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# The *Sphaerotilus-Leptothrix* Group of Bacteria

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## INTRODUCTION

Sheath formation is found in a restricted number of aquatic bacteria mainly belonging to the genera *Sphaerotilus* and *Leptothrix*. However, a recent survey of the filamentous organisms occurring in activated sludge (19, 28, 100) has revealed that, in addition to the gram-negative *Sphaerotilus* and *Leptothrix* spp., a number of differing sheathed bacteria exist. One group of less known gram-negative organisms, with a different guanine-plus-cytosine content of deoxyribonucleic acid (DNA) and initially described as *Streptothrix* spp. (102), were later renamed *Haliscomenobacter* spp. (103). A second group of unknown sheathed organisms detected in activated sludge includes gram-positive bacteria (28, 100). Their isolation is difficult, and so far no detailed description of their properties is available.

Concerning the nomenclature of the organisms of the *Sphaerotilus-Leptothrix* group, we do not agree with Pringsheim's proposal (69, 70)

† Deceased, 15 June 1977.

to insert all the members of this group into one genus, *Sphaerotilus*, recognizing only two species, *S. natans* and *S. discophorus*. This classification is not in accordance with the diversity of the various species of the group, particularly those of the genus *Leptothrix*. Therefore, the nomenclature that we have put forward in the 8th edition of *Bergey's Manual* (55, 60) is maintained. In this survey the names of organisms are given as they are used in the reviewed papers. If Pringsheim's nomenclature is followed, the corresponding names from *Bergey's Manual* are added in parentheses.

The presence of a sheath has nutritional and ecological consequences for this group of organisms. This concerns particularly their growth in slowly running water low in nutrients, where the presence of a sheath enables the bacteria to attach themselves to solid surfaces. In addition, the sheath protects the organisms against parasites and predators (104). However, cell propagation of sheath-forming bacteria is not necessarily dependent on the presence of the sheath, as can be concluded from the ability of sheathless mutants of the *Sphaerotilus-Leptothrix* group to grow and divide without this structure (57, 77, 91).

The present approach to the bacteria of the *Sphaerotilus-Leptothrix* group includes a description of the morphology and physiology of these organisms, with special attention to the oxidation of iron and manganese. Shortly before the preparation of the manuscript, a review was published by Dondero (24) predominantly dealing with *S. natans* in relation to environmental and ecological problems.

## ISOLATION PROCEDURES

Bacteria of the *Sphaerotilus-Leptothrix* group may be isolated by different procedures.

Non-manganese-oxidizing sheathed bacteria belonging to the genus *Sphaerotilus* can be isolated in the following way. Grayish to brownish tufts of sheathed bacteria, generally growing attached to stones or plants in polluted streams, are collected and subsequently washed with sterile water several times. Homogenization of the washed flocs by blending for a very short time may be useful (25). Small pieces of the flocs are then transferred with a thin, straight needle to previously dried agar plates containing low levels of nitrogen and carbon sources, up to about 1 g of each per liter (3, 57, 58, 88). The provision of nutritionally poor media limits the size of undesirable bacterial colonies, leaving large areas for the filamentous organisms. This procedure enables the successful isolation of the outgrowing filamentous microorganisms with the aid of micropipettes and low-power magnification (57, 69, 70, 88). Pure cultures may be obtained after several passages on basal media supplemented with nitrogen and carbon sources up to 2 g of each per liter in the presence of vitamin $B_{12}$ (see Growth Conditions).

In nonpolluted or slightly polluted waters, sheathed bacteria may occur in low numbers, so that for a successful isolation the use of an enrichment culture may be advantageous. The application of a solution containing low levels of sodium lactate and $NH_4Cl$ (3), diluted meat extract (13), extracted hay (109), alfalfa straw (7), pea straw (57, 58), or willow leaves (29) often results in the development of a slimy growth of sheathed bacteria on the walls of the vessels and on the solids present. The adhering flocs are used for isolation of pure cultures as described above.

Many more difficulties are generally met in the isolation of sheathed bacteria from activated sludge in which these microorganisms represent only a minority of the total bacterial population. Enrichment cultures may be applied, but homogenization procedures have to be avoided. A mild treatment of the sludge suspension with the aid of a tube stirrer, followed by plating on a poor medium, may favor the isolation of a variety of filamentous and sheathed bacteria (100). Incorporation of inhibitory compounds in the media to suppress contaminating bacteria has been unsuccessful (23, 31, 47).

Manganese-oxidizing sheathed bacteria belonging to the genus *Leptothrix* can be isolated by similar procedures as mentioned above. Flocculent cell material, encrusted with ferric or manganic oxides and obtained from natural habitats, is washed and transferred to solid media supplied with low concentrations of nitrogen and carbon sources and containing $MnSO_4 \cdot H_2O$, 25 to 50 mg/liter, or $MnCO_3$, 1 to 2 g/liter (57, 58, 77). Agar media containing $MnCO_3$ are preferred because of (i) the low levels of $Mn^{2+}$ ions in solution and (ii) the constancy of the pH of the medium. However, the turbidity of the plates may be disadvantageous, masking the location of undesirable contaminations of the filamentous brown colonies. This difficulty can easily be eliminated by supplying $MnCO_3$ in the following way. Plates with a diameter of at least 12 cm are poured and dried, whereupon a hole is cut in the center of the agar with a sterile cork borer. The washed bacterial flocs or sediments are spread out on the agar surface as usual, and a few drops of a sterile concentrated $MnCO_3$ suspension is pipetted into the hole (11, 32, 101). The $Mn^{2+}$ ions diffusing through the agar are oxidized by the filamentous colonies. This method enables

optimum examination of the colonies under low-power magnification and also allows a prolonged incubation time of the plates, because the addition of sterile water to the hole every 3 days prevents drying out of the agar medium.

Commercially available $MnCO_3$ often contains too much oxidized manganese. It is therefore advisable to prepare $MnCO_3$ from equivalent amounts of analytical-grade $MnSO_4 \cdot H_2O$ and $Na_2CO_3$.

If $MnSO_4$ is incorporated in the agar media or in the culture solutions, its concentration should be kept as low as possible to facilitate detection of the $Mn^{2+}$-oxidizing ability of the bacteria. Excessive amounts of $MnSO_4$ may adversely affect the growth of the organisms (34, 41, 69, 70, 77; A. H. Johnson, Ph.D. thesis, Washington State University, Pullman, 1966).

Enrichment of *L. ochracea* has been achieved by a procedure imitating natural conditions, i.e., slowly running ferrous iron-containing soil extract at a temperature of 20 to 25°C. We have described the apparatus constructed for this enrichment procedure in detail (57, 58). This procedure may also be used for testing growth and iron precipitation by isolates of the *Sphaerotilus-Leptothrix* group under more or less natural conditions. For that purpose the iron-containing soil extract is sterilized by Seitz filtration.

## TAXONOMY

The principal morphological characteristics of bacteria of the *Sphaerotilus-Leptothrix* group are summarized in Table 1. The features reported are derived from observations of bacteria present in samples from natural habitats and from freshly isolated pure cultures. In unsectioned and unstained preparations, sheaths of *S. natans* appear smooth, whereas the *Leptothrix* species produce a netlike structure (Fig. 1 through 3).

Bacteria of both genera may contain intracellular globules of poly-$\beta$-hydroxybutyrate (PHB), particularly under laboratory conditions (Fig. 4). Ultrathin sections of cells revealed the location of intracellular polysaccharides, stored in small, irregularly shaped bodies predominantly present in the terminal and subterminal regions of the cell (66). These bodies were described earlier (22) as polyribosomal aggregations.

Additional observations on morphology, not listed in Table 1, will be given hereafter when describing various types of sheath-forming bacteria.

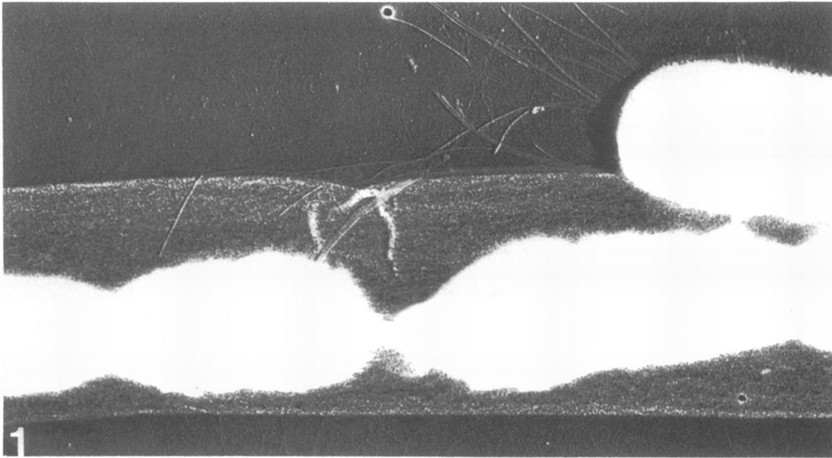
### *Sphaerotilus natans*

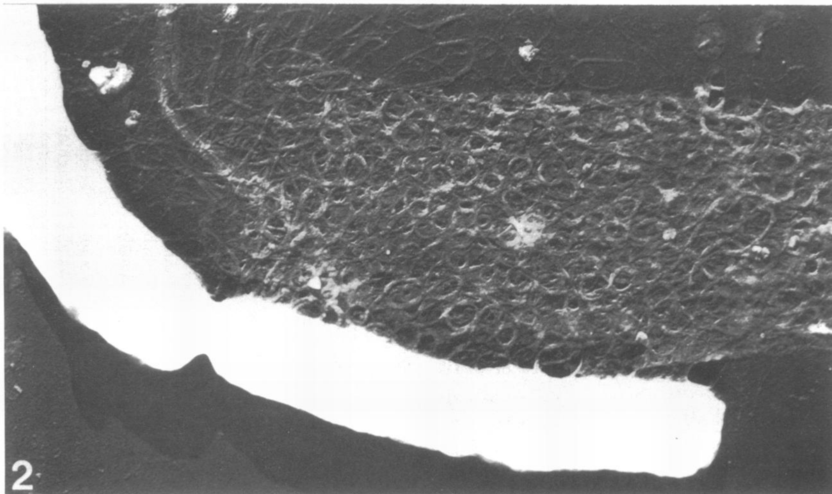
In young *S. natans* cultures, almost all of the cells are contained within sheaths; they are ar-

TABLE 1. *Differential characteristics of the species of the Sphaerotilus-Leptothrix group*[a]

| Species | Cells | | Flagella | | Holdfast in: | | False branchings | Sheaths | | Colony form on glucose (0.1%)-peptone (0.1%) agar[b] | Response to increased concn of organic nutrients[c] | $Mn^{2+}$ oxidation | Loss of $Mn^{2+}$-oxidizing capacity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Width (µm) | Length (µm) | Monotrichous, polar | Polytrichous, subpolar | Rich media | Poor media | | Surface structure[d] | Loss of formation | | | | |
| *S. natans* | 1.2–2.5 | 2–10 | − | + | − | + | + | Smooth | Sporadic | F(S), large | ++ | − | − |
| *L. lophoea* | 0.8–1.4 | 3–7 | − | + | + | + | + | Rough | Sporadic | S(F), intermediate | ± | + (retarded) | ± |
| *L. ochracea* | 1.0 | 2–4 | + | − | − | − | − | Rough | Unknown | Unknown | Unknown | + | Unknown |
| *L. pseudo-ochracea* | 0.8–1.2 | 4–12 | + | − | − | − | − | Rough | Regular | F(S), large | + | + | + |
| *L. cholodnii* | 0.7–1.3 | 2–7 | + | − | − | − | − | Rough | Frequent | S(F), intermediate | ++ | + | + |
| *L. discophora* | 0.6–0.8 | 1–4 | + | − | − | − | ± | Rough | Regular | S(F), small | ± | + | − |

[a] From reference 57 (modified).
[b] F, Filamentous; S, smooth.
[c] ++, Strong; +, moderate; ±, slight; −, absent.
[d] Electron microscopic observations.



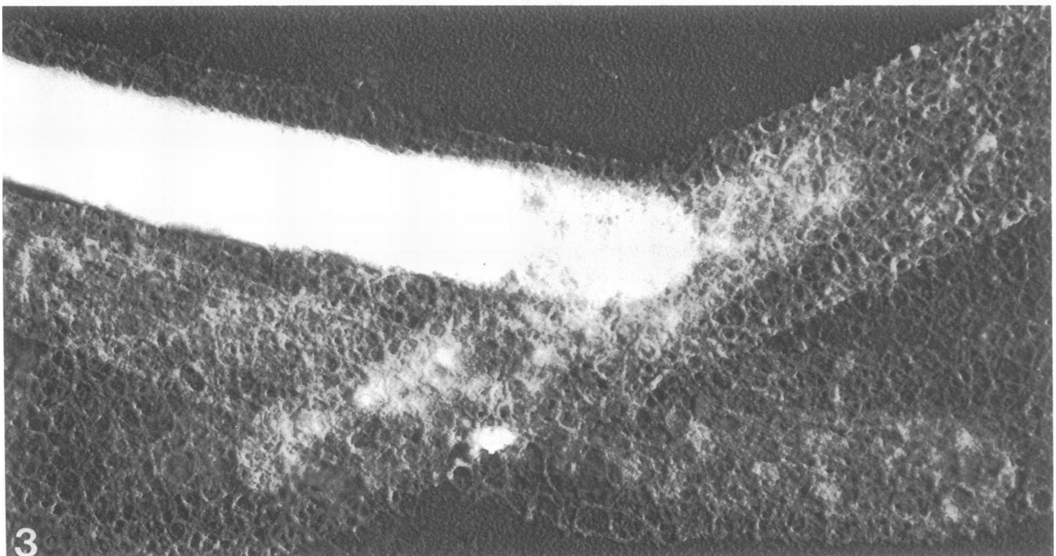




FIG. 1-3. *Surface structure of sheaths. (1) S. natans; (2) L. lopholea; (3) L. cholodnii. ×18,000.*

ranged in single rows, rarely in two or three rows (Fig. 4). In older cultures, free-swimming and nonmotile single cells or pairs of cells may be seen (57, 69, 70, 88).

Large, circular bodies resembling protoplasts have frequently been observed in pure cultures after prolonged incubation (13, 57, 67). Their formation is probably due to the production of enzymes involved in the decomposition of the cell walls during the death phase. The incorporation of 0.4 g of glycine per liter into the nutrient medium has been reported to favor this phenomenon (67).

**Flagella.** Electron microscopic examination of the flagellar appendage of *S. natans* shows a bundle of flagella, sometimes so intertwined as to give the appearance of a single "unit flagellum" (Fig. 6). The number of strands of each flagellum is given as 10 to 30 (42, 66) with the diameter of each strand given as between 12.5 and 16.0 nm (22, 36). The flagella of *S. natans* consist of three sections: a long filament, a short hook, and a basal body complex (67).

**Holdfasts.** Cells of *S. natans* swimming in liquid medium (e.g., hanging drops) become attached to solid surfaces by means of a slimy compound which soon hardens to a holdfast (Fig. 5) (67). These cellular appendages are rarely observed in common artificial media, but in a basal salts solution containing peptone, 2 g/liter, cyanocobalamin, 5 μg/liter, and a previously neutralized solution of $FeCl_3 \cdot 6H_2O$ and quinic acid in final concentrations of 1.8 and 0.75 g/liter, respectively, holdfasts may be easily observed (60). Cell material attached to the glass walls of Erlenmeyer flasks may also be used for the examination of holdfasts. Sheath synthesis proceeds from the holdfast end of the filament and presumably develops synchronously with cell division (67).

**False branchings.** Cells are released from the sheaths by expansion of dividing and growing cells and by their motility. Such free-swimming cells may sometimes adhere to existing sheaths, where they propagate and thus give rise to false branchings (Fig. 7). Strains of *S. natans* may show differences in regard to false branchings, which occur much more frequently in poor media than in rich media. The treelike growth of *S. natans* resulting from false branchings may be observed particularly in weakly polluted water and in activated sludge flocs. According to Phaup (67), false branchings may also be due to rupture of the sheath, allowing cells to protrude from the break while growth also continues in the original direction.

**Sheaths.** Sheaths cannot always be easily recognized when completely filled with cells, as in the organisms present in activated sludge samples or from natural habitats. If parts of the envelopes are clearly vacated by the cells, recognition of the organism cannot be misinterpreted (Fig. 4). However, it would be premature to identify such an organism as a *Sphaerotilus* species without isolation of the bacterium and investigation of its properties.

The sheaths are probably built up by a successive excretion of thin fibrillar layers. This may be concluded from the photographs presented by Petitprez et al. (66) and Hoeniger et al. (36), in which double or multiple layers are present. More sheath material is deposited by the cells in the presence of glucose than in its absence (36).

Analysis of envelopes prepared after treatment of cell suspensions with tris(hydroxymethyl)aminomethane buffer, tetrasodium ethylenediaminetetraacetic acid, lysozyme, and dodecyl sulfate revealed that the sheath of *S. natans* is composed of a protein-polysaccharide-lipid complex which is morphologically distinct from the cell wall and from bacterial capsules. The isolated sheaths contained 36% reducing sugars, 11% hexosamine, 27% protein, 5.2% lipid, and 0.5% phosphorus, calculated on dry matter. No muramic or teichoic acid has been detected (75).

The sheaths of *S. natans* are covered with a cohering slime layer of variable thickness, as may be seen in India ink mounts. These slime layers increase in thickness when the organism is cultivated in media with large amounts of peptone, which adversely affect filamentous growth (30). The major components of the extracellular polysaccharide are fucose, glucose, galactose, and glucuronic acid, in molar ratios of 1.0:0.77:0.77:0.80. These slime layers are qualitatively similar to polysaccharides produced by bacteria of the *Klebsiella* group (31).

**Additional characteristics.** If *S. natans* is cultivated in the presence of chelated ferric iron, such as ferric citrate or quinate, the sheaths become covered (and presumably impregnated) with ferric hydroxide, so that they resemble to some extent the sheaths of *L. ochracea* (unpublished data). However, their cell diameter is larger, and completely empty sheaths occur much less frequently than in *L. ochracea*. Covering (and, probably, impregnation) of sheaths with ferric hydroxide is also obtained upon cultivation of the organism in running ferrous iron-containing soil extract (57); manganese is not oxidized (57, 58, 69, 70, 88).

Recently, several bacteria resembling *S. natans* have been isolated from activated sludge (28, 100). However, they deviate from the common type I in that the cells are smaller and have a single polar or subpolar flagellum. Two out of

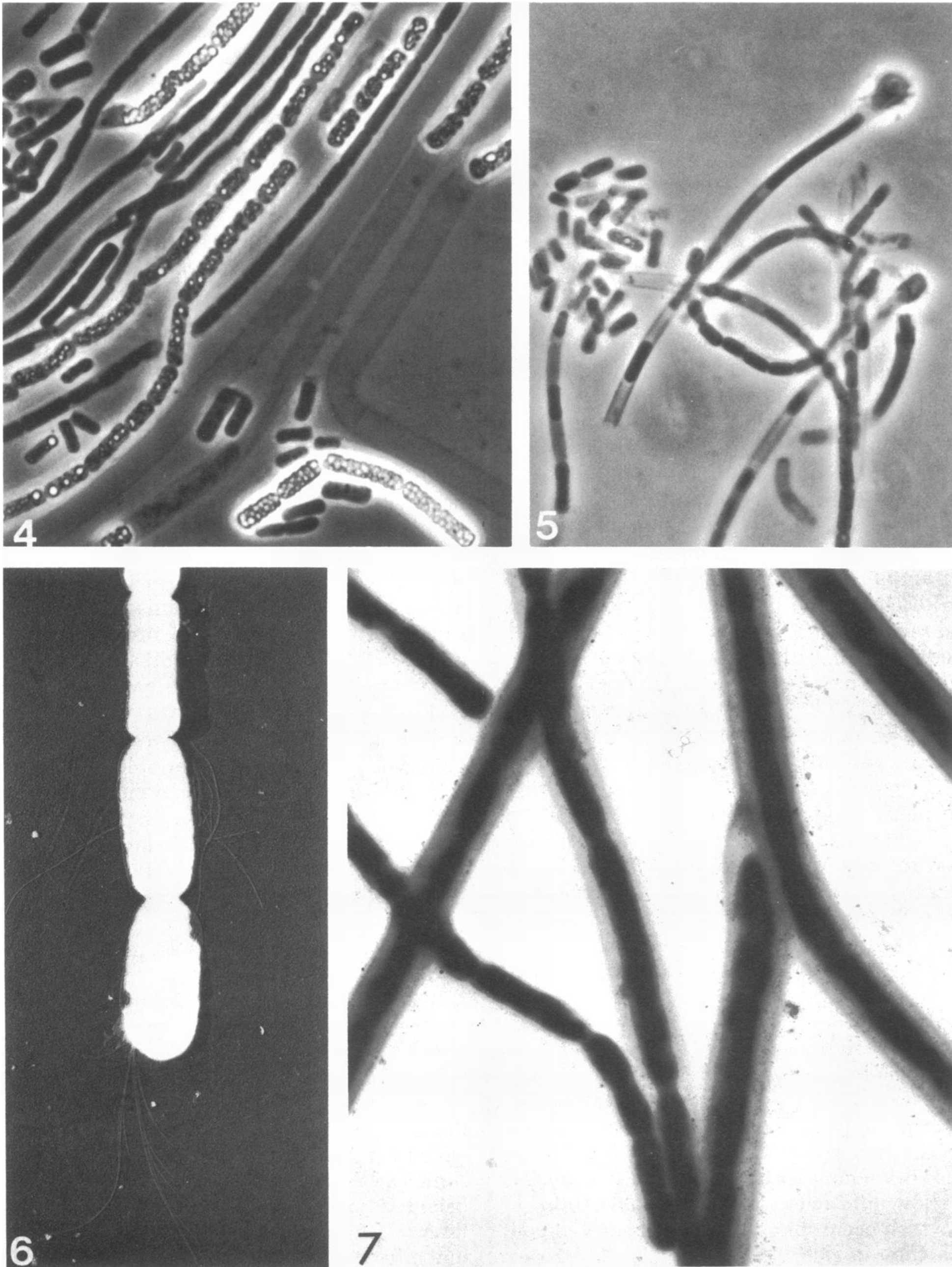



FIG. 4-7. *S. natans. (4) Cells with PHB; ×1,625. (5) Holdfasts; ×1,625. (6) Subpolar flagella; ×10,000. (7) False branchings; ×6,500. (Fig. 4 is from J. S. Poindexter, Microbiology, an Introduction to Protists, Macmillan Publishing Co., Inc., New York, 1971, with permission of the publisher.)*

10 strains produce a yellowish pigment on rich agar media. One of these strains has an abnormal slimy appearance and forms elevated colonies with lobate edges instead of the normal flat, filamentous colonies. Electron microscopic examination revealed the same, more or less smooth, sheath structure in unstained and unsectioned preparations as occurs in *S. natans*. Manganese oxides are not precipitated during growth on $Mn^{2+}$ agar or in liquid media. These

observations indicate the existence of a number of *Sphaerotilus* species with deviating morphological and physiological characteristics.

### *Leptothrix lopholea*

*L. lopholea* resembles both *S. natans* and *L. sideropous* (15, 26; M. Cataldi, Ph.D. thesis, University of Buenos Aires, Buenos Aires, Argentina, 1939). Its morphology is similar to that of *S. natans* with respect to (i) formation of holdfasts, (ii) development of a bundle of subpolar flagella, and (iii) formation of false branchings. It differs from *S. natans* in having a smaller cell diameter and in exhibiting manganese oxidation, which, however, is retarded in comparison to the manganese oxidation by other isolated *Leptothrix* species. False branchings may also be observed in rich media, which is in contrast to *S. natans*, where it only occurs in poor media.

The cells generally arise from common holdfasts when grown in relatively poor media (Fig. 8). In a basal salts medium with Casamino Acids, 2 g/liter, and supplied with a neutralized solution containing $FeCl_3 \cdot 6H_2O$ up to 2.0 g/liter and quinic acid, 0.96 g/liter, the holdfasts become impregnated with ferric hydroxide (55) and show an identical morphology to that described for *L. sideropous* (15, 26; Cataldi, Ph.D. thesis). The cells are generally slightly wider than those of *L. sideropous*. Manganese oxidation takes place either on the whole surface of the sheaths or only on the holdfasts, depending on the strain used. Colonies on poor agar media are generally smaller than those of *S. natans* and less filamentous. Although generally the response of this type of organism to increased organic nutrients is poor, some strains may exhibit an excellent response; such strains oxidize $Mn^{2+}$ more slowly and poorly (unpublished data).

### *Leptothrix ochracea*

*L. ochracea* is the most common iron-storing ensheathed bacterium, apparently occurring all over the world in slowly running ferrous iron-containing water poor in readily decomposable organic material. Under these conditions the pronounced development and activity of the organism give rise to the accumulation and sedimentation of large masses of ferric hydroxide, which presumably are responsible for the formation of bog ore.

*L. ochracea* was studied and described in detail by Cholodny (15) and Charlet and Schwartz (14) who did not succeed in isolating the organism. Authors (68; Cataldi, Ph.D. thesis) who claimed to have isolated this bacterium actually dealt with one of the other *Leptothrix* species. The same mistake was made by Pringsheim (69, 70), who considered *L. ochracea* to be a modification of *S. natans* which could occur when the latter organism was growing in Fe(II)-containing medium poor in organic nutrients. We (57) grew pure cultures of *S. natans* and crude cultures of *L. ochracea* under laboratory condi-

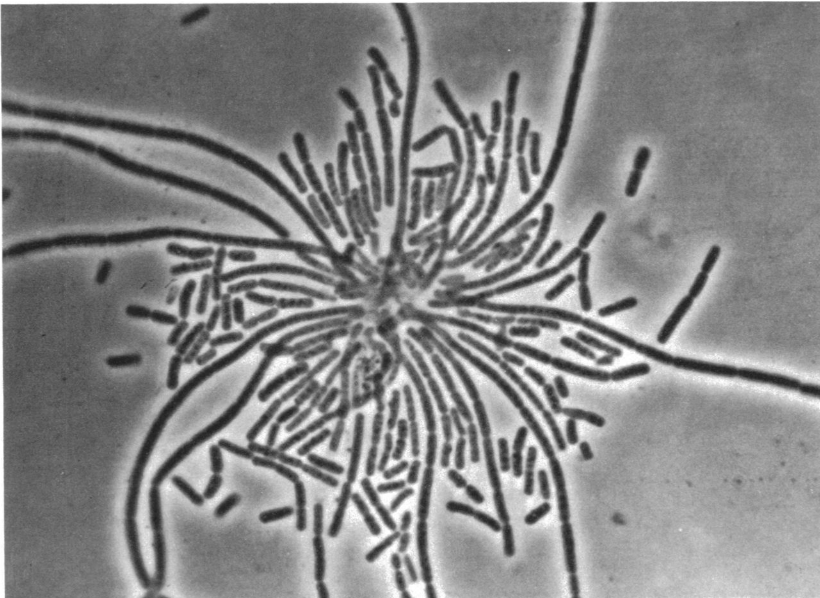


FIG. 8. *L. lopholea; many trichomes radiating from common holdfasts.* ×*1,625.*

tions in slowly running soil extract containing approximately 20 mg of Fe(II) per liter. Under these conditions the sheaths of *S. natans* on aging were covered and sometimes encrusted with iron hydroxide, giving a granular structure, as contrasted to 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, as contrasted to the envelopes of *S. natans*, which were to a larger extent filled with cells. *S. natans* was easily reisolated from its poorly developed iron-bacterium stage, but all efforts to isolate such an organism from the bulky enrichment culture of *L. ochracea* failed. One of the *Leptothrix* isolates obtained from this enrichment culture resembled *L. ochracea* when growing in iron-containing soil extract. The tendency of this organism to leave its sheath and to leave behind bulky masses of empty envelopes was much less pronounced than was observed in the crude cultures of *L. ochracea*. For that reason the name *L. pseudo-ochracea* was given to this isolate (see below).

The formation of large masses of empty sheaths by *L. ochracea* within a relatively short time was demonstrated in a microscopic study of growing crude cultures of the organism in a film of ferrous iron-containing soil extract on agar prepared with the same liquid (54, 58). A chain of about 10 cells was seen to leave their sheath at the rate of 1 to 2 μm/min, continuously producing a new hyaline sheath connected with the existing envelope. Shorter chains sometimes separated from the main chain (Fig. 9). At the end of the observation period (approximately 18 h), the originally present bacteria had increased to about 100 sheathed cells, each with a diameter of 1 μm and a length of about 3 μm, corresponding to a total cell length of approximately 300 μm. The total length of synthesized sheaths had increased to more than 10 times this value during the same period. As far as can be concluded from microscopic observations, deposition of ferric compounds on the sheaths takes place after the cells have left the envelopes. Electron microscopic observations indicated that the smooth iron hydroxide layers on the sheath consist of very small granules regularly embedded in the sheath matrix (Fig. 11). Aged sheaths of *L. ochracea* are apparently brittle, since they are often broken into relatively small pieces (Fig. 10). Nothing is known about the locomotion mechanism of the cells. A gliding movement cannot be excluded.

### *Leptothrix pseudo-ochracea*

*L. pseudo-ochracea* cells are much more slender than those of other microorganisms of the *Sphaerotilus-Leptothrix* group (Table 1). They are very motile by a single, thin, polar flagellum. Even chains of up to 6 to 10 cells show an undulatory locomotion, comparable to that of an extraordinarily long *Spirillum* sp. This characteristic may be responsible for the relatively large number of empty sheaths in culture solutions, as compared with other isolated types with the exception of *L. ochracea*, which possesses even considerably more empty sheaths.

In media with $Mn^{2+}$ ions, the sheaths become regularly encrusted with small granules of Mn oxide (57). This type survives in slowly running ferrous iron-containing soil extract as used in our apparatus (57, 58), but growth is insignificant. Under these conditions a relatively thin layer of ferric hydroxide is precipitated on the sheaths (55). Reisolation of the organism from running ferrous iron-containing soil extract on agar media can easily be accomplished.

When growing in ferric quinate-containing culture solutions, the sheaths become impregnated and poorly covered with a smooth layer of ferric hydroxide, resembling *L. ochracea* in this respect.

The colonies of this type on glucose-peptone agar are large and filamentous; the ensheathed cells often grow in concentric rings, suggesting a close relationship or identity of this organism with a sheathed bacterium isolated and wrongly described as *L. ochracea* by Cataldi (Ph.D. thesis).

### *Leptothrix cholodnii*

*L. cholodnii* can easily be isolated from nonpolluted and polluted waters as well as from activated sludge. When grown in the presence of $Mn^{2+}$ the sheaths of *L. cholodnii* become irregularly covered with granular manganic oxide (Fig. 12). At some sheath locations, the manganese oxide deposits may exceed 20 μm. In slowly running ferrous iron-containing soil extract under laboratory conditions (57, 58), the sheaths are covered with a moderately thick (10 μm), dark-brown layer of fluffy ferric hydroxide. A similar picture may be obtained in cultures supplied with ferric quinate (Fig. 14).

The behavior of this bacterium in culture solutions resembles that of *S. natans* as to growth rate, amount of cell material produced, and amounts of intracellular storage compounds formed. However, it differs from this organism in cell diameter as well as sheath morphology, flagellation (Fig. 15), $Mn^{2+}$-oxidizing ability, and its tendency to form sheathless mutants spontaneously (Fig. 13; see Mutations and Modifications). From the descriptions of *L. winogradskii* by Cataldi (Ph.D. thesis) and of *S. discophorus* by Rouf and Stokes (77), we suggest that these

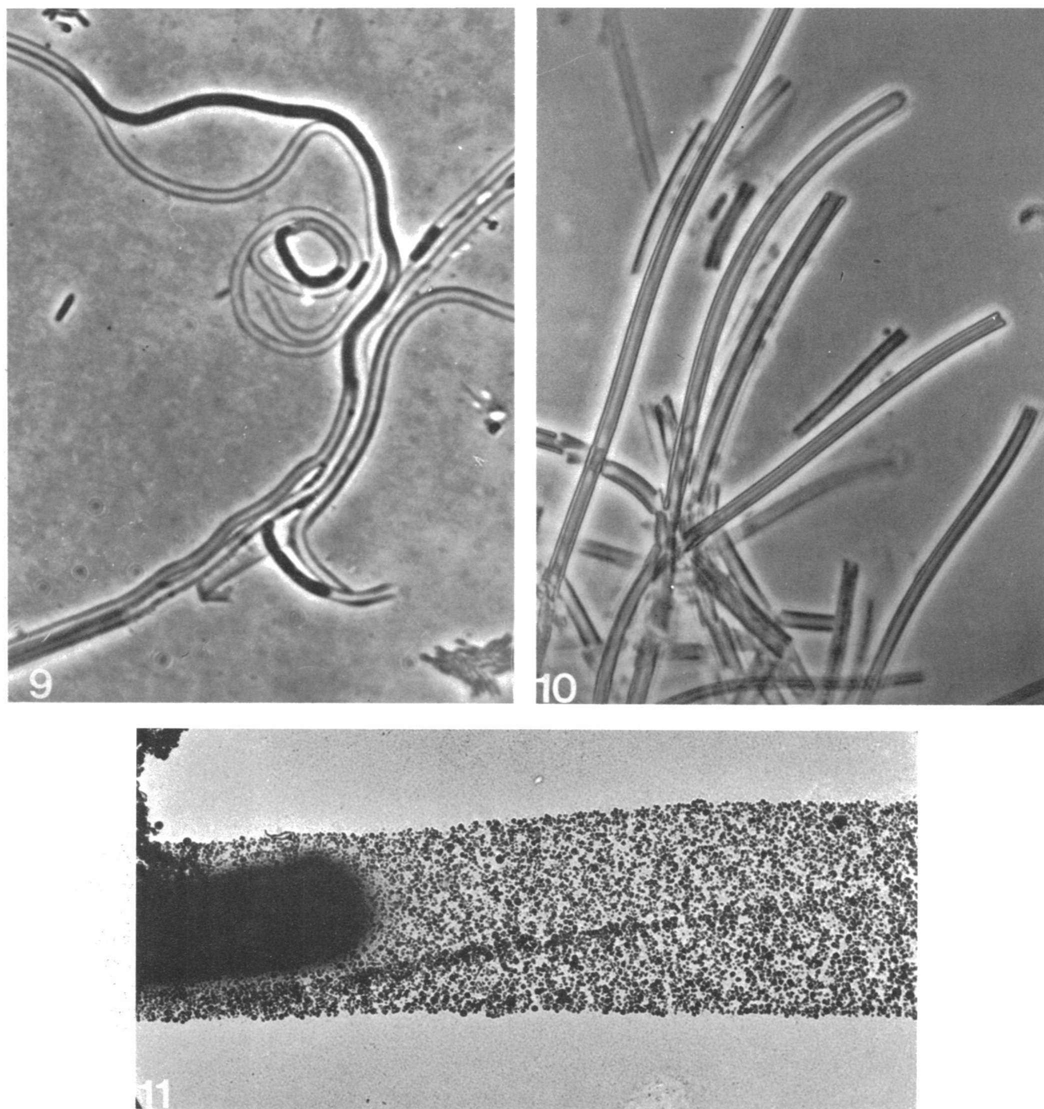


FIG. 9-11. *L. ochracea (no pure culture). (9) Cells moving out of sheaths and subsequently forming new sheaths at a rate of 1 to 2 μm/min; ×1,625. (10) Broken old sheaths covered and impregnated with ferric hydroxide, in slowly running iron(II)-containing soil extract; ×1,625. (11) Sheath impregnated with ferric hydroxide; ×21,000. (Fig. 10 is from reference 57, with permission of the publisher; Fig. 11 is from reference 54, with permission of the publisher.)*

authors were dealing with *L. cholodnii* species.

### *Leptothrix discophora*

*L. discophora* cells are the smallest among the isolated strains of the *Sphaerotilus-Leptothrix* group (Table 1). The motile cells may either be surrounded by a thin sheath or be free-swimming. When the microorganisms are grown in nutrient media containing $Mn^{2+}$, the sheaths become irregularly encrusted with granular manganic oxide (Fig. 16). *L. discophora* grows poorly in slowly running iron(II)- and manganese(II)-containing soil extract (53, 57); the sheaths become covered with a thick, dark-brown, fluffy layer of ferric hydroxide and $MnO_2$ (Fig. 18), increasing the diameter of the bare sheaths 10- to 25-fold, up to about 20 to 25 μm (53, 54, 57, 58). Under these conditions the sheaths may taper towards the growing tips (15, 29). According to Cholodny (15), false branchings may occur in natural habitats, but we have never observed this property in culture solutions.

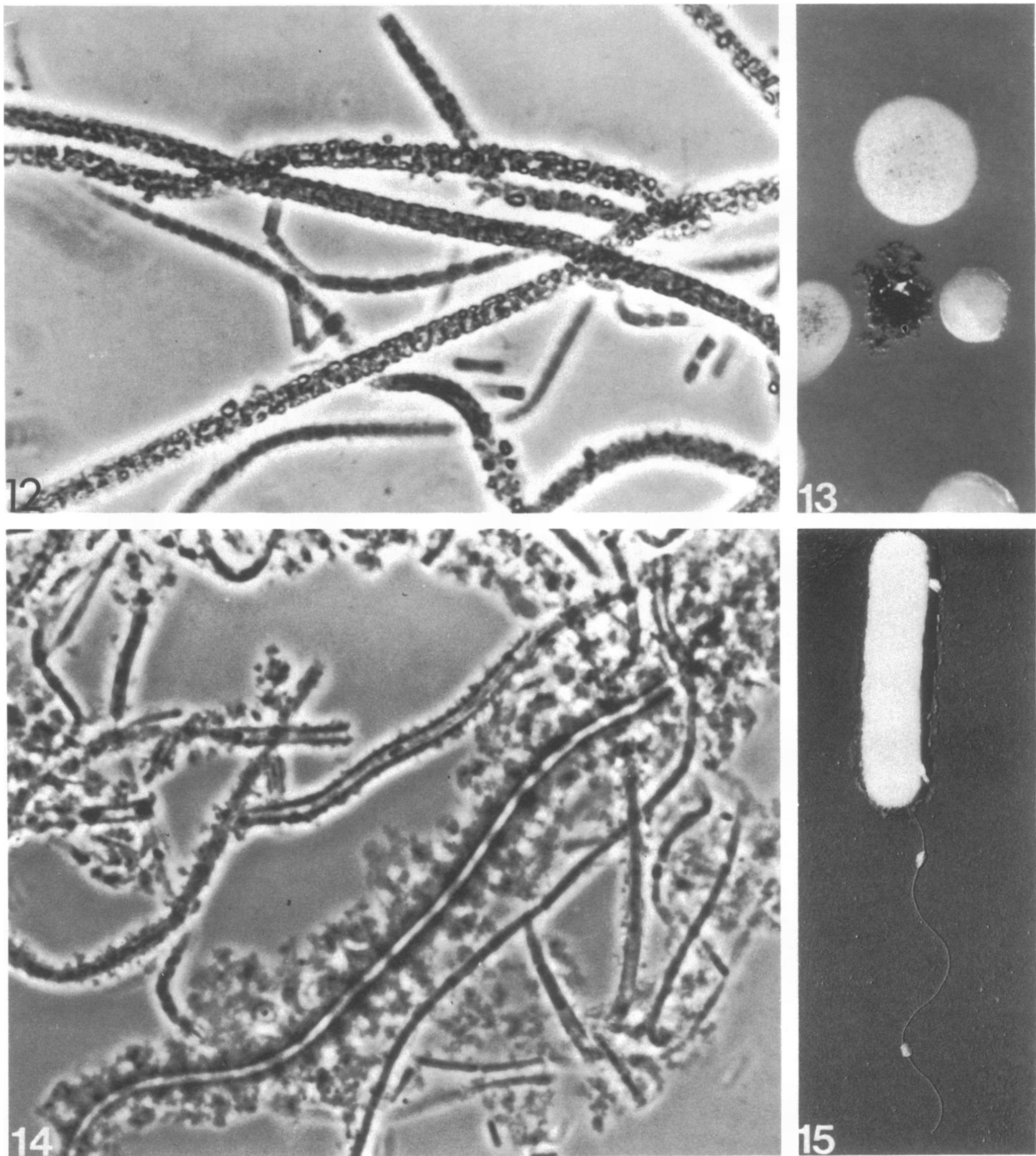



FIG. 12–15. *L. cholodnii. (12) Granules of $MnO_2$ on the sheaths; ×1,625. (13) Rough, black (original type) and smooth (mutant) colonies; ×19. (14) Grown in nutrient medium enriched with iron(III) quinate, sheaths covered with ferric hydroxide; ×1,625. (15) Cell with one polar flagellum; ×10,000. (Fig. 12 and 13 are from reference 57 with permission of the publisher.)*

On solid media with limited nutrients, the colonies are much smaller (0.1 to 0.3 mm in diameter) and generally show less filamentous development than do the other types of the *Sphaerotilus-Leptothrix* group (53, 57). Enrichment of the media with high levels of glucose and peptone only slightly increases growth, even when the media are supplied with methionine, purine bases, vitamin $B_{12}$, biotin, and thiamine.

On manganese(II)-containing nutrient agar (57), the black-brown, widely spaced colonies are frequently surrounded by either a dark-brown halo of pinpoint granules (Fig. 17) or by a diffuse light-brown halo of oxidized manganese. This phenomenon is strain dependent, and some strains form no halo at all. Crowded colonies are more filamentous (Fig. 19). Strains of this type have only been isolated from unpol-

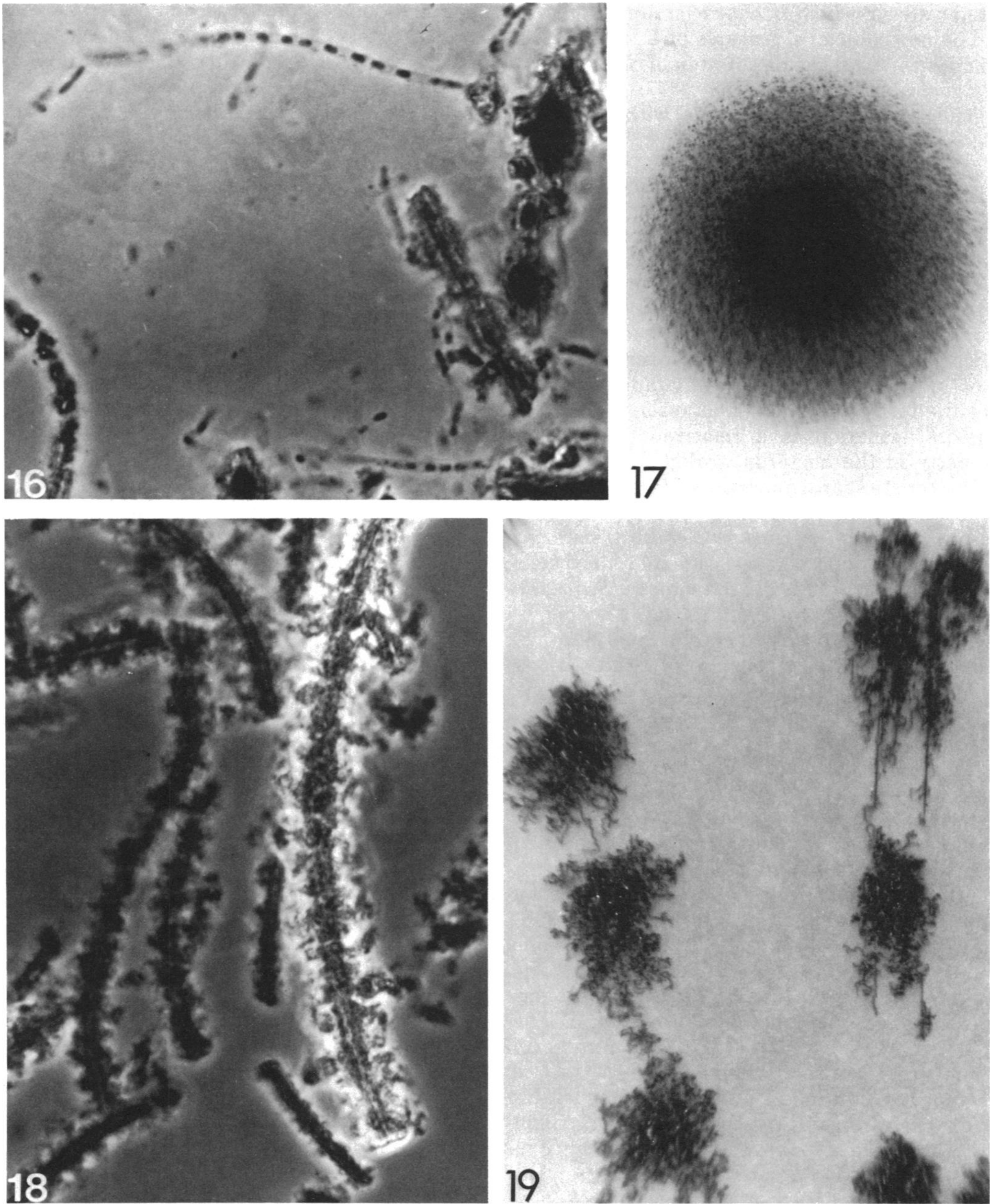



FIG. 16-19. *L. discophora. (16) Young sheathed bacteria without $MnO_2$ deposition, growing in $MnSO_4$-containing nutrient solution, and old, presumably empty, sheaths with encrustations of $MnO_2$; ×1,625. (17) Smooth colony (widely spaced) on $MnSO_4$-containing agar; $MnO_2$ is present within the colony and in a halo which contains no bacteria; ×45. (18) Sheaths covered with ferric hydroxide and $MnO_2$ in running iron(II)- and manganese(II)-containing soil extract under laboratory conditions; ×1,625. (19) Crowded filamentous colonies on $MnCO_3$-containing agar; ×35. (Fig. 16 is from reference 53, with permission of the Society for Applied Bacteriology; Fig. 17 and 18 are from reference 57, with permission of the publisher.)*

luted water in which *L. ochracea* predominates.

The morphological features of *L. discophora* do not fit in with the description of *S. discophorus* given by Rouf and Stokes (77). The latter organism is presumably identical with *L. cholodnii*.

## NOMENCLATURE AND GENETIC RELATIONSHIPS

### Nomenclature

The sheath-forming bacteria of the *Sphaerotilus-Leptothrix* group possess a number of common morphological and physiological characteristics. These include (i) the formation of a sheath, (ii) the requirement of vitamin $B_{12}$, and (iii) the formation of PHB as reserve material. Sheath formation is a relatively uncommon property in the bacterial world; the two other characteristics are much less uncommon. An additional factor substantiating the assumed relationship of the organisms of both genera includes the guanine-plus-cytosine content of their DNA, which is practically the same for *S. natans* and *Leptothrix* species tested so far. The following values were obtained in our laboratory: *S. natans*, 69.7%; *L. cholodnii*, 69.6%; and *L. discophora*, 71.2% (17). These values agree with the earlier estimates by Mandel et al. (51), viz., *S. natans*, 70%, and *S. discophora* (presumably *L. cholodnii*), 69.5% guanine plus cytosine. Hybridization experiments with *S. natans* and *L. discophorus* carried out in our laboratory so far have not indicated a close relationship between the two organisms (unpublished data).

Although the above-mentioned similarity of a number of important characteristics might support Pringsheim's proposal (69, 70) to create one genus, *Sphaerotilus*, for all the members of the *Sphaerotilus-Leptothrix* group, several other arguments favor maintaining both genera. (i) The ability to oxidize Mn(II) to $MnO_2$ is a property found in all of the isolated *Leptothrix* species but not in *S. natans*. Upon aging, some strains of the latter organism may show a very slight oxidation, which, however, is negligible in comparison with the pronounced activity of the *Leptothrix* spp. (ii) *S. natans* possesses a pronounced response to organic nutrients, producing high yields of cell material. It is a typical wastewater organism and is rarely found in natural waters. This is in contrast with most of the *Leptothrix* spp., which hardly respond to added nutrients and which are typical organisms of uncontaminated natural waters. The behavior of one species, *L. cholodnii*, is at variance with this general rule; it is found not only in uncontaminated water but also in activated sludge, owing to its response to increased nutrient supply. (iii) The cells of *S. natans* are much larger than those of most of the *Leptothrix* spp. This applies particularly to *L. discophora* (Table 1). (iv) *Leptothrix* cells, except those of *L. lopholea*, possess one polar flagellum; free cells of *S. natans* and those of *L. lopholea* are motile by means of a tuft of several subpolar flagella. (v) False branching only occurs in *S. natans*, particularly in relatively poor media, and in *L. lopholea*. (vi) Holdfasts are found in *S. natans* but not in the *Leptothrix* spp. with the exception of *L. lopholea*.

A serious mistake of Pringsheim's nomenclature, which has unfortunately been adopted in some handbooks (2, 80), is the relocation of all of the *Leptothrix* spp. in one species, *S. discophorus*. This causes much confusion, since this name is used in the literature for organisms of the *L. discophora* type as well as for bacteria resembling *L. cholodnii* (77, 91) and presumably other Mn(II)-oxidizing species. The presumed reason for the failure of the simplified identification scheme is that this scheme was based on a very small number of isolates originating from only a few habitats.

A further error in Pringsheim's scheme, the assumption that *S. natans* and *L. ochracea* are identical, was presumably due to the fact that laboratory experiments had been performed by Pringsheim only with *S. natans* and not with *L. ochracea* (70).

According to Lewin (48), the organisms of the *Sphaerotilus-Leptothrix* group might be more conveniently relocated in the order *Pseudomonadales*. The development of a subpolar tuft of flagella or one polar or subpolar flagellum and the high percentage of guanine plus cytosine in the DNA of the organisms would support this proposal.

### Mutations and Modifications

Upon their isolation and preservation under laboratory conditions, the described species of the *Sphaerotilus-Leptothrix* group may sometimes lose some easily observable morphological and physiological features by mutation. The irreversible loss of the capacity to synthesize sheaths and the disappearance of the ability to oxidize $Mn^{2+}$ are the most remarkable mutations encountered in this group of bacteria (57, 91). The strains of *S. natans*, which are unable to oxidize $Mn^{2+}$, do not rapidly lose their sheath-forming capacity, and mutants in this respect are observed only rarely (88). The same is true of the strains of *L. lopholea*. *L. pseudo-ochracea*, originally growing in concentric circles and spreading over large areas of agar plates, forms smaller colonies on prolonged storage of the

culture. Ultimately, only small, smooth, circular colonies result, containing nonsheathed cells with a poor or nonexistent Mn-oxidizing capacity. However, the cells do not lose their very rapid motility and even may exhibit chains of three to five nonsheathed cells (unpublished data).

Irreversible alteration of sheathed into nonsheathed cultures with simultaneous loss of the capacity to oxidize $Mn^{2+}$ can be observed under laboratory conditions in *L. cholodnii* soon after its isolation (57). The colonies of the sheathless mutants have lost their hairy (rough) appearance and are smooth (Fig. 13). Other strains of this type may give rise to nonsheathed mutants which maintain their $Mn^{2+}$-oxidizing character on agar plates. The appearance of mutants forming smooth colonies and lacking the ability to oxidize $Mn^{2+}$ was readily observed in aging broth cultures of *S. discophorus* (probably identical with *Leptothrix cholodnii*) (70, 77, 91).

The strains of *L. discophora* did not change their pronounced Mn-oxidizing capacity during a cultivation period of 10 years on agar slants. However, sheath formation was strongly reduced, so that sheathed cells were only occasionally present in liquid media.

Discontinuation of sheath formation may also be due to nongenetic factors. This is particularly true of organisms maintained under rich nutritional conditions (30, 53, 57, 88). When grown on a basal medium containing 1 g of glucose and 1

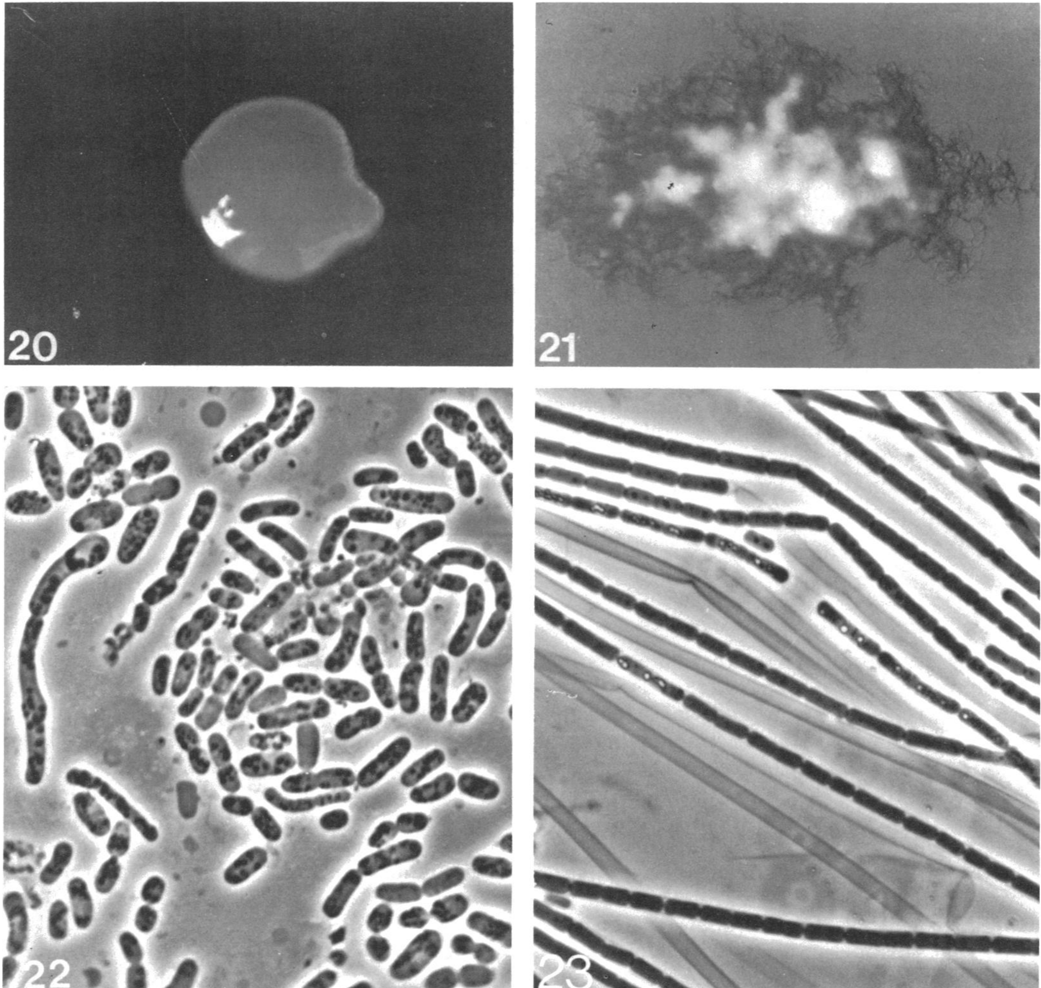


FIG. 20-23. *S. natans. (20) Smooth colony on basal agar containing 5 g of glucose and 5 g of peptone per liter; ×22. (21) Rough colony on basal agar containing 1 g of glucose and 1 g of peptone per liter; ×22. (22) Sheathless cells from a smooth colony; ×1,625. (23) Sheathed cells from a rough colony; ×1,625. (Fig. 20 through 22 are from reference 57, with permission of the publisher.)*

g of peptone, *S. natans* forms large, hairy (rough) colonies, as contrasted to the smooth, almost circular (particularly when widely spaced) colonies that are formed in the presence of 5 g of glucose and of peptone. Under these conditions no sheaths are formed (Fig. 20 through 23). Transfer of the organism to a poor medium restores sheath formation.

More nonfilamentous colonies of *S. natans* were found with increased concentrations of various sugars as well as with increased peptone concentrations. The effect of the latter nutrient was much more pronounced than that of sugars (30).

Glucose, galactose, mannitol, sucrose, or salicin in concentrations of 0.2 to 1% in the presence of 0.5% peptone favored the formation of the smooth colony type of *S. discophorus* (presumably identical with *L. cholodnii*). This effect was not observed with raffinose and glycerol. Phytone and tryptone were partially effective in smooth colony formation, whereas proteose-peptone and tryptose apparently were ineffective (91).

The $MnO_2$ deposition in the smooth colonies was greatly decreased in the presence of 1% of glucose, galactose, mannitol, or sucrose and slightly decreased with salicin (91). Since the pH of poorly buffered nutrient media containing glucose and peptone in a ratio above 1 falls considerably, the above-mentioned adverse effect of sugars on $Mn^{2+}$ oxidation may have been due to the unfavorable pH effect. Such an effect was observed with a manganese-oxidizing *Arthrobacter* sp. (9). In experiments with washed cells of *L. cholodnii*, preincubation of the bacteria with glucose did not affect the $Mn^{2+}$ oxidation rate (see Oxidation of Manganous Ions).

# GROWTH

## Determination of Growth

The determination of growth of members of the *Sphaerotilus-Leptothrix* group has its special problems. Numbers of cells in suspension cannot be evaluated by plate counts upon serial dilution or nephelometric or spectrophotometric methods, due to the flocculent and filamentous growth of the organisms. Savel'eva (78) developed a method for counting *L. discophora* and *L. sideropous* cells on a double-layer agar plate, but she did not mention the problems in separating sheathed cells. It must, therefore, be concluded that this method may be useful for the isolation of sheath-forming microorganisms from enrichment cultures or natural habitats but not for cell counting. Measuring cell mass by nephelometric or spectrophotometric procedures may give more reproducible results if the cultures are homogenized by sonic oscillation (unpublished data).

Cell dry weight is not always a good parameter of growth, since sheath-forming bacteria of the *Sphaerotilus* type may contain large amounts (up to 70%) of reserve material, such as PHB and glycogen. Therefore, cell protein or cell nitrogen and possibly DNA of the culture are better parameters.

The application of continuous cultures may be a promising method for studying the growth of sheath-forming bacteria in relation to ecological factors. However, the attached growth of sheathed bacteria interferes strongly with reliable determination of different parameters. Experiments on the attached growth of *S. natans* in a series of continuously fed vessels did not correspond with steady-state conditions and did not allow theoretical interpretation of the results (20). An improved method to control filamentous growth has been developed (72). If sheathless mutants differ only in sheath synthesis or attached growth, but not in growth rate, from their ensheathed parent strains, they might be studied in continuous culture.

Methods have been described to estimate the growth rate of filamentous bacteria, including *S. natans*, in natural habitats by using slide immersion and ultraviolet radiation (6).

## Growth Conditions

**Mineral requirements.** Excellent growth of most of the isolated *S. natans* and *L. cholodnii* strains was obtained by the authors 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 organic or inorganic nitrogen source, and vitamin $B_{12}$, 0.005 mg/liter. Most of the other *Leptothrix* strains grow moderately in this medium (57).

Although no detailed experiments have been carried out to determine the essentiality of the above-mentioned elements, it may be assumed that all of them (except, probably, boron) are required when purified salts and mineral-free water are used for preparing the nutrient media. An effect of $Ca^{2+}$ on sheath synthesis of *S. natans* has been observed when cultivating this organism in a series of flasks continuously fed with a nutrient solution containing complex organic nitrogen sources. In media with Ca concentrations below 0.1 mM the bacteria grew as single cells, but at higher calcium levels they developed sheaths and became attached to the

walls of the vessels (21).

**Carbon sources.** Sheathed bacteria are able to utilize a great variety of organic 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 representatives of the *Sphaerotilus-Leptothrix* group (37, 38, 47, 53, 57, 88).

No growth of *S. natans* was obtained by a number of authors with cellobiose, starch, or inulin (37, 38, 47, 79). In testing 10 strains of both non-manganese-oxidizing and manganese-oxidizing organisms, we found 1 strain of the former group and 2 of the latter group able to utilize starch as the sole carbon and energy source (unpublished data).

Depending on the type of ensheathed bacterium used, sugars may generally be added to the media in amounts of up to about 10 g/liter without exhibiting any appreciable limitation of growth (53, 88). Heat sterilization of glucose together with the basal salts solution at a neutral pH may result in the formation of inhibitory compounds. Much lower concentrations of both alcohols and organic acids can be incorporated as carbon and energy sources in nutrient media for the sheathed bacteria (88).

Although amino acids may serve as the source of both carbon and nitrogen, an inhibitory effect on growth has been reported for some of these compounds (see below).

*S. natans* and *L. cholodnii* show a much more pronounced response to increased supply of organic nutrients than do the other *Leptothrix* strains that we have studied. This was found to be particularly true of *L. discophora*, which grows satisfactorily in running iron- and manganese-containing soil extract, but hardly responds to added organic nutrients (see Table 1). This result is in agreement with the occurrence of *L. discophora* in unpolluted water, in contrast with *S. natans*, which prefers polluted water, and *L. cholodnii*, which is found in natural waters as well as in activated sludge (57). These data were obtained with freshly isolated strains. Upon prolonged cultivation under laboratory conditions, the *L. discophora* strains responded better to an increased supply of organic nutrients.

**Nitrogen sources.** Although conflicting results have been obtained as to the ability of sheath-forming bacteria of the *Sphaerotilus-Leptothrix* group to utilize inorganic N compounds, convincing evidence is available that *S. natans* (88) and at least some *Leptothrix* species can use $NH_4^+$ and $NO_3^-$ as sole N sources. We confirmed this conclusion with *S. natans* and *L. cholodnii* (57). However, the growth of these organisms with the inorganic N sources was less luxuriant than it was with a mixture of aspartic and glutamic acids, peptone, or other complex N compounds (57, 88). The inferior results with inorganic N compounds, particularly $NH_4$ salts, compared with organic N sources, were presumably caused by a pronounced shift of the pH of the culture solution after uptake of $NH_4^+$ or $NO_3^-$. This assumption is confirmed by the more satisfactory results with inorganic N sources when *S. natans* was grown in continuous culture (20) or in a pH-controlled batch culture (unpublished data). In these experiments excellent growth of the organism was obtained with $NH_4^+$ or $NO_3^-$ as the N source and glucose as the carbon and energy source.

A second factor that may interfere with the comparison of different N sources in sheathed bacteria is the requirement of these organisms for vitamin $B_{12}$ when they are growing in media with $NH_4^+$, $NO_3^-$, or a mixture of aspartic and glutamic acids. No vitamin $B_{12}$ is required when the organisms are growing with Casamino Acids, peptone, or other complex N compounds that contain methionine (40, 57, 89).

Apart from the inhibitory effect of inorganic salts present in commercially available complex nutrients, individual amino acids present in such nutrients may interfere with the growth of bacteria of the *Sphaerotilus-Leptothrix* group (40). The manganese-oxidizing strains are, presumably, even more sensitive than those not oxidizing $Mn^{2+}$ (57). Addition of 10 g of Casamino Acids (Difco) per liter, 5.4 g of simulated casein hydrolysate per liter, or 2 g of a uniform mixture of 18 amino acids per liter to the basal medium exerted complete suppression on the growth of a number of strains of *S. discophorus* (presumably *L. cholodnii*) (40; Johnson, Ph.D. thesis). L-Arginine, L-glutamic acid, L-leucine, L-lysine, and L-proline were not inhibitory or slightly inhibitory at 1.8 to 3.6 g/liter. A second group of amino acids, including L-aspartic acid and L-methionine, were moderately inhibitory, whereas a third group, consisting of L-alanine, glycine, L-histidine, L-cystine, L-isoleucine, L-tyrosine, L-phenylalanine, L-tryptophan, DL-valine, DL-serine, and DL-threonine, inhibited growth either completely or severely in the above-mentioned concentrations. The toxicities of the individual amino acids may account for the toxicity of Casamino Acids.

*S. discophorus* (presumably *L. cholodnii*) may tolerate peptone up to about 2 g/liter, but proteose peptone, phytone, tryptone, and tryptose were inhibitory to growth at this concentra-

tion. This may have been due to a higher content of free amino acids in these complex nutrients (Johnson, Ph.D. thesis).

A relationship has been observed between the N source required by *S. natans* and the type of carbon compound supplied. Excellent growth of *S. natans* was obtained with inorganic N compounds when sucrose, glycerate, or succinate was the carbon source, but poor growth was obtained when glucose had been supplied (88). We were unable to confirm this result, since we obtained good growth of *S. natans* with inorganic N compounds when glucose was the carbon source (53, 57).

**Growth factors.** We found a requirement of vitamin $B_{12}$ (cyanocobalamin) for all of our *S. natans* and *Leptothrix* strains when the organisms were cultivated in media free from methionine. In the presence of sufficient amounts of this amino acid, no vitamin $B_{12}$ is required (53, 54, 57). These results were confirmed with *S. natans* (61) and with several *S. discophorus* strains (presumably *L. cholodnii* strains) (40, 89). Methionine may be supplied as the L or D isomer, but at increased levels the latter is more toxic (40).

In addition to cyanocobalamin, biotin and thiamine have been shown to be essential growth factors for *S. discophorus (Leptothrix* sp). (77, 89). We did not find such requirements in our *Leptothrix* strains (57). Of 11 strains of *S. discophorus (Leptothrix* sp.) tested, 2 required adenine or guanine (89).

**Temperature.** Growth of the bacteria of the *Sphaerotilus-Leptothrix* group has generally been reported to occur at temperatures between 15 and 40°C, with an optimum at about 30°C (23, 57, 88) but with exceptions (98). Therefore, it is advisable to cultivate these organisms at temperatures between 20 and 25°C.

**pH.** Reliable experiments to determine the growth rate in automatically pH-controlled media have not yet been reported. *N*-2-Hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid and tris(hydroxymethyl)aminomethane buffers have been used to keep the pH values of culture solutions constant. However, the buffer capacity of these solutions may be inadequate due to the composition of the medium as well as to cultural conditions. Buffer solutions may also interfere with $Mn^{2+}$ oxidation (99). The introduction of $KNO_3$ or $(NH_4)_2SO_4$, sugars, or neutralized organic acids into nutrient media involves a rapid change of the pH which cannot be corrected by low concentrations of buffer solutions. Media containing equal amounts of both sugars and peptone in moderate concentrations are suitable for growing the organisms without a drastic change of the pH (57). It is generally assumed that the sheathed bacteria grow rapidly at pH values between 6.5 and 8.1 (57, 77, 88), with an optimum at pH 7.5.

**Salt tolerance.** The bacteria of the *Sphaerotilus-Leptothrix* group generally do not tolerate high salt concentrations. Depending on the strain tested, the growth of *S. natans* may be affected adversely by inorganic salts at concentrations between 0.3 and 2 g/liter (47, 79, 88). Despite the susceptibility to salts, sheathed bacteria occur in sea water (65). This implies the existence of salt-tolerant or halophilic types. Suppression of growth by increased amounts of complex organic nutrients may be due partly to the relatively high concentrations of salts present in such nutrients as Casamino Acids, nutrient broth, and Trypticase soy broth.

The inhibitory effect of phosphate on manganese oxidation by *Leptothrix* cells (see Oxidation of Manganous Ions) indicates an activity of this compound on the enzyme level (99). A nonsheathed mutant of a manganese-oxidizing *Leptothrix* strain was found to be very sensitive to 0.02 M phosphate buffer, which caused lysis of cells (J. C. Makemson, Ph.D. thesis, Washington State University, Pullman, 1970). These facts may explain why the application of buffer solutions for keeping the pH of the media constant may give erroneous results (30).

Sheathed bacteria often fail to grow in media containing more than 100 mg of $MnSO_4 \cdot H_2O$ per liter (70; Johnson, Ph.D. thesis). Several manganese-oxidizing strains exhibited stimulation of growth in the presence of 50 mg of $MnSO_4 \cdot H_2O$ per liter, but 150 mg/liter suppressed cell synthesis (Johnson, Ph.D. thesis). Evidence was obtained that non-manganese-oxidizing species are poisoned at lower $Mn^{2+}$ concentrations than are those oxidizing $Mn^{2+}$ (70).

The iron level in a synthetic sewage medium may be a critical factor in the growth of *S. natans.* This concerns not only the reduced growth as a result of limiting amounts of iron but also the inhibition by excessive amounts. A decrease of the $FeCl_3$ concentration from 25 to 2 mg/liter was found to increase cell yield (106). The adverse effect of relatively small amounts of $FeCl_3$ was probably due to a chemical reaction between iron and one or more essential medium components, rendering the latter insoluble after heat sterilization. In the presence of complexing compounds, much higher concentrations of iron can be withstood. The authors succeeded in growing *S. natans* in a nutrient medium containing 1.8 g of $FeCl_3 \cdot 6H_2O$ per liter and 0.75 g of quinic acid per liter upon neutralization and sterilization by Seitz filtration (unpublished data).

**Oxygen.** All isolated species of the *Sphaero-*

*tilus-Leptothrix* group are obligate aerobes (20, 77, 88). Although in strongly aerated culture solutions the final cell yield is often hardly enhanced, as compared with stationary cultures (1, 53), the growth rate of the former cultures is much increased. Electrochemically determined oxygen uptake by cell suspensions exposed to different $pO_2$ values often deviated from linearity with decreased oxygen supply. It was a result of the flocculent character of the washed cell suspensions causing limited oxygen diffusion into the flocs (43). This effect becomes particularly clear when the residual $O_2$ content has fallen below 1 to 2.5 mg/liter.

Low oxygen levels in a continuously fed system adversely affected the attached growth of pure cultures of *S. natans*. However, in mixed populations the development of *S. natans* was favored by very low oxygen concentrations, indicating that the accompanying bacteria were more seriously affected by the low oxygen supply than was *S. natans* (20). The competition for substrates, which at optimum $O_2$ supply was dominated by the non-sheath-forming bacteria, was apparently much more favorable to *S. natans* at low $O_2$ supply, at which the aerobic sheathless organisms were hardly growing. Part of the latter organisms may have died and autolysed under these conditions, releasing cell constituents which may have additionally favored the growth of *S. natans*. These observations demonstrate that *S. natans* may behave differently under natural conditions, as compared with pure culture conditions ( 1, 20).

The relatively good growth of *S. natans* under conditions of restricted oxygen supply, possibly caused by overloading, may sometimes cause dominance of this organism in activated sludge. Owing to the protruding filaments, the settling of the sludge flocs is much retarded, so that separation of sludge and effluent is poor (bulking sludge). Improving the oxygen supply of the flocs may stimulate the growth of the nonfilamentous bacteria, which may lead to elimination of *S. natans* (1).

## Metabolism

The bacteria of the *Sphaerotilus-Leptothrix* group are characterized by the formation of a sheath, often surrounded by a slime layer which is closely connected with the iron-accumulating and manganese-oxidizing capacities of the organisms. These properties lead to the formation and accumulation of large amounts of ferric and manganese oxides, which are characteristic of most of the *Leptothrix* species. Details concerning these abilities are given below.

Other common physiological characters of this group of bacteria include their tendency to form globules of PHB as reserve material, their ability to grow at low $pO_2$ values, their requirement of vitamin $B_{12}$ (which can be replaced by methionine), and their sensitivity to relatively low concentrations of mineral salts, particularly phosphate.

All of the isolated types of the *Sphaerotilus-Leptothrix* group are obligately aerobic, heterotrophic bacteria. The statement sometimes met with in the literature that at least some of these organisms would be able to utilize the energy of oxidation of $Fe^{2+}$ and $Mn^{2+}$ has so far not been confirmed (see details below).

Experiments with washed cultures of *S. natans* supplied with labeled glucose in Warburg vessels indicated that the Entner-Doudoroff and pentose-phosphate pathways function in the breakdown of the sugar. Subsequent degradation of the pyruvate proceeds according to the tricarboxylate and/or glyoxylate cycles (J. M. Robertson, Ph.D. thesis, Oregon State University, Corvallis, 1968).

**Synthesis and degradation of poly-β-hydroxybutyrate.** The formation of internal PHB globules as reserve material in amounts of 30 to 40% of the dry cell weight is a common property of both *Sphaerotilus* and *Leptothrix* species (53, 56, 57, 76, 90, 92). Synthesis of PHB is often thought to be due to nitrogen limitation of the bacteria, provided that adequate amounts of a consumable carbon source are available.

We showed this in nonshaken batch cultures of *S. natans* growing in a mineral salts medium containing peptone (1 g/liter) and different amounts of glucose (1, 2.5, 5, 10, and 15 g/liter). After 3 days of incubation at 25°C, the cells of all the treatments were packed with PHB globules. Two days later the PHB content of the cells grown at the lowest glucose supply had largely disappeared, presumably due to the exhaustion of the external carbon supply and subsequent utilization of the reserve material as an internal source of energy. Cells supplied with more glucose were unaltered, owing to the presence of residual glucose in the medium, which prevented the utilization of the PHB reserves (53, 56, 57).

In addition to nitrogen deficiency, sulfur, phosphorus, and, particularly, oxygen limitation have been found to favor the storage of PHB in microorganisms (18, 81, 107). The effect of oxygen limitation on the storage of PHB was studied in detail with cells of *Azotobacter beijerinckii* growing in continuous culture (18). A sudden drastic reduction of the oxygen supply of a slowly growing chemostat culture of this organism resulted in a pronounced rise of the PHB content until it became more than 50% of the dry weight of the cells. This was thought to be

due to the restricted oxidation of nicotinamide adenine dinucleotide phosphate, reduced form, (owing to oxygen limitation), giving rise to accumulation of the reduced coenzymes followed by inhibition of citrate synthase and isocitrate dehydrogenase, thus slowing down the tricarboxylate cycle. The resulting accumulation of acetyl-coenzyme A, the key compound of PHB synthesis, favored the formation of the reserve material via acetoacetyl-coenzyme A and $\beta$-hydroxybutyrate-coenzyme A, a step promoted by nicotinamide adenine dinucleotide phosphate, reduced form.

Stimulation of PHB synthesis by reduced oxygen supply of nitrogen-deficient *S. natans* was shown in our laboratory to occur in stationary batch cultures, where PHB formation was much more pronounced than in shaken cultures (unpublished data).

The stimulatory effect of nitrogen limitation on PHB synthesis might be explained by assuming that cessation of protein synthesis, an endergonic process coupled to adenosine 5'-triphosphate production via the electron transport chain, leads to an increase of the nicotinamide adenine dinucleotide phosphate, reduced form, concentration of the cell, which favors PHB synthesis as it was described for oxygen-limited bacteria (18).

Degradation of PHB and utilization of the resulting acetyl-coenzyme A for energy supply of *A. beijerinckii* commenced when the available exogenous carbon and energy sources were exhausted. It was strongly favored by relaxation of the oxygen limitation of the PHB-containing bacteria. Under such conditions, the excess of nicotinamide adenine dinucleotide phosphate, reduced form, was oxidized, and the tricarboxylate cycle resumed its functioning (18).

Accumulations of PHB, such as occurred in our batch cultures with nitrogen-limited *S. natans* (53, 56, 57) and in Stokes and Parson's cultures of *S. discophorus (Leptothrix* sp.) (90), rapidly disappeared upon exhaustion of the external supply of carbon compounds without relaxation of the nitrogen limitation. The utilization of the degradation products of PHB for the energy supply of the *Sphaerotilus* and *Leptothrix* cultures enabled these organisms to survive for longer periods of time. *S. discophorus (Leptothrix* sp.) cells grown in a peptone-glucose medium and containing 35% PHB were incubated in a phosphate buffer solution for 8 h and then tested for viable cells and PHB content. Viability had fallen to 44%, and PHB had fallen to 19% during this period. Cells grown without glucose and containing no PHB had a viability of 19% after 8 h (90). These viability values are low compared with those of starvation experiments with glycogen-storing arthrobacters and lipid-storing yeasts of the *Rhodotorula* type carried out in our laboratory (56; M. H. Deinema, Ph.D. thesis, State University at Groningen, Groningen, The Netherlands; L. P. T. M. Zevenhuizen, Ph.D. thesis, University of Amsterdam, Amsterdam, The Netherlands). The *Arthrobacter* cells contained about 40% glycogen as reserve material when the starvation period began; their viability was maintained at almost the same level during a period of 3 weeks, after which the glycogen reserves gradually had fallen to about zero. Addition of ammonium sulfate resulted in a ready drop of the glycogen content accompanied by a fourfold increase of bacterial numbers. The lipid-storing and -consuming yeasts behaved in the same way. These experiments demonstrate that both glycogen and lipid reserves were serving as carbon and energy sources in the respective organisms and, upon addition of a nitrogen compound, were also used for the synthesis of cell material. So far, it is unknown if PHB reserves of sheathed bacteria, in addition to functioning as an energy store, can be used as a carbon source for cell synthesis upon adding a nitrogen source.

The fact that, under the conditions of the reported experiments, both *Sphaerotilus* and *Leptothrix* spp., upon exhaustion of the external organic carbon supply, degrade PHB at a high rate without concomitant synthesis of new cell material suggests that these organisms are wasting most of the energy contained in their PHB reserves. It is interesting to note that this property could be involved in the elimination of *S. natans* from "bulking" activated sludge by applying discontinuous loading of the sludge. During the short periods of increased nutrient supply, *S. natans* is thought to absorb considerable amounts of compounds which can lead to PHB formation. During subsequent periods of poor nutrient supply, PHB will be oxidized without stimulating cell growth. Other organisms, not possessing this system of PHB storage, will respond to the improved nutrient supply by increased cell growth and gradually will eliminate *S. natans*.

An important observation made by Stokes and Powers (92) was that the addition of $Mn^{2+}$ in amounts of 0.5 to 2.5 mM to PHB-containing cells of *S. discophorus (Leptothrix* sp.), suspended in phosphate buffer solution, brought about a pronounced rise of endogenous respiration as well as of PHB degradation. Since the respiration of cells without PHB did not respond to the added $Mn^{2+}$, it can be assumed that $Mn^{2+}$ ions are responsible, possibly by functioning as electron carriers, for the increased oxidation of PHB. This assumption is corroborated by the

fact that endogenous respiration and PHB degradation of *S. natans*, which is unable to oxidize $Mn^{2+}$, were not affected by added $Mn^{2+}$. Magnesium ions had an even more pronounced stimulatory effect on endogenous respiration but hardly promoted PHB degradation of starving cells suspended in phosphate buffer solution (92).

That the $Mn^{2+} \rightleftarrows Mn^{4+}$ reaction may function as an electron carrier in oxidation reactions of *Leptothrix* cells is in agreement with the observation made in our laboratory that the oxygen uptake of logarithmic-phase cells supplied with $Mn^{2+}$ is much higher than that of cells without added $Mn^{2+}$ (see Growth Conditions). Visible Mn oxidation (brown color) was not observed, obviously because the $Mn^{4+}$ upon its formation was readily reduced to $Mn^{2+}$ by electrons supplied by the cells.

**Glycogen as reserve material.** In addition to PHB, sheathed bacteria may store large amounts of polysaccharides of the glycogen type. This can be seen from the analysis of cell material of an *S. natans* culture grown for 6 days in a basal culture solution supplemented with 5 g of glucose per liter and 1.5 g of peptone per liter. The general composition of the cells was as follows: 38% polysaccharides, 29% PHB, 4% crude fat, and 25% protein (56, 57). Nitrogen and oxygen limitations are supposed to be the main factors involved in the accumulation of glycogen. A comparison of stationary and shaken cultures of *S. natans* made in our laboratory (unpublished data) revealed that, similar to the accumulation of PHB, much more polysaccharide was stored in the cells of stationary cultures than in those of shaken cultures, suggesting a stimulating effect of oxygen limitation on the synthesis of this polymer. After the glucose of the nutrient medium had been used up, a rapid fall of both polysaccharide and PHB occurred; polysaccharide was even more readily degraded than PHB. In shaken cultures these processes proceeded more rapidly than in stationary cultures (unpublished data). Robertson (Ph.D. thesis) found that during endogenous respiration of *S. natans* PHB was used in preference to other cellular carbon sources.

A much slower utilization of glycogen and lipid, as compared with PHB and glycogen in *S. natans*, was observed in a *Pseudomonas* strain which had stored large amounts of these compounds. It enabled the organism to survive for a much longer time than did *S. discophorus (Leptothrix* sp.). A 50% survival time of the *Pseudomonas* sp. was obtained after a starvation period of 60 days (111), as compared with 12 h for *S. discophorus (Leptothrix* sp.) (90). It is unknown if the latter organism contained glycogen in addition to PHB, as was observed in *S. natans* (56, 57).

**Deposition of sulfur.** Upon exposure of *S. natans* cells to $H_2S$, the organism deposits sulfur intracellularly (85). This reaction presumably indicates a detoxication mechanism enabling *S. natans* to grow in a medium containing toxic amounts of $H_2S$ (106).

## OXIDATION OF IRON

Oxidation of ferrous ions is often thought to be one of the most typical characteristics of bacteria of the *Sphaerotilus-Leptothrix* group. Some authors even assumed that *Leptothrix* spp. would grow autotrophically or mixotrophically, utilizing the energy liberated upon the oxdation of $Fe^{2+}$ (15, 49, 68, 108,109). Therefore, the *Sphaerotilus-Leptothrix* strains are often classified in the literature as the third group of iron bacteria, in addition to the autotrophic, obligately aerobic, acidophilic *Thiobacillus ferrooxidans* and the microaerophilic *Gallionella ferruginea*, which probably also grows autotrophically (44).

However, Winogradsky's hypothesis concerning autotrophy has never been confirmed. The main experimental difficulty in testing *Leptothrix* species for autotrophy is that these organisms grow between pH 6 and 8. At these pH values, $Fe^{2+}$ is readily oxidized to $Fe^{3+}$ non-biologically, so that it is even difficult to decide whether or not the bacteria contribute to the oxidation of $Fe^{2+}$, irrespective of their ability to grow autotrophically. In the case of *T. ferrooxidans* no difficulties are encountered in demonstrating autotrophic growth, since this organism thrives in a ferrous salts-containing medium below pH 3, where spontaneous oxidation of ferrous ions does not occur (16, 71, 93).

The second way to exclude non-biological iron oxidation, a very low $pO_2$, has so far not been used to demonstrate biological iron oxidation, probably because the ensheathed bacteria grow poorly under such conditions. This method may be used for showing the autotrophic growth of the microaerophilic *Gallionella* species (33, 44).

If autotrophic growth of sheathed bacteria exists, it might occur in *L. ochracea*, because natural habitats of this organism generally contain few living cells amongst large amounts of iron-containing empty sheaths (14, 15, 53, 57). However, microscopic observations revealed that cells as well as newly synthesized sheath material of this organism were not impregnated or covered with ferric hydroxide, suggesting a nonautotrophic propagation (58). Analyses of organic material (cell dry weight) in ferric hydroxide precipitates from laboratory cultures do not support the existence of an autotrophic me-

tabolism of ensheathed bacteria of the *Leptothrix* type. Ratios of cell dry weight to ferric iron, which in autotrophic iron bacteria of the *T. ferrooxidans* type are given as 1:200 to 1:500 (4, 93, 95), are reported for *Leptothrix* spp. as 1:4 (68). Such deviating values can only be explained by assuming that the ferrous compounds used were contaminated with organic nutrients which were responsible for heterotrophic growth.

The oxidation of $Fe^{2+}$ contained in chelates (citrate, quinate) probably proceeds spontaneously after utilization of citric or quinic acid by the bacteria (unpublished data).

From the above considerations it may be concluded that the bacteria of the *Sphaerotilus-Leptothrix* group most probably are unable to derive energy from the oxidation of ferrous ions; it is even uncertain if they can catalyze this oxidation. It could therefore be argued that the name "iron bacteria" is wrong for this group of organisms.

The only specific connection between bacteria of the *Sphaerotilus-Leptothrix* group and iron known so far concerns the tendency of these organisms to deposit large amounts of ferric iron in or on their sheaths. This can be seen in slowly running water rich in iron and manganese, where the occurrence of flocculated masses of ferric and manganic oxides is often accompanied by masses of filamentous bacteria of *L. ochracea* (Fig. 10 and 11) and *L. discophora* (Fig. 18) or one of the other *Leptothrix* species (e.g., *L. cholodnii* [Fig. 14]) described in Taxonomy.

The specific ability of *Leptothrix* spp. to deposit large amounts of ferric iron on or in their sheaths was demonstrated by growing *S. discophorus* (presumably identical with *L. cholodnii* or *L. discophora*) at different concentrations of added $^{59}FeCl_3$ (73, 74). The amount of iron deposited on or in the sheaths increased with raised $Fe^{3+}$ concentration in the medium. At the highest concentration used (4 mM), the amount of ferric iron deposited on or in the sheaths was 10 to 100 times that precipitated on the cells of a non-sheath-forming mutant strain of *S. discophorus* (*Leptothrix* sp.) and on those of a number of other nonsheathed bacteria, including *Klebsiella aerogenes, Escherichia coli*, and *Pseudomonas denitrificans*. Deposition of iron was found to be most pronounced at the onset of the stationary growth phase; during the exponential phase it was many times lower (73). The possibility that during the latter phase a ready reduction of $Fe^{3+} \rightarrow Fe^{2+}$ could have prevented the precipitation of iron was not considered. Also, no efforts were made to study the effect of ferrous ions on the iron-precipitating capacity of *Leptothrix* strains.

Although under natural conditions *S. natans* is seldom found to be accompanied by flocculated masses of ferric hydroxide, this bacterium is able to deposit Fe(III) in or on its sheaths. We showed this upon growing the organism under laboratory conditions in slowly running ferrous iron-containing soil extract. It resulted in heavy encrustations of aged sheaths (53, 57, 58). Resting cells of *S. natans* supplied with $^{59}$Fe(II) citrate, were found to take up 40% of the $^{59}$Fe within 1 h. Upon fractionation of the organism into sheaths and cell components, nearly all of the label was found in the sheath fraction (S. S. Husain, J. C. Towne, and G. W. Changus, Bacteriol. Proc., p. 84, 1965). In both types of experiments it was unknown if the ferrous iron supplied was oxidized during or before uptake by the ensheathed organism. If it is assumed that the deposition of iron on or in the sheaths of the filamentous organisms depends on the binding of $Fe^{3+}$ to certain components of the sheaths, the spontaneous oxidation of $Fe^{2+} \rightarrow Fe^{3+}$ might be the rate-limiting reaction in the iron precipitation. The presence of a protein of enzymatic character accelerating the conversion of $Fe^{2+} \rightarrow Fe^{3+}$, as has been found in human blood serum (62, 63), might be active in the oxidation of ferrous ions by sheath-forming bacteria. We found such a protein to be active in $Mn^{2+}$ oxidation by *Leptothrix* species (see Oxidation of Manganous Ions).

Formation of flocculated masses of iron(III) hydroxide is not only observed in slowly running iron-containing water of ditches and brooklets, but also in drain pipes used for discharge of excessive amounts of water from wet soils (33, 45, 46, 87). Under certain soil conditions (high iron content, presence of organic matter, and poor aeration of the soil owing to excess of water), considerable amounts of Fe(III) are reduced and, in periods of high rainfall, translocated as soluble Fe(II) compounds to the drain pipes. The better supply of oxygen in and outside the drain pipes causes oxidation of Fe(II) and precipitation of iron(III) hydroxide together with slimy masses of bacteria, particularly of *Gallionella* spp. and sheath-forming organisms of the *Leptothrix* type. The sludgelike deposit of iron hydroxide, bacteria, and soil organic matter may hamper or even entirely block the functioning of the drainage system.

Translocation of reduced iron in soil and water and its subsequent precipitation as ferric compounds may proceed in different ways. In soils poor in organic matter, most of the Fe(II) occurs as inorganic compounds or as $Fe^{2+}$ adsorbed to soil colloids. Upon improved oxygen supply, such as occurs in and around drain pipes and in slowly running water of ditches and brooklets, $Fe^{2+}$ may be oxidized spontaneously and precip-

itated as ferric hydroxide, or it may be converted biologically (under microaerophilic conditions) by such microorganisms as *Gallionella* spp. and, possibly, *Leptothrix* spp. (12). Both types of organisms require a neutral to slightly alkaline reaction. At pH values of 3 to 4, oxidation of Fe(II) can only be performed by the chemoautotrophic *T. ferrooxidans*.

In soils rich in organic matter, most of the Fe(II) may occur as soluble organic complexes with humic acid and other organic soil constituents derived from breakdown of plant material, like gallic acid, tannic acid, etc. Some of these compounds not only keep iron in the reduced state, but they are even able to reduce Fe(III) to Fe(II) (33, 39, 46, 52). When arriving in such aerated areas as drain pipes, part of the organic iron compounds are precipitated, apparently after oxidation of Fe(II). This can be concluded from the high content of organic matter in the ochreous precipitate found in and around the drain pipes (64, 87). A further amount of Fe(III) in the precipitate occurs as ferric hydroxide. This is thought to be derived from iron liberated after bacterial degradation of the complexing organic compound as Fe(II) and subsequently oxidized spontaneously. A remaining amount of organic Fe(II) compounds disappears with the drain water into ditches and brooklets, where a similar type of bacterial flora is responsible for the precipitation of Fe(III).

This phenomenon may also be observed in water supplies containing ground water with considerable amounts of organic Fe compounds. Water from soils bearing a vegetation of eucalypts, as is found in water catchment areas of Australia, is thought to contain soluble iron complexes with gallic acid, which is present in appreciable amounts in these plants. Upon decomposition of iron gallate by common bacteria of water supplies, precipitation of ferric hydroxide occurs (50).

As was stated earlier in this paper, sheath-forming bacteria of the *Leptothrix* type accumulate in slowly running water containing considerable amounts of iron complexes with organic matter, particularly humic acid. Such accumulations not only occur in the above-mentioned natural environments, but they have also been observed under laboratory conditions in our apparatus in which ferrous iron-containing soil extract continuously passes the flocculated organisms (57, 58). As Fe(II) in combination with humus complexes is only very slowly oxidized, one might suppose that the *Leptothrix* spp. are able to utilize humic acid, or part of it, as a carbon and energy source, thus liberating Fe(II), which subsequently is spontaneously oxidized to Fe(III). Such a reaction has been observed with *Leptothrix* spp. in the case of iron(II) citrate and iron(II) quinate (unpublished data) and with *Pseudomonas* spp. in the case of iron(II) gallate (50). However, so far no evidence is available concerning the functioning of humate as a substrate for *Leptothrix* spp. An alternative explanation of the enrichment of these organisms in the above-mentioned environments would be that the organisms in some way are profiting from the presence of large amounts of precipitated ferric hydroxide and organic iron complexes.

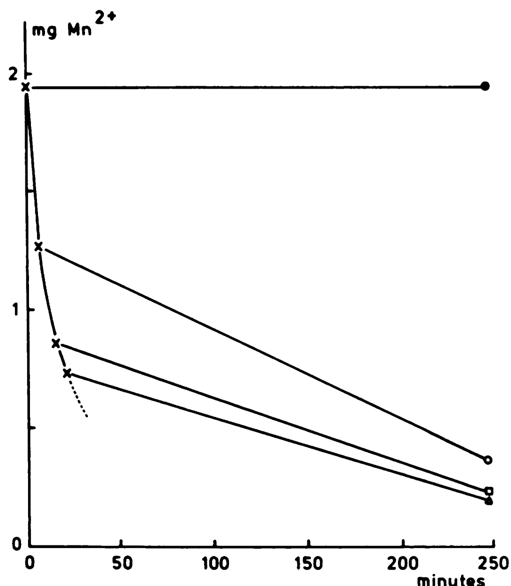



FIG. 24. *Manganous ions oxidized by washed cell suspensions of L. cholodnii, strain 1, cultivated without added $Mn^{2+}$, incubated in the presence of $MnSO_4$, 0.8 mM; sodium malonate, 8 mM; Sörensen phosphate buffer (pH 7.0), 7.4 mM. Symbols: ●, cells treated with phosphate buffer before the addition of $MnSO_4$ and malonate; ×, phosphate omitted; ○, □, and △, phosphate added after 7, 14, and 21 min, respectively. (From reference 99, with permission of the publisher.)*

## OXIDATION OF MANGANOUS IONS

Three different processes can be distinguished in the oxidation of $Mn^{2+}$, viz., (i) a rapid non-biological oxidation in solutions above pH 9, (ii) a slow non-biological oxidation catalyzed by hydroxycarboxylic acids above pH 7.5 (11, 59, 86), and (iii) a biological oxidation, proceeding optimally between pH 6.5 and 7.3, independent of the presence of hydroxycarboxylic acids (5, 8-10, 41, 53, 57, 59, 77, 82-84, 96, 97, 105, 110)

### Non-Biological Oxidation of $Mn^{2+}$

(i) The first non-biological reaction can almost

be neglected in bacterial cultures and in natural habitats, presumably with the exception of algal cultures, which in the light may attain pH values above 9.

(ii) The second reaction proceeds in nutrient media containing relatively large amounts of $MnSO_4$ and of neutralized hydroxycarboxylic acids, such as citric acid and malic acid, as carbon and energy sources (0.5 to 10 g of each per liter). Microorganisms consuming the carbon compounds raise the pH of the medium to 7.5 to 8.0, whereupon the remaining hydroxycarboxylic acids exert a catalytic effect on $Mn^{2+}$ oxidation, establishing a soluble, brown manganese complex presumably containing $Mn^{3+}$ (54, 86, 101). Subsequent utilization of the manganese-hydroxycarboxylate complex results in the precipitation of manganese oxides, mainly $MnO_2$. Under such conditions appreciable amounts of $Mn^{2+}$ also may be precipitated, presumably as manganese hydrocarbonate.

It has been suggested that reaction (ii) would be important in manganese transformations in natural habitats, for instance, in soils (32, 86) and polluted water (7). However, the levels of both $Mn^{2+}$ and hydroxycarboxylic compounds in nature are generally too low to initiate this non-biological conversion of $Mn^{2+}$ (54, 59). It may be assumed that this type of $Mn^{2+}$ oxidation reaction occurs only under particular laboratory conditions. It is performed by all of those microorganisms which consume the added hydroxycarboxylic acid (86). Therefore, counting of such bacteria as manganese oxidizers, as is often done on citrate-agar plates (32, 94), is wrong.

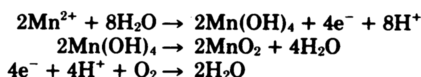
## Biological Oxidation of Manganous Ions

(iii) Biological $Mn^{2+}$ oxidation is a property of several heterotrophic microorganisms, bacteria, fungi, and yeasts (54, 59). All of the tested *Leptothrix* spp., as contrasted with *S. natans*, are able to oxidize $Mn^{2+}$ (see Taxonomy). Investigations of this phenomenon are less problematic, compared with $Fe^{2+}$ oxidation, because at a pH near neutrality non-biological oxidation of $Mn^{2+}$ does not interfere with the biological reaction (101). However, quantification of the clearly visible oxidation reaction from $Mn^{2+}$ to brown manganese oxides, for instance, by measuring the amount of $Mn^{2+}$ disappearing from the bacteria-containing medium, is no simple task because of a number of complications. (i) $Mn^{2+}$, instead of being oxidized, can be adsorbed by cells, sheaths, extracellular slime layers, or by $MnO_2$ produced at an earlier stage (10, 27). (ii) The proteinaceous compound responsible for the catalysis of $Mn^{2+}$ oxidation is co-precipitated proportionally to the amount of manganese dioxide produced (54, 99). (iii) The manganese dioxide produced during the exponential growth phase of the microorganisms may be partly reduced by the actively metabolizing cells, thus masking part of the manganese-oxidizing capacity.

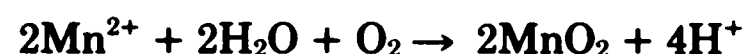
## Factors Affecting Manganese Oxidation by *Leptothrix* spp.

**Effects of heat, enzyme inhibitors, and Pronase.** Heat treatment of cell suspensions of *S. discophorus* (presumably *L. cholodnii*) (5 min at 93°C) eliminated the manganese-oxidizing capacity of the cells. $HgCl_2$ inhibited endogenous respiration but did not affect $Mn^{2+}$ oxidation (41). The latter observation is different from that on an *Arthrobacter* species whose manganese-oxidizing capacity was inhibited by a number of enzyme poisons, including $HgCl_2$, $CuCl_2$, KCN, and $NaN_3$ (8). The inhibiting effect of the latter two compounds was strongly increased by adding small amounts of $H_2O_2$, which in itself did not affect Mn oxidation owing to the catalase activity of the bacteria. It was thought that KCN and $NaN_3$ were inhibiting this activity so that $H_2O_2$, if formed or added, could prevent the oxidation of $MnO_2$ by reducing it as fast as it was formed (8).

Spent cell-free culture solutions of *L. cholodnii*, exhibiting a manganese-oxidizing capacity, failed to oxidize $Mn^{2+}$ after we had maintained the solution for 5 min at 90°C. The same effect was obtained by preincubation of the used culture solution with Pronase, a protein-degrading enzyme (99). These observations indicate that a protein of enzymatic character catalyzes $Mn^{2+}$ oxidation, which presumably proceeds as follows:



$$2Mn^{2+} + 8H_2O \rightarrow 2Mn(OH)_4 + 4e^- + 8H^+$$
$$2Mn(OH)_4 \rightarrow 2MnO_2 + 4H_2O$$
$$4e^- + 4H^+ + O_2 \rightarrow 2H_2O$$



$$2Mn^{2+} + 2H_2O + O_2 \rightarrow 2MnO_2 + 4H^+$$

**Effect of phosphate.** *S. discophorus* (presumably *L. cholodnii*) grown in an $Mn^{2+}$-containing nutrient medium readily oxidizes $Mn^{2+}$ upon centrifugation, washing, and suspension in a phosphate buffer. If the cells have been grown without added $MnSO_4$, they are unable to oxidize $Mn^{2+}$ upon suspending the collected bacteria in a phosphate buffer. From this result it was concluded that $Mn^{2+}$ oxidation would be mediated by an inducible enzyme (41). However, similar experiments carried out in our laboratory (99) showed that the formation of the protein catalyzing $Mn^{2+}$ oxidation is independent of the presence of $Mn^{2+}$ ions. *Leptothrix* cells grown without added $Mn^{2+}$ readily oxidized $Mn^{2+}$ upon

TABLE 2. *Effect of different media at pH 7.0 on the apparent oxidation of $Mn^{2+}$ ions by washed cells of L. cholodnii strain 1 grown without added $Mn^{2+}$*[a]

| Medium used for cell suspension | Incubation time (min) | $MnSO_4 \cdot H_2O$ added (mM) | Sodium malonate[b] added (mM) | $Mn^{2+}$ oxidized (μg of Mn/mg of dry cells per min) |
|---|---|---|---|---|
| Basal salts solution[c] | 180 | 0.4 | 0 | 0.01 |
| | 180 | 0.4 | 3.5 | 0.43 |
| Spent culture solution[d] | 30 | 0.4 | 0 | 0.11 |
| | 30 | 0.4 | 3.5 | 2.69 |
| Glass-distilled water | 30 | 0.4 | 0 | 1.97 |
| | 30 | 0.4 | 3.5 | 2.77 |

[a] From reference 99, with permission of the publisher.
[b] Relieves the inhibitory effect of phosphate on $Mn^{2+}$ oxidation.
[c] Similar to basal salts medium (see text), except that $Fe^{2+}$ was omitted.
[d] Basal salts medium supplied with (in milligrams per liter): glucose, 1,000; peptone, 1,000; $FeCl_3 \cdot H_2O$, 3; and vitamin $B_{12}$, $5 \times 10^{-3}$. Cells were transferred after 3 days.

collecting, washing, and resuspending them in distilled water. However, if they were incubated in the basal salts medium, no oxidation of $Mn^{2+}$ took place (Table 2). The inhibitory effect of the inorganic salts solution was due to the presence of phosphate. This explains the absence of manganese-oxidizing activity upon suspending *S. discophorus (Leptothrix* sp.) cells grown without added $Mn^{2+}$ in phosphate buffer (41). The inhibitory effect of phosphate on $Mn^{2+}$ oxidation only occurs with cells which have not started $Mn^{2+}$ oxidation. This is clearly seen in Fig. 24, taken from reference 99. Cells of *L. cholodnii* grown without added $Mn^{2+}$ and supplied with $Mn^{2+}$ shortly before the addition of phosphate continue to oxidize manganese, though at a reduced rate. This explains why *Leptothrix* cells grown with added $Mn^{2+}$ continue to oxidize $Mn^{2+}$ when suspended in a phosphate buffer.

The inhibitory effect of phosphate on manganese oxidation is not due to reduced solubility of $Mn^{2+}$, since the major part of the latter remained soluble. Some divalent organic acids, particularly malonic acid, counteracted the inhibitory effect of phosphate (see Table 2).

**Effect of biological reduction of $MnO_2$.** If reduction of manganese dioxide by *Leptothrix* spp. might occur under certain conditions, visible (i.e., measurable) $Mn^{2+}$ oxidation would give a wrong idea of the manganese-oxidizing capacity of these bacteria. Reduction of previously produced manganese dioxide by a manganese-oxidizing *Arthrobacter* sp. was shown (10) to occur when this organism was transferred from a shallow vessel, in which $MnO_2$ was produced, to a narrow tube. The adverse effect of increased density of unwashed cell suspensions on the manganese oxidation rate of the *Arthrobacter* sp. (10) and of *L. cholodnii* (unpublished data) was probably due to the excretion of organic compounds that were able to reduce $MnO_2$. More convincing evidence concerning the release of such compounds by a dense suspension of *L. cholodnii* cells was obtained in oxygen uptake experiments in Warburg vessels. Uptake of oxygen was strongly stimulated by the added $Mn^{2+}$, which apparently functioned as electron carrier in the oxidation of the released unknown compounds. This was concluded from the fact that after 9 h of incubation no visible manganese oxidation had occurred in spite of the fact that more than five times the amount of $O_2$ required for complete oxidation of the added $Mn^{2+}$ had been taken up. At this time oxygen uptake continued at almost the same rate (unpublished data).

The reduction of manganese oxides by released cellular material may explain the observation that the oxidation of $Mn^{2+}$ starts at the end of the exponential phase of growth (10, 35, 99). Old cells and even empty sheaths of *Leptothrix* spp. show a much more pronounced tendency to produce and accumulate manganese dioxide than do growing cells (53, 54). During the exponential growth phase, release of organic compounds reducing manganese dioxide is apparently responsible for the retarded or prevented visible oxidation of $Mn^{2+}$ (see Fig. 16).

**Effect of adverse pH values.** Deviation of the pH of the medium from optimum values may often be the reason for the poor oxidation of $Mn^{2+}$ by *Leptothrix* cells. For instance, batch cultures of such organisms provided with considerable amounts of peptone often show a rise in pH from neutrality (which is optimal for biological Mn oxidation) to between 8.5 and 9.0. In sugar-containing media, production of organic acids causes a considerable drop in pH, which may be responsible for a decreased manganese-oxidizing capacity (see the results of experiments

with a manganese-oxidizing *Arthrobacter* strain [9]).

### Contribution of $Mn^{2+}$ Oxidation to the Energy Supply of *Leptothrix* spp.

Although manganese-oxidizing sheath-forming bacteria are unable to grow in media containing only inorganic compounds, the possibility that the oxidation of $Mn^{2+}$ would contribute to the energy supply of these organisms should not be entirely excluded. The probability of the utilization of the energy released by the oxidation of $Mn^{2+}$ is often thought to be very low for the following reasons. (i) The amount of energy released upon the oxidation of $Mn^{2+}$ to $Mn^{3+}$ or $Mn^{4+}$ is very small (95). (ii) The maximum concentration of $Mn^{2+}$ tolerated by *Leptothrix* spp. is very low (54, 70), compared with the high concentration of $Fe^{2+}$ required for the autotrophic growth of *T. ferrooxidans* (16, 71); it is hardly higher than the minimum concentration of $Fe^{2+}$ supporting growth of *T. ferrooxidans* (93). (iii) Oxidation of $Mn^{2+}$ by *Leptothrix* spp. proceeds mainly outside the cells, sometimes even outside the colonies (see Fig. 16 and 17). The protein (enzyme) catalyzing the oxidation of $Mn^{2+}$ 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.

In spite of these arguments, some authors believe they have shown that ensheathed bacteria of the *Leptothrix* type can utilize the energy of Mn(II) oxidation for growth (34). These authors used cell nitrogen, protein, and DNA contents of the culture as measures for growth of their *S. discophorus* strain (*Leptothrix* sp.); they found considerably higher cell yields in cultures with added $MnSO_4$ than in those without added manganese. This was true both of media with organic nutrients and of inorganic basal media, indicating autotrophic growth of the organism. In the latter case the amount of cell material, calculated from the amount of cell nitrogen, equaled 160 mg/liter obtained upon the oxidation of 140 mg of $Mn^{2+}$. This amount of cells is many times higher than the cell yield which might have been expected if the amount of energy derived from $Mn^{2+}$ oxidation approximated that derived from $Fe^{2+}$ oxidation. This result can only be explained by assuming that the basal nutrient medium had been contaminated with considerable amounts of consumable organic matter. Similar improbable yield responses to added $MnSO_4$ had previously been reported for a *Leptothrix* strain supplied with low amounts of organic nutrients (68).

That yield data derived from the cellular nitrogen of *Leptothrix* cultures grown with and without added $Mn^{2+}$ should be carefully checked is clearly shown in the following experiments (53, 54). One strain of *L. pseudo-ochracea* and two strains of *L. cholodnii* were cultivated in basal medium, including vitamin $B_{12}$, and supplied with small amounts of glucose and nitrogen in the form of aspartic and glutamic acids, peptone, or yeast extract. One-third of the total number of cultures of each series received $MnCO_3$, and one-third received $CaCO_3$, whereas the remaining cultures were left without carbonate. At the conclusion of the experiment, the precipitate of the culture, including sheathed bacteria, manganese dioxide, and some manganous carbonate, was collected by centrifugation, washed twice with distilled water, and analyzed for total nitrogen, which was thought to be cellular nitrogen. The analyses of this experiment (53, 54) showed that the cultures with $MnCO_3$ contained considerably more nitrogen than those with $CaCO_3$ and those without carbonate. From these results one might conclude that the higher amount of nitrogen in the cultures with added $MnCO_3$ indicated higher amounts of cellular material due to the utilization of the energy of oxidation of $Mn^{2+}$. An alternative explanation might be that, in the cultures without $Mn^{2+}$ oxidation, a certain amount of soluble nitrogen, either originally present in the medium or released by the *Leptothrix* cells during growth, was lost during the centrifugation and washing procedure. In the presence of $MnO_2$ this nitrogenous fraction, which included the proteinaceous catalyst responsible for the $Mn^{2+}$ oxidation, would have been bound to the $MnO_2$ precipitate.

Evidence concerning the occurrence of the latter phenomenon was obtained with spent culture solutions of ensheathed bacteria grown without added $Mn^{2+}$. These cell-free solutions, which contained the proteinaceous catalyst, were supplied with $MnSO_4$ (final concentration, 0.3 mM) and sodium malonate (final concentration, 13 mM) at pH 7.0. Upon incubation for 2 to 3 h, the precipitated manganese dioxide was collected, washed with distilled water, and analyzed for nitrogen (99). After removal of the manganese-protein precipitate, a second addition of $Mn^{2+}$ to the spent solution gave no further formation and precipitation of $MnO_2$. These observations suggest that the catalyst (enzyme) is bound to the precipitated manganese dioxide, resulting in elimination of its activity. A paper chromatographic analysis of the $MnO_2$-protein complex upon hydrolysis with HCl revealed the

TABLE 3. *Nitrogen content of cells and of manganic oxide-protein compounds produced in spent culture solutions of L. cholodnii strain 8*[a]

| Growth conditions | Time of harvest (days) | Nitrogen (μg/25 ml of culture)[b] in: | | |
|---|---|---|---|---|
| | | Cells + $MnO_2$ precipitate | Manganic oxide produced in: | |
| | | | Spent culture solution[c] | Washing solution[c] |
| Without $MnCO_3$ | 5 | 467 | 18 | 5 |
| | 8 | 395 | 3 | 6 |
| With 0.3 g of $MnCO_3$ per liter | 5 | 419 | 50 | 8 |
| | 8 | 349 | 70 | 12 |

[a] Unpublished data.
[b] Mean values of quadruplicate cultures.
[c] Soluble protein precipitated upon treatment with 0.35 mM $Mn^{2+}$ and 12 mM sodium malonate during 2 h at 40°C.

presence of a variety of amino acids, as in other proteins.

It is clear that a continuous excretion of the catalyst by the *Leptothrix* cells during a growth period of several days brings about a considerable increase of insoluble protein in the presence of $Mn^{2+}$, whereas in the control cultures without added $Mn^{2+}$ this protein remains soluble and is discarded with the spent culture solution upon cell harvest (Table 3). The amount of protein bound by $MnO_2$, which could be mistaken for autotrophic growth, depends on: (i) the $Mn^{2+}$ concentration in the culture solution, (ii) the rate of release of the catalyst, (iii) the growth rate of the cells, and (iv) the time of incubation of the culture.

Summarizing, it can be stated that so far no evidence has been provided concerning the ability of manganese-oxidizing microorganisms to use the energy of oxidation of $Mn^{2+}$ ions.

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