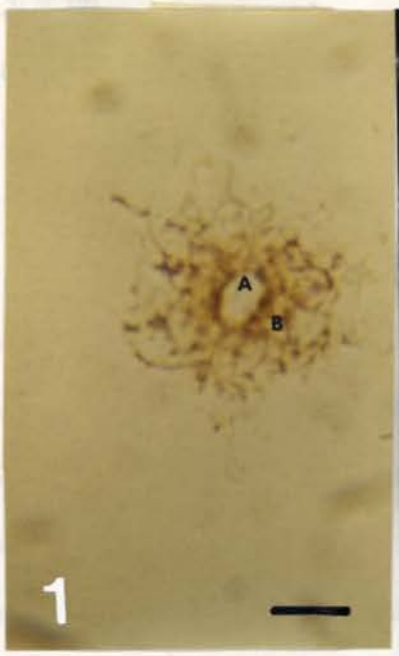
Molisch described Siderocapsa treubii as spherical organisms 0.4-0.6 μm wide. Usually 1 to 6 cells were found to occur in a primary capsule 5-20 μm in diameter. Molisch found the clear central well, where the cells are located, to be from 1.8-3.6 μm in diameter. S. major is described by Molisch as being similar in size except that anywhere from 2 to 100 short rods, 0.7-1.8 μm long, are found in the central well. Scanning electron micrographs (Figs. 3 and 4) show rod-shaped cells corresponding to Molisch's description of S. major. It is difficult to imagine 80 to 100

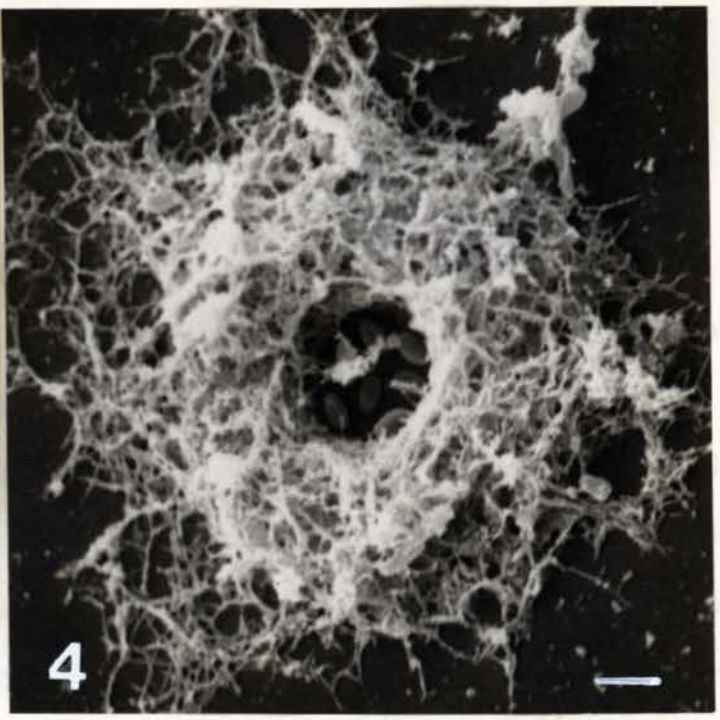
Fig. 1. Light micrograph of a typical Siderocapsa microcolony (from submerged slide tech.). Crystal violet stain. (A) Clear central well with cells; (B) deposition area. Bar= 5.0 µm.

Fig. 2. Light micrograph of Siderocapsa. Note area of deposition seems to have taken up stain better than the microcolony in Fig. 1. Crystal violet; glass slide. Bar= 5.0 µm.

Fig. 3. SEM of Siderocapsa major. Parducz's fixative; glass coverslip. Bar= 2.0 µm.

Fig. 4. SEM of Siderocapsa major. Parducz's fixative; glass coverslip. Bar= 2.0 µm.


A
B
1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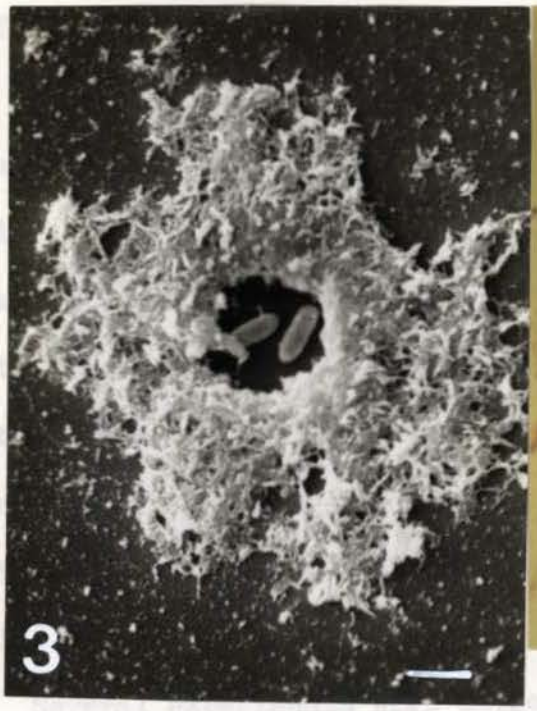
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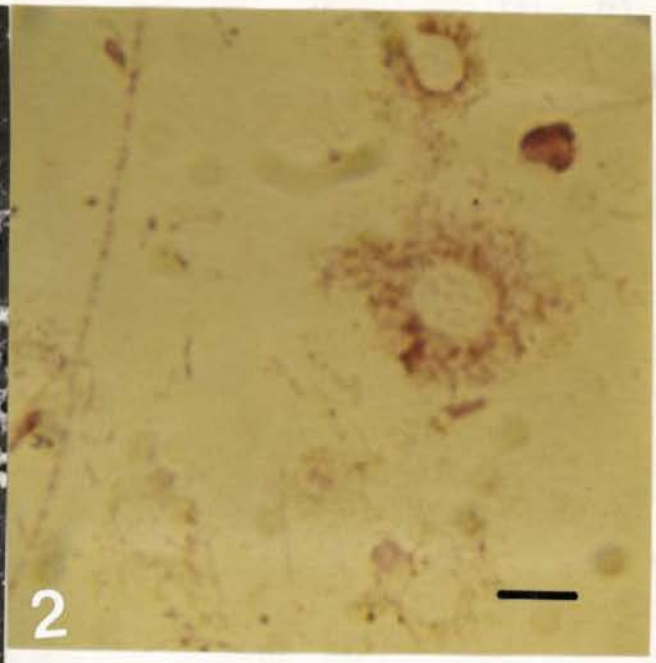

2

short rods, the type of which Molisch described, fitting into a central well of 3.6 μm in diameter.

In addition to Siderocapsa several different types of filamentous bacteria were found to attach to the glass slides. Sphaerotilus natans (Fig. 5) was often seen. This bacterium was identified according to Pringsheim (1949a, 1949b) and by demonstration of the presence of PHB granules through the use of Sudan Black B stain (Fig. 6). It is important not to confuse these granules with ensheathed coccoid cells which they resemble. Other filamentous bacteria with sheaths more heavily encrusted than typical S. natans sheaths were also observed (Fig. 7). These organisms were identified as members of the Sphaerotilus-Leptothrix group (Pringsheim, 1949a, 1949b). The holdfasts appear different than those of S. natans filaments. This difference is seen in Fig. 8 where two types of filamentous bacteria are shown. The more heavily incrusted filament has a holdfast differing in morphology from its neighbor and from other types (Fig. 7).

Siderocapsa species have been commonly referred to as iron-depositing because deposits surrounding these cells are brown to golden-brown. Dubinina and Zhdanov (1975) state that these cells (S. eusphaera) are capable of precipitating iron and manganese in vitro, however, there is little experimental evidence supporting the contention that these bacteria oxidize iron and/or manganese under natural conditions. This is not so for members of the Sphaerotilus-Leptothrix group. They give a positive Prussian blue Reaction, indicating the presence of ferric compounds (Pringsheim 1949a, 1949b).

Several experiments were employed in attempts to determine if iron or manganese was present in Siderocapsa. Prussian blue staining methods on Siderocapsa microcolonies failed to demonstrate the presence of iron

Fig. 5. Light micrograph of Sphaerotilus natans. Crystal violet; submerged slide technique. (A) Holdfast; (B) sheath with cells. Bar= 5.0 µm.

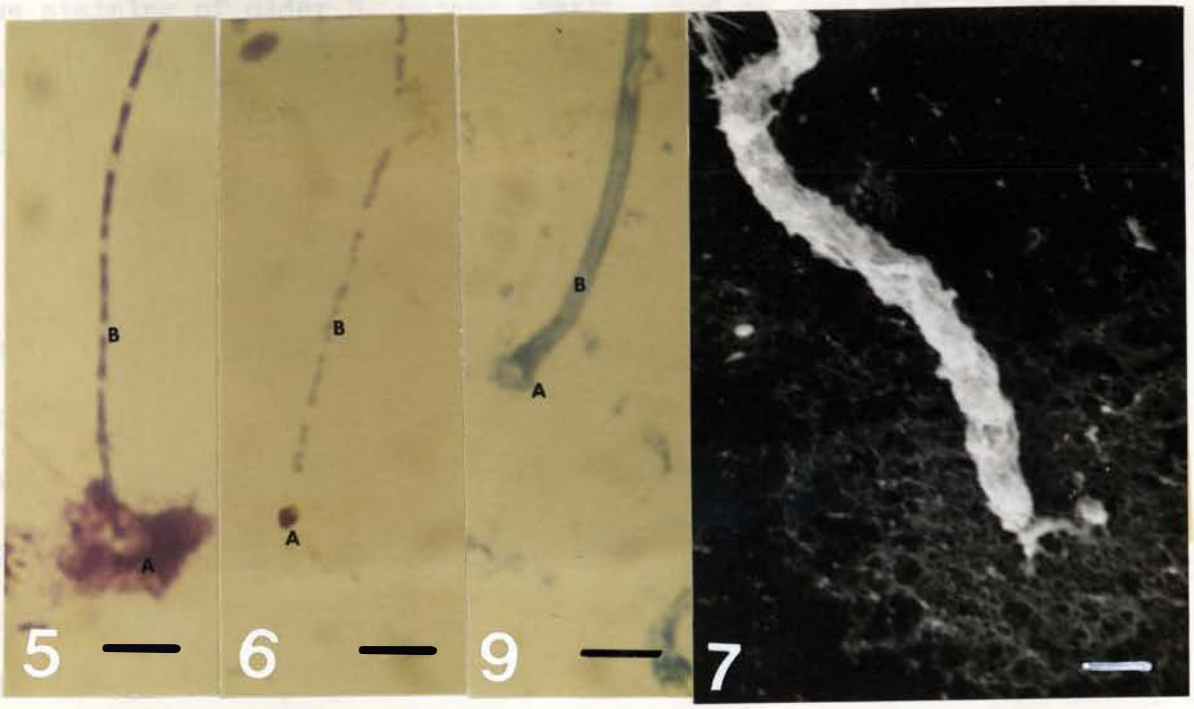
Fig. 6. Light micrograph of S. natans. Sudan Black B; showing the presence of PHB. (A) Holdfast; (B) PHB granules inside ensheathed cells. Bar= 5.0 µm.

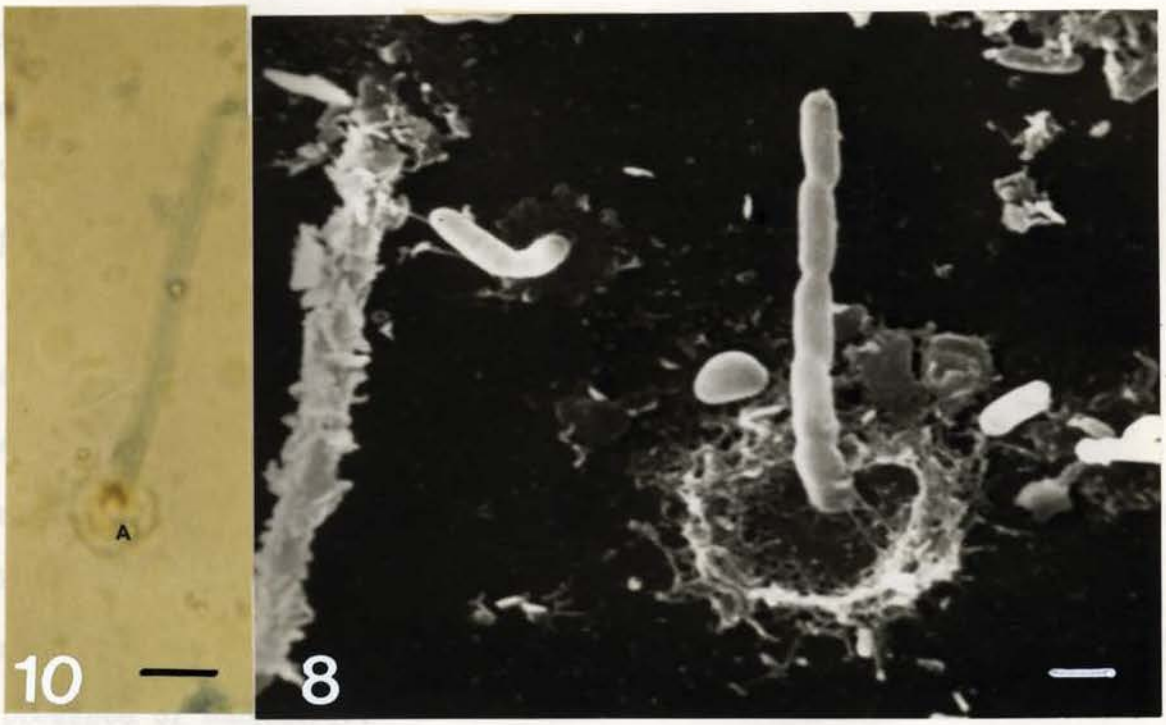
Fig. 7. SEM of a filamentous member of the Sphaerotilus-Leptothrix group; note heavy incrustation of the sheath. Parducz's fixative; glass coverslip. Bar= 5.0 µm.

Fig. 8. SEM of several filamentous members of the Sphaerotilus-Leptothrix group. Note differences in holdfast and sheath morphology. $OsO_4$ fixation; glass coverslip. Bar= 2.0 µm.

Fig. 9. Light micrograph of S. natans (old). The blue color indicates a positive Prussian blue Reaction demonstrating the presence of iron. Glass slide substrate. (A) Holdfast; (B) sheath. Bar= 5.0 µm.

Fig. 10. Light micrograph of Prussian blue staining of S. natans. Results here are similar to Fig. 9 except that no iron is present in the holdfast (A). Glass slide substrate. Bar= 5.0 µm.


B
A
5
B
A
6
B
A
9
7


A
10
8

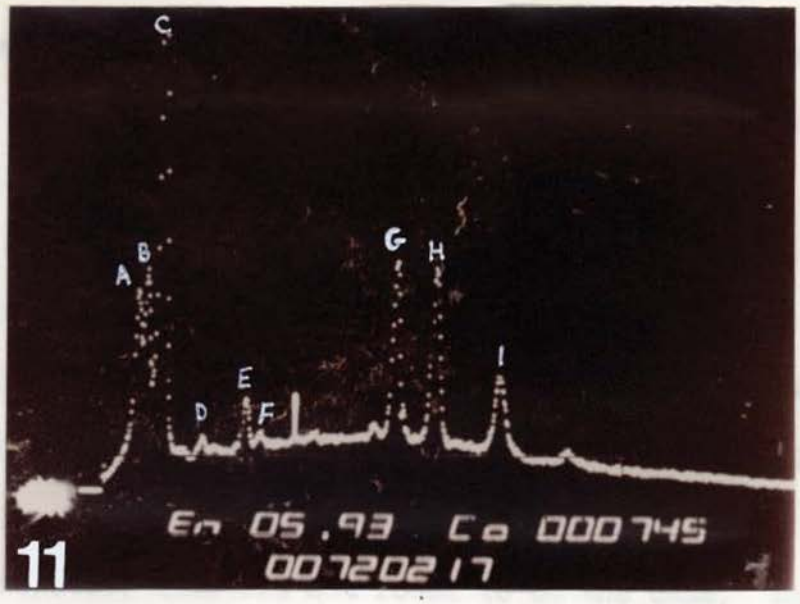
in or around the cells (Appendix A). Fig. 9 shows the results of Prussian blue staining of older S. natans sheaths used as controls. Iron was not always found to be present in the holdfasts of this organism (Fig. 10).

X-ray microanalysis performed on an S. natans filament (similar to the one in Fig. 9) and a Siderocapsa colony (similar to Fig. 1) demonstrated no iron on the sheaths of S. natans, however a peak corresponding to a small amount of manganese was seen (marker, Fig. 11). Peaks A, C, and B correspond to the presence of aluminum and gold (sputtercoating) and silicon (glass coverslip), respectively. Peak D corresponds to calcium (a necessary sheath constituent). Peaks E and F are presumably system's peaks (they correspond to no element). Peak G corresponds to osmium ($OsO_4$ fixation). Peaks H and I correspond to the $L\alpha$ and $L\beta$ shells of gold, respectively. X-ray analysis of the holdfast (not shown) did not indicate the presence of any iron or manganese. This must be assumed to be a young S. natans filament that has not begun to accumulate iron (Pringsheim, 1949a, 1949b). The results for Siderocapsa are shown in Fig. 12. Peaks A thru I correspond to the same elements as seen in Fig. 11. However much more manganese was found to be present in Siderocapsa (marker). Peak J corresponds to either the presence of iron and/or manganese. Due to the overlap of the $K\beta$ and $K\alpha$ energy levels of iron and manganese, it cannot be accurately ascertained which element is present in the J peak. However since high levels of manganese are known to be present (marker) this peak (J) probably indicates the presence of manganese.

Some workers (Pringsheim, 1949a; Hirsch and Pankratz, 1970) have suggested that Siderocapsa may be an artifact caused by bacterial cells which have become entrapped in the wells of the holdfasts of Sphaerotilus-

Fig. 11. X-ray microanalysis of a young S. natans filament. $OsO_4$ fixation. Peaks correspond to Al (A), Si (B), Au (C, H, I), systems peaks (E, F) and Os (G). Marker corresponds to the presence of Mn.

Fig. 12. X-ray microanalysis of a Siderocapsa microcolony (deposition area around the cells). $OsO_4$ fixation. Peaks A-I correspond to peaks A-I as seen in Fig. 11. Marker corresponds to Mn levels. Presence of Mn is most likely indicated by peak J (see text).


C
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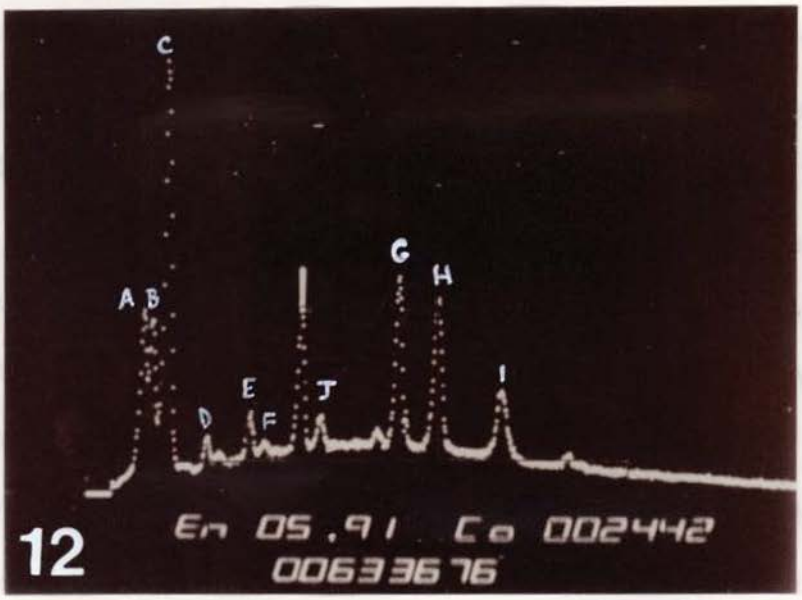
00720217


C
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00633676

Leptothrix species whose filaments have become dislodged in some way. A developmental study by Loos in our laboratories (unpublished) has shown that the central well area remains constant throughout the development of the microcolony. Results here confirm that the size of the central well is constant among differing microcolonies and does not vary with varying diameter of manganese deposition (Table 1). A direct relationship exists between the amount (area) of deposition and the number of cells present. Analysis by linear correlation (Steel and Torrie, 1960) shows a significant positive r value ($0.5317, \alpha=0.05$). If a young Sphaerotilus filament had broken off, occasionally large numbers of cells would be seen "entrapped" within the newly formed "holdfast". However, this was not found to be the case. In smaller, presumably younger colonies, lower numbers of cells were always found.

Scanning electron micrograph studies have shown what appears to be developmental stages in the growth of these colonies (Figs. 13-16). Fig. 13 shows a young colony with only two cells. A colony with four cells is shown in Fig. 14. Not only can a heavier deposition of material be seen around the latter microcolony but strands of material are beginning to cross over the top of the central well area. Fig. 15 shows a completely covered colony alongside a younger one. In additional to developmental stages, cells undergoing binary fission have been observed in these microcolonies (Fig. 16).

Generally the area around the filament at the base of the holdfast of S. natans was found to be less well-defined and often appeared "dirtier" than in a typical Siderocapsa microcolony (Figs. 17-21). Often bacteria were seen aggregating in and around the deposition of a microcolony (Fig. 14) and it is highly likely that this may serve as a point of attachment for swarm cells of Sphaerotilus and other filamentous bacteria.

TABLE 1. Microcolony morphology of *Siderocapsa major*.

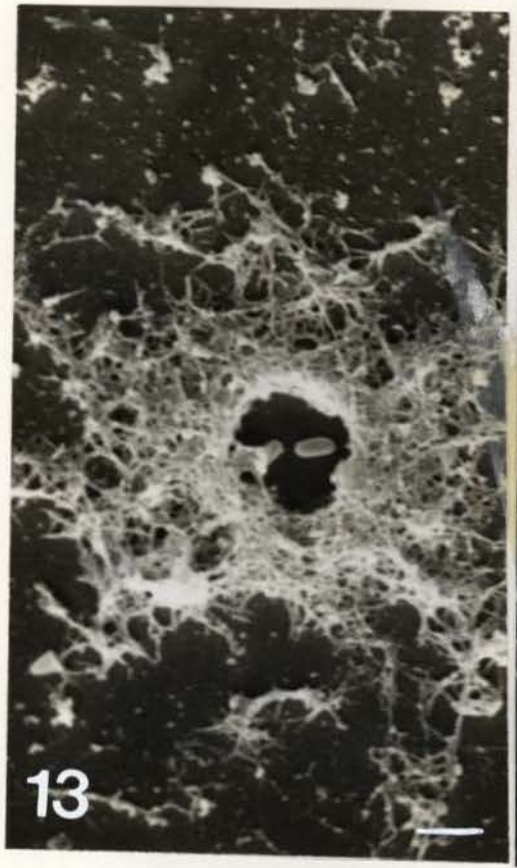
| Colony | Deposition diameter (µm) | Central well diameter (µm) | Total no. of cells in colony |
|---|---|---|---|
| 1 | 17.0 | 2.93 | 6 |
| 2 | 9.82 | 2.25 | 2 |
| 3 | 8.04 | 2.26 | 2 |
| 4 | 17.23 | 2.28 | 5 |
| 5 | 20.72 | 2.6 | 4 |
| 6 | 14.08 | covered | ? |
| 7 | 15.29 | 2.78 | 7 |
| 8 | 17.04 | 2.27 | 4 |
| 9 | 11.89 | 3.19 | 1 |
| 10 | 11.74 | 2.31 | 7 |
| 11 | 9.87 | covered | ? |
| 12 | 10.94 | 2.7 | 2 |
| 13 | 10.61 | 3.26 | 2 |
| 14 | 7.36 | 1.42 | 3 |
| 15 | 8.4 | 2.37 | 1 |
| 16 | 18.17 | 2.74 | 2 |
| 17 | 9.19 | 2.66 | 1 |
| 18 | 9.06 | 3.12 | 1 |
| 19 | 14.08 | 2.62 | 4 |
| 20 | 6.9 | 3.49 | 1 |
| 21 | 14.29 | 2.94 | 3 |

Fig. 13. SEM of a young Siderocapsa major microcolony. Parducz's fixative; glass coverslip. Bar= 2.0 µm.

Fig. 14. SEM of Siderocapsa major. In comparison with Fig. 13, this colony is slightly older. Note strands of material beginning to cover the central well area. Parducz's fixative; glass coverslip. Bar= 2.0 µm.

Fig. 15. SEM of Siderocapsa major. Two microcolonies may be seen; a moderately developed colony (A) and a fully developed, completely covered one (B). Parducz's fixative; glass coverslip. Bar= 2.0 µm.

Fig. 16. SEM of Siderocapsa major. Cells undergoing binary fission (A) may be observed here. Parducz's fixative; glass coverslip. Bar= 2.0 µm.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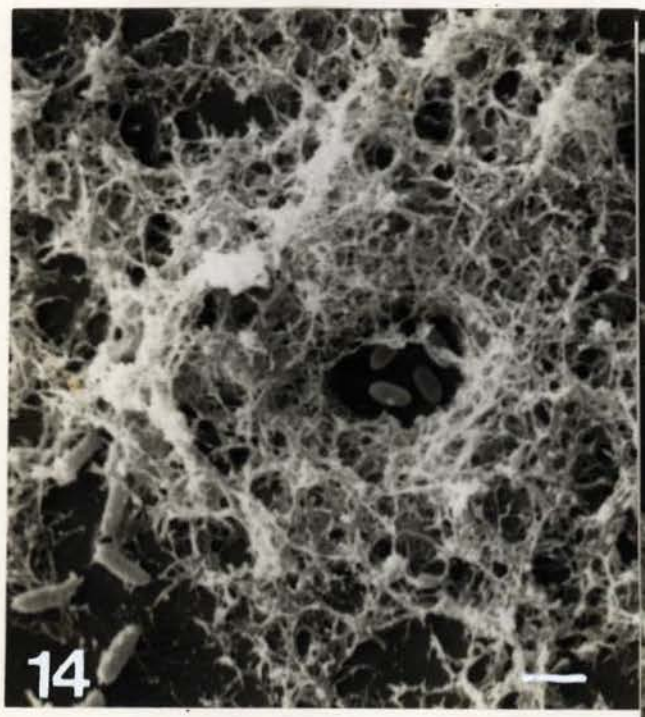

14

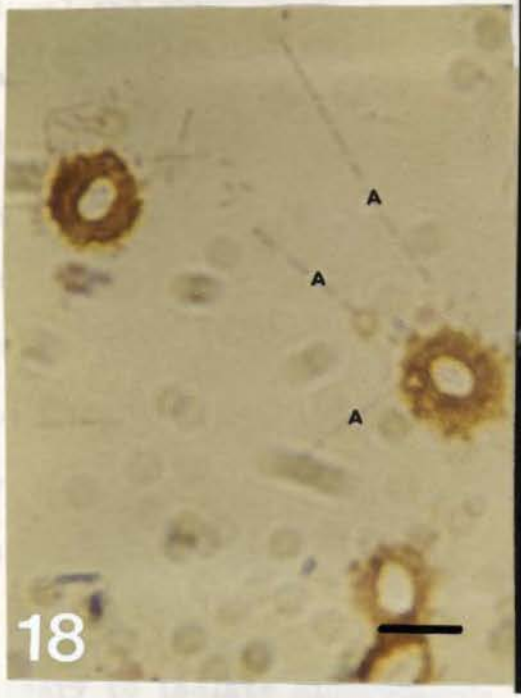

A
B
15

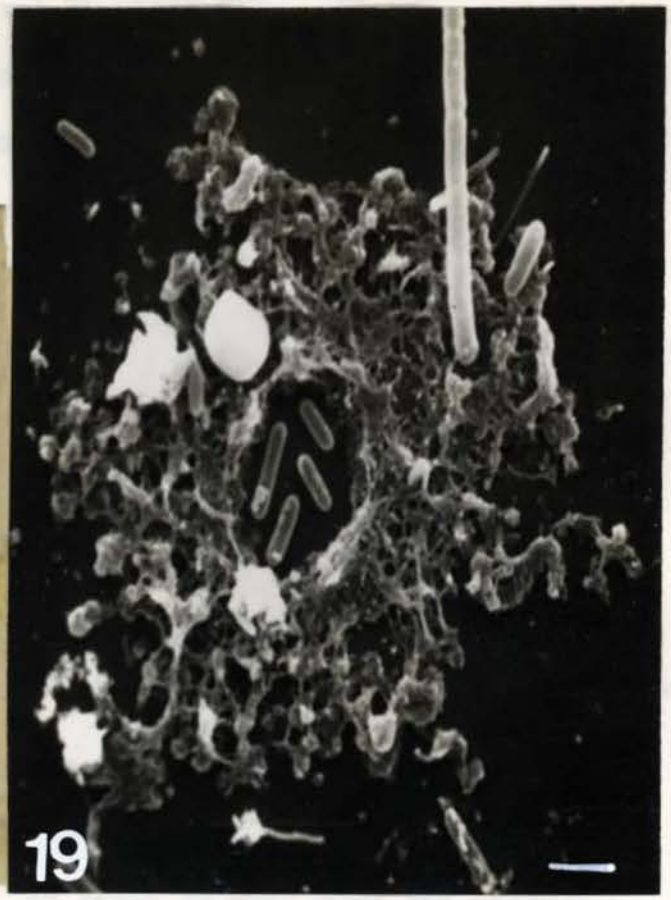
Fig. 17. SEM of S. natans. $OsO_4$ fixation; glass coverslip. Bar= 2.0 µm.

Fig. 18. Light micrograph of Siderocapsa microcolonies. Note bacterial filaments (A) emerging from the deposition area. Crystal violet; glass slide. Bar= 5.0 µm.

Fig. 19. SEM of S. major microcolony. The filament growing from the colony is of the same type as those seen in Fig. 18. $OsO_4$ fixation; glass coverslip. Bar= 2.0 µm.

Fig. 20. SEM of S. major microcolony. The filament is similar to the ones seen in Figs. 18 and 19. $OsO_4$ fixation; glass coverslip. Bar= 2.0 µm.


A
A
A
18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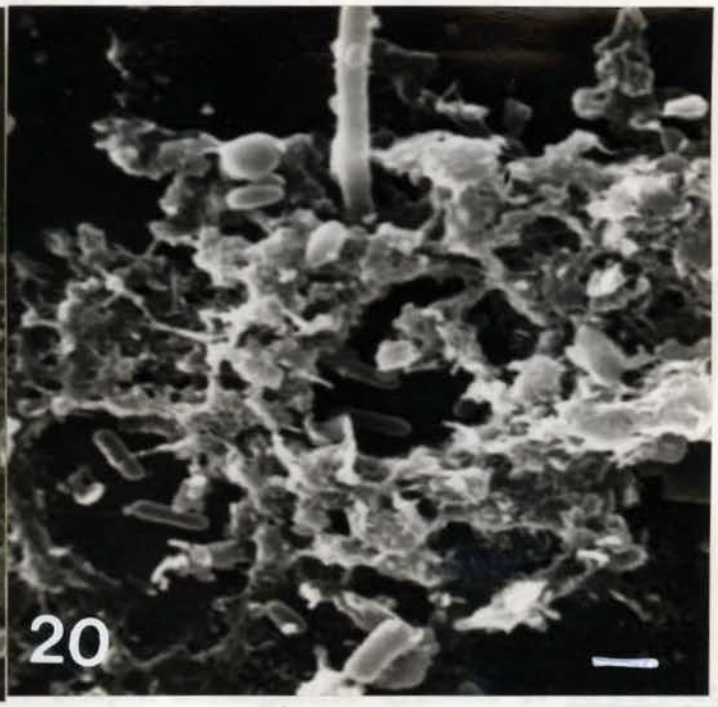
17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This may be the case in Figs. 20 and 21 where filaments are seen growing from the deposition of a microcolony.

Dubinina and Zhdanov (1975) on the basis of data provided from cultural and physiological studies have suggested that Siderocapsa is related to Arthrobacter. They propose that the genus Siderocapsa be abolished as an independent taxonomic entity and that the name Arthrobacter siderocapsulatus be substituted for this group of bacteria. They claim to have isolated Siderocapsa eusphaera, the first member of this genus to be grown in pure culture. They observed polymorphism and the V-shaped snapping division characteristic of Arthrobacter. In addition, they demonstrated that this bacterium oxidizes ferrous iron and manganese when grown under the proper conditions.

In an attempt to test Dubinina and Zhdanov's thesis, it is first necessary to isolate Siderocapsa. In order to show that the isolate is indeed Siderocapsa, these cells should be capable of producing characteristic microcolonies in pure culture.

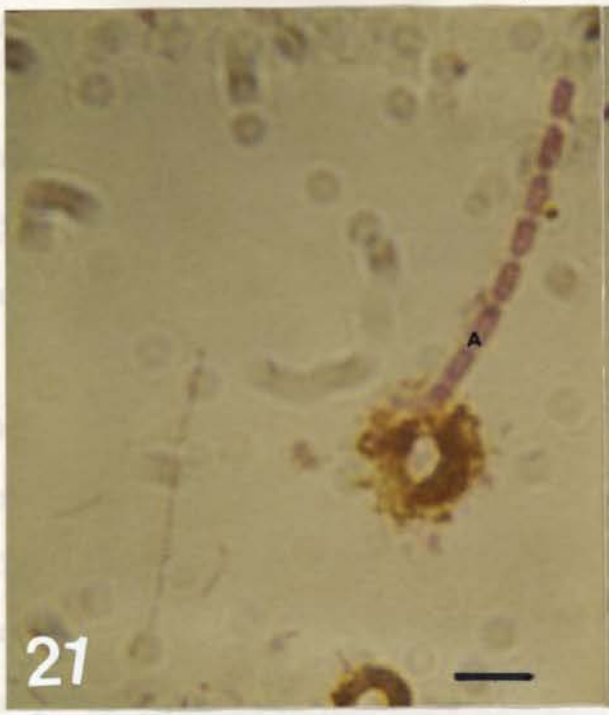
Several procedures were employed in the attempt to isolate and culture Siderocapsa. Clean glass slides from the Campus pond containing Siderocapsa major as well as Sphaerotilus natans and other members of the Sphaerotilus-Leptothrix group were pressed onto petri dishes containing either Pringsheim medium or an iron-containing medium. Reddish colonies (iron-containing) were isolated from the iron medium. Upon microscopic observation, cells in these colonies appeared identical. The bacteria are Gram-negative rods which characteristically undergo morphological changes as cultures age. Often V-shaped arrangments typical of Arthrobacter were seen in cultures 48-96 hours old (Fig. 22). Upon prolonged incubation (more than 4 weeks) these rods were observed to become more

Fig. 21. Light micrograph of a Siderocapsa microcolony. An S. natans filament (A) may be seen growing from the area of deposition. Crystal violet; glass slide. Bar= 5.0 µm.

Fig. 22. Light micrograph of the iron-oxidizing isolate from 72 hour PCA culture. V-shaped Arthrobacter-like arrangements (A) may be observed. Crystal violet; glass slide. Bar= 5.0 µm.

Fig. 23. Light micrograph of cells of the iron-oxidizing isolate, 72 hour PCA culture. Addition of calcium caused some sheath formation (A). Crystal violet; glass slide. Bar= 5.0 µm.

Fig. 24. Light micrograph of cells from an older PCA culture (more than 4 weeks) of the isolate. Note rods more elongate than those seen in Fig. 22. Crystal violet; glass slide. Bar= 5.0 µm.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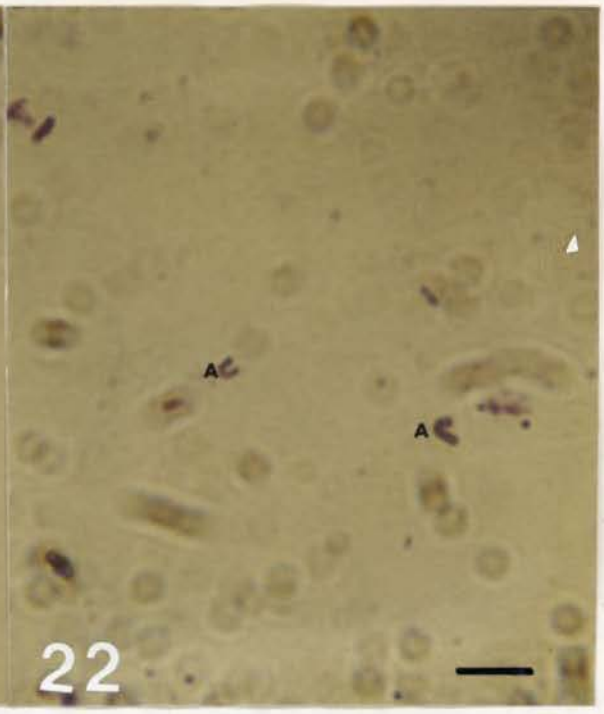
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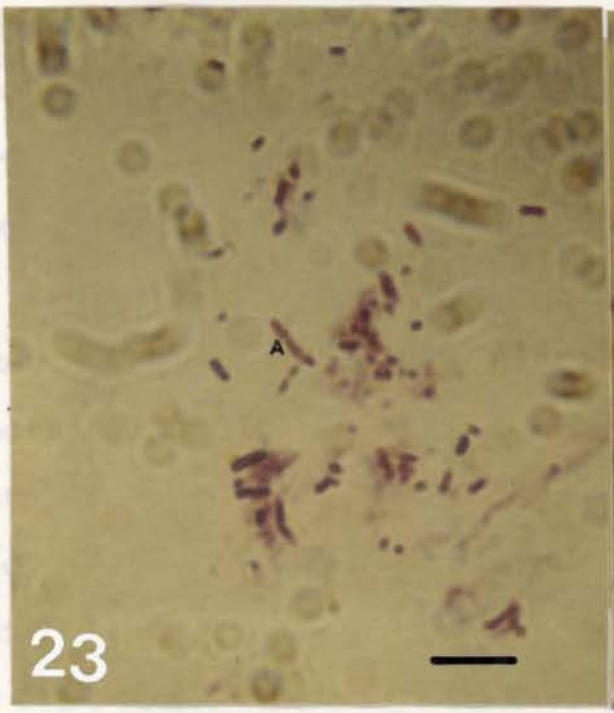
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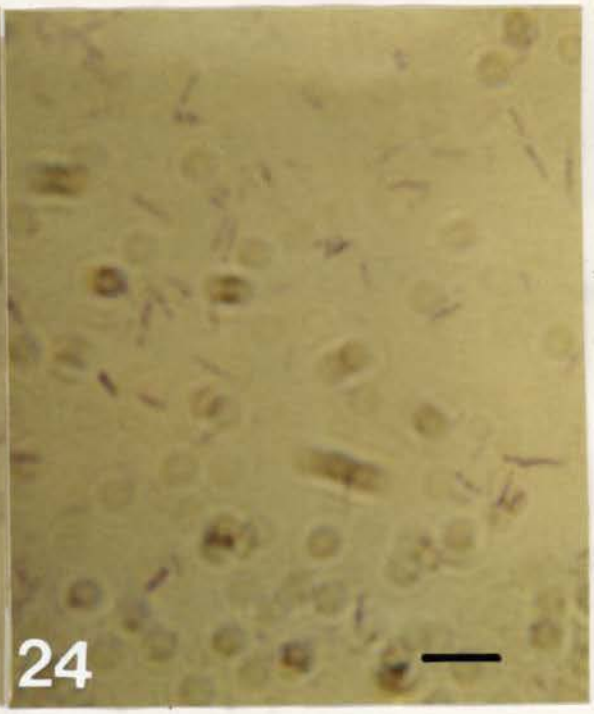
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elongate (Fig. 23). Addition of calcium to PCA and the iron-containing medium induced some sheath formation (Fig. 24) although no filamentous growth could be detected on agar cultures. In broth cultures the cells aggregated into a slime composed of filaments.

Microscopic comparisons revealed that cells of the isolated iron oxidizer were not the same size as cells regularly found in Siderocapsa microcolonies. Such variation in size is not unexpected in bacterial cultures in general.

After cultures were isolated, experiments were attempted to initiate the development of characteristic microcolonies on slides from cells grown in pure culture. Coplin jars containing sterile pond water with clean glass slides were inoculated with bacteria from agar cultures. No characteristic colonies appeared on these slides. Addition of several drops of 10% ferric ammonium sulfate and/or 10% manganese sulfate to the coplin jars had no effect on microcolony formation. These experiments were repeated using nutrient broth instead of sterile pond water to provide added nutrients which would promote microcolony formation. However, typical microcolonies were not obtained.

These results suggest that either the pure culture cultures isolated were not Siderocapsa or that pure cultures of Siderocapsa rapidly lose the ability to form the microcolonies on glass slides. To test the latter possibility, slides which were known to contain Siderocapsa microcolonies were placed adjacent to (but not touching) 3 clean, sterile slides in a coplin jar of filter-sterilized water. After a period of 48 hours microcolonies were observed on the previously clean slides. However, only one transfer was obtainable; with the addition of iron and manganese, in the forms of drops of 10% ferric ammonium sulfate,

respectively, to the coplin jars had no effect on transfer ability. Enlargment of the environment, using a 5 liter aquarium, as well as the addition of $O_2$ through an aquarium stone, had no effect. Evidently, these bacteria somehow lose the ability to transfer after a short period of time.

As a comparison, similar tests were run on S. natans (ATCC 15291). Clean glass slides were placed in nutrient broth cultures containing S. natans filaments. After 48 hours, in addition to many single cells, filaments were observed on these slides. Some of these filaments were found to have holdfasts resembling those from earlier light microscopy studies (e.g. Fig. 5).

Separation of Siderocapsa into species is based on morphological characters including their ability to oxidize and accumulate iron and/or manganese. Such distinctions are often acknowledged as arbitrary (Bergey's Manual of Determinative Bacteriology, 8th ed.). Dubinina and Zhdanov (1975) propose the elimination of the genus Siderocapsa on the basis of similarity in colony morphology and certain aspects of physiology of their isolate (S. eusphaera) to Arthrobacter species. While such a relationship may exist, these researchers have not demonstrated that all species of Siderocapsa are related to Arthrobacters. Indeed the relationship of S. eusphaera to S. treubii is in itself questionable, since the latter is sessile while the former is planktonic. Planktonic iron accumulaters include not only the known Arthrobacters which sequester iron but also any heavily encapsulated bacterium which utilizes the organic portion of an iron chelate. Such organisms as Enterobacter, Klebsiella and Serratia would be expected to be similar in microcolony morphology due to their abilities to utilize certain organo-iron compounds, such as ferric ammonium citrate.

In addition, Dubinina and Zhdanov (1975) point out similarities in colony morphology between S. eusphaera and Arthrobacter. In doing so they appear to misinterpret Molisch's illustration (Dubinina and Zhdanov, 1975, p. 347, Fig. 10b) which refers to microcolony formation rather than macrocolony formation (Dubinina and Zhdanov, 1975, p. 347, Fig. 10a). These two figures can not be compared. Included in their paper are some rather vague transmission electron micrographs of Siderocapsa microcolonies. If the validity of the relationship of S. treubii to S. major is questionable then the relationship of S. treubii to Arthrobacter is also in doubt.

From comparisons of physiological test data (Table 2) and behavioral characteristics in culture media, S. natans does not appear to show any relation to the isolate. Cultural behavior of the isolate has been shown to be somewhat similar to Arthrobacter globiformis but physiological tests seem to indicate no relationship. A. globiformis stains Gram-positive while the isolate is Gram-negative. Some physiological relationship may be seen between Enterobacter aerogenes and the isolate. E. aerogenes stains Gram-negative as well. Test results showed E. aerogenes to produce colonies similar to the isolate when cultured on the iron-containing medium.

TABLE 2. Physiological test results.

| Medium | Bacterial species | | | |
|---|---|---|---|---|
| | *E. aerogenes* | *S. natans* | "Siderocapsa" | *A. globiformis* |
| Glucose | + | - | - | - |
| Sucrose | + | - | - | - |
| Mannitol | + | - | - | - |
| Lactose | + | - | - | - |
| Indole | - | - | - | - |
| Methyl Red | - | - | - | - |
| V-Proskauer | + | - | + | - |
| Citrate | + | - | + | - |
| Nutrient Gelatin | + | - | - | + |
| Nitrate Reduction | + | + | + | + |
| Catalase | + | + | - | + |
| Lysine Decarboxylase | + | - | - | - |
| Phenylalanine Deaminase | - | - | - | - |
| $H_2S$ Production | - | - | - | - |
| Motility | + | + | + | - |

# CONCLUSIONS

1. Photomicroscopic examination of submerged glass slides and coverslips has revealed bacteria which correspond to Molisch's (1909, 1910) description of *Siderocapsa major*. Scanning electron microscopy has shown cells, in a clear central well, surrounded by an amorphous material which accumulates with the age of the colony.
2. Most previous authors have failed to explain how they have concluded that the material sequestered by *Siderocapsa* microcolonies is iron. Initial work with stains and x-ray microanalyses failed to demonstrate the presence of iron, however significant amounts of manganese has been found to be present.
3. Size and morphology of the holdfast of *Sphaerotilus-Leptothrix* members does not correspond to the size of the deposit around the *Siderocapsa* microcapsule. In addition, a definite developmental pattern appears which relates the total number of cells in the microcapsule to the total amount of material deposited.
4. Morphological behavior in culture has shown that the isolate might be related to *Arthrobacter globiformis*. Physiological test results indicate a possible relation to *Enterobacter aerogenes*.

# APPENDIX A

# APPENDIX A

## Stains, Fixatives and Reagents

### Stains

1. Prussian blue Reaction (Gurr, 1966).

   Solution A: 2.0 g potassium ferrocyanide to 99 ml deionized (or distilled) water.
   Solution B: 5.0 ml glacial acetic acid to 95 ml deionized water.
   Procedure: Allow a slide containing iron bacteria to dry, then heat fix. Heat a mixture of 25 ml each of solutions A and B to the boiling point. Transfer the slide to the mixture of solutions A and B, just below the boiling point. Rinse the slide with deionized water. Drain and allow to air dry thoroughly. Iron present in the sheaths of the bacteria will be a blue color.

2. Microchemical test for iron (Johansen, 1940).

   Solution A: 1% aqueous HCl.
   Solution B: Concentrated aqueous solution of potassium ferrocyanide.
   Procedure: After heat fixing transfer slides containing iron bacteria to a mixture containing 3 drops of solution B (freshly prepared) to every 25 ml of solution A; immerse for 5 minutes. Rinse the slide with deionized (or distilled) water. Any iron present will turn a blue color.

### Fixatives

1. Parducz's Instantaneous Fixation Technique (Parducz, 1967).

   Solution A: 2% aqueous $OsO_4$.
   Solution B: Saturated aqueous $HgCl_2$.
   Mix: 6 parts solution A with 1 part solution B.

### Reagents

1. 10% $MnSO_4 \cdot 7H_2O$

   | | |
   |---|---|
   | $MnSO_4 \cdot 7H_2O$ | 10 g |
   | Deionized (or distilled) water | 100 ml |

2. 10% ferric ammonium citrate

   | | |
   |---|---|
   | Ferric ammonium citrate | 10 g |
   | Deionized (or distilled) water | 100 ml |

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