Archiv für Mikrobiologie 49, 132—149 (1964)

Department of Bacteriology and Public Health, Washington State University
Pullman, Washington

Morphology, Nutrition and Physiology of Sphaerotilus discophorus*

By
M. A. ROUF and J. L. STOKES

With 11 Figures in the Text

(Received April 6, 1964)

According to PRINGSHEIM (1949a) the genus *Sphaerotilus*, the filamentous, sheathed bacteria, includes three species: *S. natans*, *S. discophorus* and *S. trichogenes*. Only the first two species have been investigated extensively (PRINGSHEIM 1949a, 1949b; STOKES 1954; HÖHNL 1955; DONDERO 1961; MULDER and VAN VEEN 1963). The *Sphaerotilus* group is of considerable interest as iron bacteria, as slime formers in polluted streams and rivers, and as unusual morphological types.

Early investigations of *S. discophorus*, under the names of *Leptothrix ochracea*, *L. crassa*, *L. sideropous* and *L. winogradskii*, were made by WINOGRADSKY (1888, 1922), MOLISCH (1910), LIESKE (1919), CHOLODNY (1926) and CATALDI (1939). All of these organisms differ from *S. natans* primarily in that they oxidize manganous salts. The manganic oxide formed is deposited on the sheaths and colors them golden yellow or dark brown. PRINGSHEIM (1949a) considers all of these filamentous, sheathed, manganese oxidizing organisms to be strains of *S. discophorus* and we have adopted his logical and simple system of classification. These early investigations supplied much information on the ecology, isolation, morphology, nutrition and physiology of *S. discophorus* and on whether the organisms can utilize energy from the oxidation of ferrous and manganous ions. The latter aspect, despite extensive experimentation, remains controversial (see also GANTER and SCHWARTZ 1956; PRÄVE 1957; MULDER and VAN VEEN 1963).

The present investigations were undertaken to obtain additional information on the morphological, nutritional and physiological properties of *S. discophorus*. Our data confirm, differ from, and extend previously published information on *S. discophorus*. A preliminary report of some of our data has been made (ROUF and STOKES 1962b).

* Based in part on a thesis presented by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Washington State University, 1963.

Materials and Methods

Many different media and techniques were used. It seems best, from the standpoint of clarity, to present technical details at the time the specific experiments in which they were used are described.

Isolation of S. discophorus

The hay infusion enrichment method of WINOGRADSKY (1888) modified by the addition of manganous carbonate (MOLISH 1910) was used to isolate *S. discophorus* from natural bodies of water. The enrichment medium contained a mixture of thoroughly extracted alfalfa hay (STOKES 1954), freshly precipitated $Fe(OH)_3$ prepared by adding NaOH to a saturated solution of $FeSO_4$, and also solid $MnCO_3$. Care was taken to wash the $Fe(OH)_3$ free of alkali. The mixture was placed in glass cylinders, of 100 ml capacity, and filled almost to the top with samples of water from streams, rivers and lakes. These enrichment cultures were incubated at 25° C.

Within about a week, reddish-brown flakes were seen on the sides of the cylinders near the surface of the water. Microscopically, the flakes consisted primarily of filaments of *S. discophorus* strikingly encrusted with a thick, golden-brown layer of iron and manganese oxides. The filaments were 10—15 μ wide and most of the thickness was due to the inorganic oxide deposit.

Filaments from enrichment cultures were washed in several changes of sterile, distilled water to remove adhering bacterial contaminants. Between washings the filaments were dried on filter paper. The washed filaments were streaked on an agar medium which contained 0.5% peptone, 0.05% ferric ammonium citrate, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2$, 0.005% $MnSO_4 \cdot H_2O$, 0.001% $FeCl_3 \cdot 6H_2O$ and 1.2% agar in tap water. The plates were incubated at 25° C.

In 2—3 days a variety of colonies developed. Interspersed among colorless colonies were a number of easily distinguishable light to dark-brown colonies, about 2 mm in diameter, which had filamentous or hair-like projections. Such colonies are characteristic of *S. discophorus*. Preliminary identification was made by microscopic examination of portions of the brown colonies. This showed the presence of chains of rod-shaped cells within metallic oxide encrusted sheaths. Confirmation that the isolates were indeed *S. discophorus* was obtained later on the basis of other morphological and cultural characteristics of the purified cultures as described below. Pure cultures were obtained by restreaking well isolated colonies from the initial plate cultures. A total of 11 pure cultures of *S. discophorus* was isolated, each from a different sample of natural water.

Stock cultures were maintained on agar slants of the same composition as the isolation agar medium. The cultures were stored either at room temperature or in the refrigerator and subcultured at monthly intervals onto freshly prepared moist slants. Growth may not occur if the slants are allowed to become dry before inoculation. Survival appears to be better at room temperature. Although slant cultures may remain viable for as long as 2 months, sometimes subculturing is not successful after that period of time.

Morphology

Colonies

These are easily recognized by their characteristic filamentous form and their light to dark brown coloration due to the deposition of iron and manganese oxides (Fig. 1). The hairlike edges of the colonies (Fig. 2) resemble those of *S. natans* (STOKES 1954) although, usually, they are not as extensive. Occasionally the extruding filaments are quite short so that the edge of the colony appears almost smooth (Fig. 3).

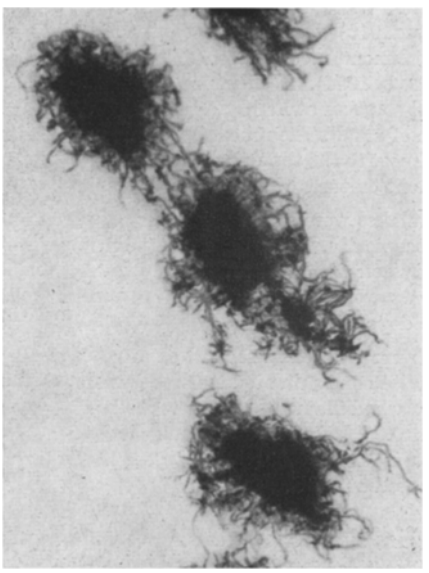

Fig. 1. Colonies of *Sphaerotilus discophorus*, strain *21* grown on peptone-glucose-salts agar with $MnSO_4$. Incubated for 2 days at 25 C; ×132

S. discophorus may dissociate spontaneously to give rise to an S or smooth type colony as contrasted to the R or filamentous form (PRINGSHEIM 1949a). Dissociation occurred frequently in our stock cultures and re-isolations from R colonies were made to maintain the cultures in the R phase. The S colony has a smooth edge, is colorless or only slightly brown and consists of short rods. Smooth colonies are formed usually on rich media. Dissociation was noted also by MULDER and VAN VEEN (1963). They suggested that two different types of R to S dissociations may occur; one dependent on nutrition and the other on mutation. The R to S dissociation also occurs in *S. natans* (PRINGSHEIM 1949a; STOKES 1954).

Filaments

These are composed of chains of rod-shaped cells enclosed in tightly-fitting sheaths. Their length is quite variable and may be as much as 970 μ according to GANTER and SCHWARTZ (1956). When *S. discophorus* is grown without manganous salts, the sheaths appear colorless and transparent and the internal cells are readily visible (Fig. 4). The narrow width of the filaments, about 1 μ, and the transparency of the sheaths make it more difficult to see the sheaths of *S. discophorus* than those of *S. natans* which are 1—2 μ wide. Because of the tight fit between cells and sheath, the latter can be seen only in areas of the filament vacated by the cells.

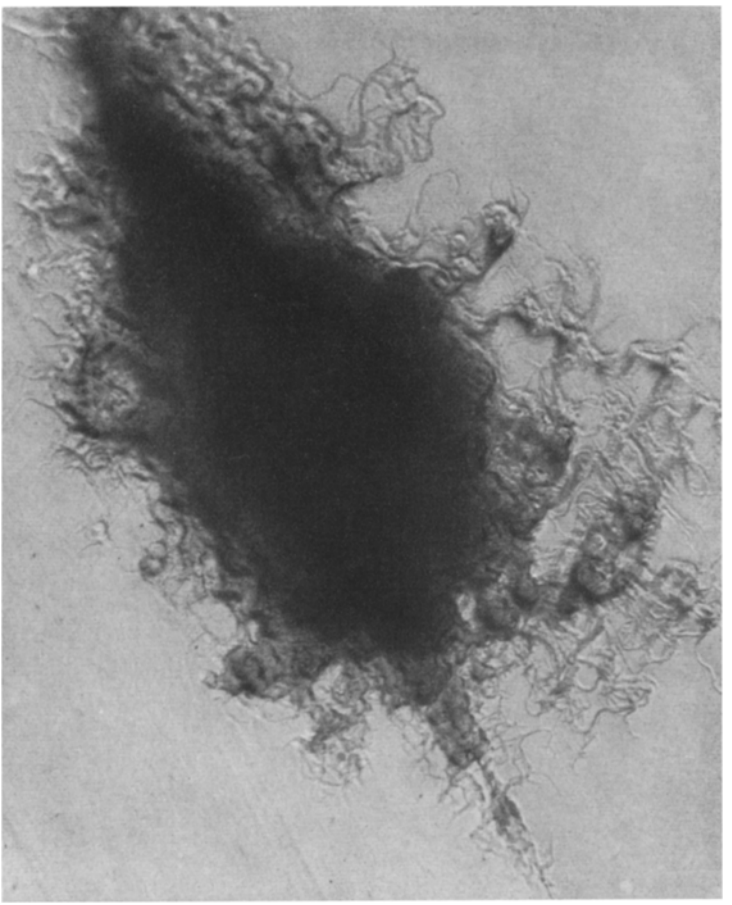

Fig. 2. Enlarged colony of *Sphaerotilus discophorus* strain *11* which shows the hair-like structure of the edge of the colony. Grown on peptone-glucose-salts agar with $MnSO_4$ for 4 days at 25 C; ×132

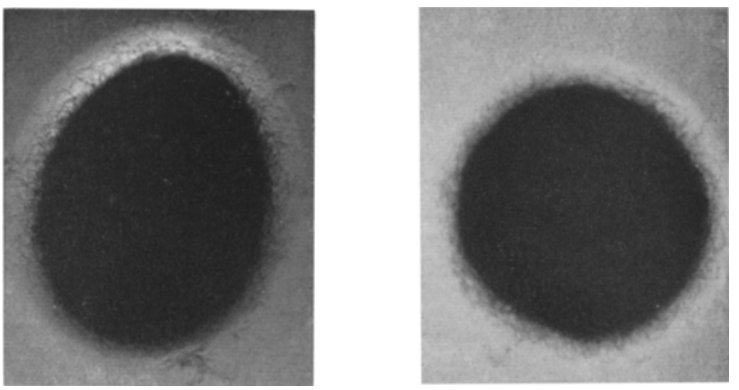

Fig. 3. "Short-haired" colonies of *Sphaerotilus discophorus*, strain *31*. Grown on peptone-glucose-salts agar with $MnSO_4$ for 2 days at 25 C; ×132

Filaments from cultures grown with iron and manganese salts are heavily encrusted with oxides of these metals (Fig. 5). The shape of the inorganic deposit is irregular, somewhat hemispherical and discontinuous (Fig. 6). In young cultures the deposits are relatively thin and golden yellow; but as the cultures age, the deposits thicken and turn dark brown. Further details of this process will be given later.

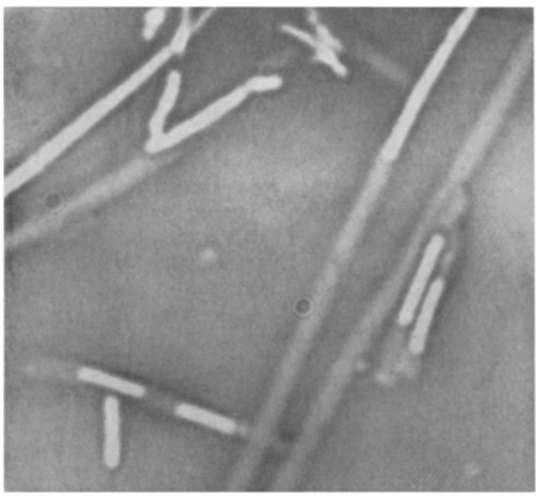

Fig. 4. Filaments of *Sphaerotilus discophorus*, strain *16*, negatively stained with nigrosin to show the sheaths and internal cells; ×2,000

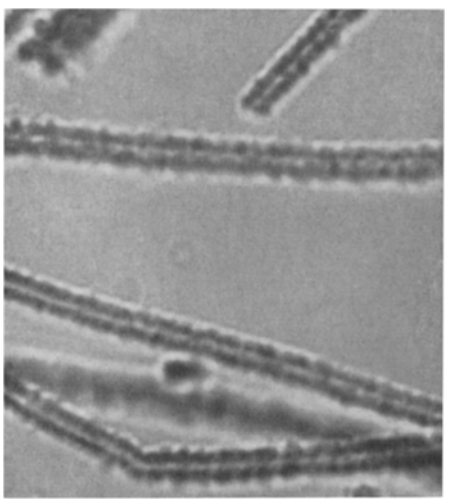

Fig. 5. Iron and manganese encrusted filaments of *Sphaerotilus discophorus*. strain *39*; ×2,800

Cells

Chains of rod-shaped cells can be seen within the sheaths of the filaments. In young cultures, the cell chains can be seen moving out from the ends of the oxide encrusted sheaths. The individual cells are about 0.8 to 1.0 μ wide and 2.5 to 9.8 μ long in nigrosin stained preparations (Fig. 7). When Gram stained, the cells are thinner, 0.5—0.9 μ, which indicates some distortion of the cells by the staining procedure. CATALDI (1939) reported that the cells of *S. discophorus (Leptothrix crassa)* are 0.5 to 0.8 μ wide, and PRINGSHEIM (1949a) stated that the cells are 5—10 μ or more in length. According to GANTER and SCHWARTZ (1956) the cells are 0.6—0.9 μ wide and 1.3—2.6 μ long. The cells of the manganese

oxidizing strains of MULDER and VAN VEEN (1963) ranged from 0.6—1.4 μ in width to 1—12 μ in length. In general there is reasonable agreement among different investigators as to the dimensions of the individual cells. It appears that the cells of *S. discophorus* are narrower than those of *S. natans* which are 1.2—2.4 μ wide (STOKES 1954; MULDER and VAN VEEN 1963).

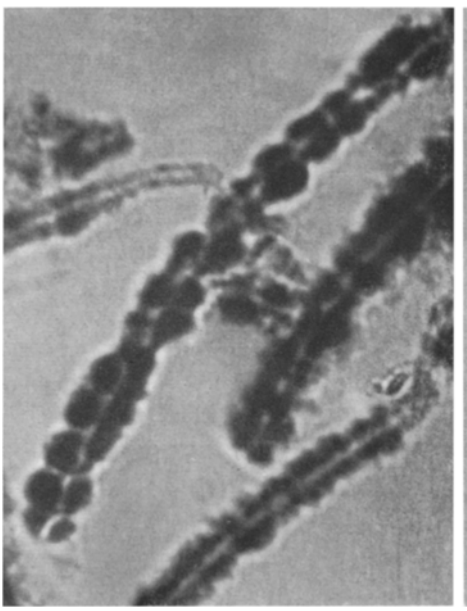

Fig. 6

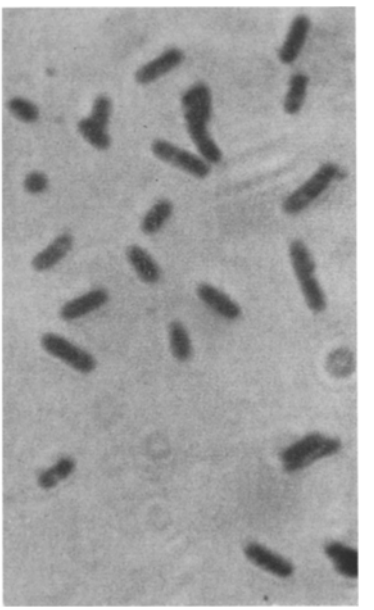

Fig. 7

Fig. 6. Iron and manganese encrusted filament of *Sphaerotilus discophorus*, strain *32*; ×2,800
Fig. 7. Cells of *Sphaerotilus discophorus*, strain *11*; ×5,600

The cells contain numerous refractile, sudanophilic granules which are somewhat less prominent than those of *S. natans*. The chemical nature of the granules will be discussed later. All of our strains were Gram negative, in agreement with the findings of CATALDI (1939) for her manganese oxidizing strains. The cells of *S. natans* also are Gram negative. Also, like *S. natans*, the cells of *S. discophorus* are motile and posses a single, polar flagellum (Fig. 8). Such flagellation has been reported also by PRINGSHEIM (1949a) and GANTER and SCHWARTZ (1956).

Nutrition

Although the matter of autotrophy in *S. discophorus* is controversial, there is general agreement among investigators that the organism can grow well heterotrophically with organic substrates. Our primary concern in the present paper is with the heterotrophic nutrition of *S. discophorus*.

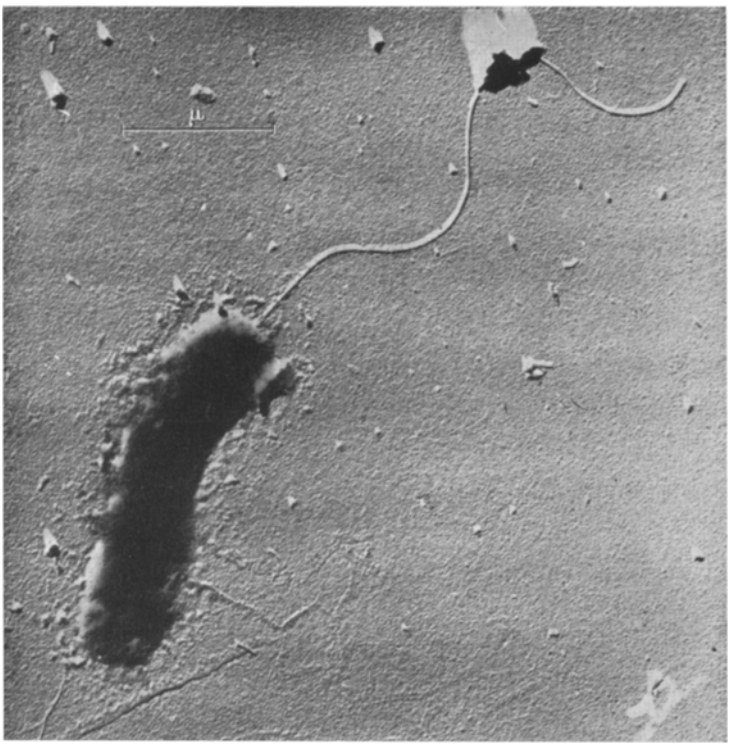

Fig. 8. Polar flagellation of *Sphaerotilus discophorus*; electron micrograph

Most of the experiments were made with 5 strains of *S. discophorus* selected at random from the 11 strains available, i.e., *32*, *35*, *36*, *39* and *41*. For growth experiments 10 ml of liquid medium, pH 7.0, in 50 ml Erlenmeyer flasks was used. The flasks were inoculated by loop transfer from 2 or 3 days old agar slant cultures. The liquid cultures were incubated at 30°C and growth was estimated visually during about 1 week of incubation. Growth was usually evident after 1 day and maximum in 2 or 3 days. Further incubation resulted in a clearing of the cultures due to lysis.

The peptone-mineral salts medium used for the isolation of *S. discophorus* supports good growth when used either as a broth or agar medium. Reduction of the peptone concentration from 0.5—0.1% reduced growth somewhat but an increase to 1.0% did not increase growth. With 0.1% peptone the addition of 0.2—0.5% glucose increased growth. At the 0.5% peptone level, glucose was not stimulatory and it markedly reduced the oxidation of $MnSO_4$, i.e. there was a sharp reduction in the brown pigmentation of the cultures. The mineral salts were essential for growth since growth did not occur with peptone alone.

Common laboratory media, such as nutrient broth, brain heart infusion, trypticase soy and yeast extract (1%), did not support growth of *S. discophorus*. However growth could be obtained in these media when the nutrient concentrations were lowered. Nutrient broth supported growth when diluted 1:2, trypticase soy broth diluted 1:15, and yeast extract at 0.1 or 0.2% concentration. The inhibitory effect of the usual concentrations of these media is due probably to excessive amounts of free amino acids which they provide (DONDERO 1961; MULDER and VAN VEEN 1963). Peptone appears to be low in free amino acids, since good growth of *S. discophorus* can be obtained even with 3% peptone, although higher concentrations are toxic.

Vitamin requirements

Vitamin-free acid hydrolyzed casein (Bacto casamino acids) could not replace peptone for growth. When the casein hydrolysate was used, the basal medium was modified by the addition of 0.01% tryptophane, 0.0025% cysteine, 0.3% glucose and 0.05% K_2HPO_4. Phosphate was not required in the peptone medium probably because it was supplied as an impurity by the peptone. Also $MnSO_4$ was omitted since the formation of manganic oxide interfered with the visual estimation of growth. The medium was adjusted to pH 7.1.

The addition, however, of a mixture of B-vitamins to the casein hydrolysate medium permitted growth. The vitamins used, in micrograms per ml of medium, were thiamin, riboflavin, nicotinic acid and pantothenic acid, 0.2; pyridoxin, 1.0; *p*-aminobenzoic acid, 0.4; pteroylglutamic acid, 0.01; and biotin, 0.02. Further experimentation showed that of the 8 vitamins, only thiamin and biotin were required for growth (Table 1). There was slight growth of some strains without biotin but this was due probably to carry-over of traces of biotin with the inoculum since use of washed inocula usually eliminated all growth.

Growth did not take place with either thiamin or biotin alone but only when both vitamins were present in the casein hydrolysate medium. For good growth 0.02 μg of thiamin and 0.002 μg of biotin per ml of medium were required but we have used routinely ten times as much of each vitamin per ml of medium in order to be certain that sufficient amounts woud be available in the cultures.

This is the first time that a requirement for thiamin and biotin by *S. discophorus* has been reported. MULDER and VAN VEEN (1963) found that some of their *Leptothrix* or manganese oxidizing strains require vitamin B_{12} (cyanocobalamin) for growth and that this requirement could be replaced by methionine. Although the effect of other vitamins including thiamin and biotin was tested, only vitamin B_{12} was essential for growth. Some of their strains however, failed to grow or grew very poorly with

Table 1. *Vitamin requirements of Sphaerotilus discophorus*

Media	Strain			
	32	*36*	*39*	*43*
Basal[1]	4+[2]	4+	4+	4+
Basal minus thiamin	—	—	—	—
Basal minus riboflavin	4+	4+	4+	4+
Basal minus nicotinic acid	4+	4+	4+	4+
Basal minus pantothenic acid	4+	4+	4+	4+
Basal minus pyridoxine	4+	4+	4+	4+
Basal minus para-amino benzoic acid	4+	4+	4+	4+
Basal minus pteroylglutamic acid	4+	4+	4+	4+
Basal minus biotin	1+	1+	±	±

[1] Contained 0.5% vitamin-free casamino acids, 0.01% tryptophane, 0.0025% cysteine, 0.3% glucose, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2$, 0.001% $FeCl_3 \cdot 6H_2O$, 0.05% ferric ammonium citrate, 0.05% K_2HPO_4, and all of the vitamins mentioned in the table.

[2] — = no growth; ± = very slight growth; 1+ to 4+ = increasing amount of growth. Results recorded after 3 days of incubation at 30° C.

casamino acids but apparently the vitamin mixture was not tested with the casamino acids medium. If this had been done perhaps the thiamin and biotin requirement might have been observed. Strain variation as well as methodology also may account for these differences. We did not observe a vitamin B_{12} requirement with our strains apparently because methionine which can substitute for the vitamin was supplied by the casamino acids present in our medium.

The vitamin requirements of *S. discophorus* may have an important bearing on the controversy concerning the autotrophy of this organism. All of the numerous attempts by past investigators to grow pure cultures of *S. discophorus* autotrophically were made with inorganic media without added vitamins. Virtually all of these attempts ended in failure (PRINGSHEIM 1949b). If vitamins are required for autotrophic growth then these negative results are understandable and positive results can be expected only in inorganic media supplied with the essential vitamins.

Carbon and energy sources

A variety of organic compounds were tested. The basal medium contained 0.05% casamino acids, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2$, 0.001% $FeCl_3 \cdot 6H_2O$, 0.05% ferric ammonium citrate, 0.05% K_2HPO_4, 0.2 µg/ml thiamin, 0.02 µg/ml biotin and distilled water. $MnSO_4$ was omitted. The medium was adjusted to pH 7.1 and distributed in 10 ml amounts into 50 ml Erlenmeyer flasks and autoclaved. The small amount of casamino acids, 0.05%, was sufficient to supply the nitrogen requirements for growth but not sufficient for the carbon and energy needs of

S. discophorus. Therefore the basal medium supported only barely visible growth unless a utilizable source of carbon and energy was added. To each flask was added 1 ml of a separately autoclaved solution of the energy source to give a final concentration of 0.3 % in the medium. Acids were neutralized prior to use and ethyl alcohol was filter sterilized. In some experiments 0.05 % peptone replaced casamino acids as the nitrogen source. Nine strains were used. The inoculated flasks were incubated at 30°C and growth was judged by visual observations. The basal medium without added energy source served as the control.

All strains grew with glucose, fructose, galactose, mannitol, sorbitol, maltose, glycerol, salicin, raffinose, dulcitol and ethanol. Only five strains were tested with the latter 4 compounds. Raffinose, sorbitol and glycerol supported the most growth. Sucrose, erythrytol and lactate were utilized by 6 of the 9 strains. Lactose and xylose were utilized poorly and by only a few strains. The carbon and energy sources which did not support growth of *S. discophorus* included arabinose and the organic acids, formate, acetate, propionate, butyrate, malate, succinate, citrate and benzoate.

The *Leptothrix* strains of CATALDI (1939) utilized glucose, citrate, oxalate and asparagine as carbon sources.

The *Leptothrix crassa* strain of GANTER and SCHWARTZ (1956) utilized glucose, maltose and sucrose. Also, asparagin and the ammonium salts of oxalic, lactic and citric acids could serve as sources of both energy and nitrogen. MULDER and VAN VEEN (1963) reported that glucose, fructose and sucrose were especially good carbon sources for some of their *Leptothrix* strains. Also, acetate, lactate, malate, glycerol and β-hydroxybutyrate but not citrate supported growth.

The carbon and energy sources for *S. discophorus* are similar to those utilized by *S. natans*. The latter also can grow with glucose, galactose, mannitol, sorbitol, sucrose, maltose, glycerol and ethanol but not with arabinose, propionate or benzoate (STOKES 1954).

Nitrogen sources

There is general agreement among investigators that manganese oxidizing strains, *S. discophorus* or *Leptothrix* species, can grow well with peptone, beef extract and other complex nitrogenous substances provided that relatively low concentrations are used. Casein hydrolysate or a mixture of amino acids simulating casein hydrolysate also is a satisfactory source of nitrogen (MULDER and VAN VEEN 1963). Asparagine (CATALDI 1939; PRINGSHEIM 1949a; GANTER and SCHWARTZ 1956), the ammonium salts of oxalic, lactic and citric acids (GANTER and SCHWARTZ 1956) and $(NH_4)_2SO_4$ or NH_4NO_3 (LIESKE 1919; CATALDI 1939; MULDER and VAN VEEN 1963) may be utilized by some strains.

Our strains grew well with 0.5% peptone, in diluted nutrient broth, brain heart infusion and trypticase soy broths, with 0.2% yeast extract and with 0.5% casein hydrolysate. They did not grow with $(NH_4)_2SO_4$ in a glucose-mineral salts medium supplied with thiamin and biotin possibly because vitamin B_{12} was absent.

Physiology

Growth temperatures

Molisch's strain of *L. ochracea* grew optimally at 23—25°C and failed to grow at 5°C and 40°C. Some of Cataldi's *Leptothrix* strains grew best at 25—28°C and others at 37°C. All of her strains had a minimum growth temperature of 15°C.

Five of our strains were examined for their cardinal growth temperatures in the peptone-mineral salts isolation medium (liquid) and in the same medium without $MnSO_4$ but fortified with 0.2% glucose, thiamin and biotin. Similar results were obtained with both media and all strains. The cultures grew well in the range of 10—35°C although growth was slow at 10°C. Growth was most rapid at 25—35°C. There was very slight or no growth at 37°C and no growth at 40°C. On prolonged incubation, for 1 month, 4 of the 5 strains grew at 5°C but not at 0°C.

In general, *S. discophorus* grows at somewhat lower temperatures than *S. natans*. The latter grows in the range of 15—40°C (STOKES 1954).

pH limits for growth

The peptone-glucose-mineral salts-vitamin liquid medium was used in 10 ml amounts per 50 ml Erlenmeyer flasks. The pH was adjusted to various values in the range of pH 4.0—9.8. McIlvaine's standard buffer solution (citric acid disodium phosphate) at 1/10th the usual concentration, was used for pH 4.0 through pH 7.9. For pH 8.2, 8.6, 9.0 and 9.8, 0.01 M NH_4OH—HCl buffer was used. The results obtained with 5 strains of *S. discophorus* are shown in Table 2. No growth appeared at pH 4.0, 5.0, 9.0 and 9.8. Slow and less than maximal growth occurred at pH 6.0, 6.4, 8.2 and 8.6. Rapid and extensive growth occurred at pH 7.0 and 7.9. Thus the pH range for growth of all strains was pH 6.0—8.6 inclusive with an optimum at about pH 7.0—8.0. Similar results were obtained in media adjusted to various pH levels with HCl and NaOH. Use of 0.02 M phosphate buffer to establish pH levels war unsatisfactory because the buffer markedly inhibited growth.

MOLISCH and LIESKE used slightly alkaline media to grow their strains of *Leptothrix*. Most of CATALDI's strains grew in the range of pH 6—10 and the optimum was pH 8.0—8.5. PRINGSHEIM adjusted his growth media to pH 7.0—7.5. GANTER and SCHWARTZ were able to grow *L. crassa*

Table 2. *Effect of pH on the growth of Sphaerotilus discophorus*

pH	Strain									
	32		*35*		*36*		*39*		*41*	
	2D[1]	4D	2D	4D	2D	4D	2D	4D	2D	4D
4.0	−[2]	−	−	−	−	−	−	−	−	−
5.0	−	−	−	−	−	−	−	−	−	−
6.0	1+	2+	2+	2+	−	2+	2+	2+	1+	2+
6.4	1+	2+	2+	2+	−	2+	1+	2+	1+	2+
7.0	3+	4+	3+	4+	3+	4+	3+	4+	3+	4+
7.9	3+	4+	3+	4+	3+	4+	3+	4+	3+	4+
8.2	2+	2+	2+	4+	2+	4+	2+	4+	2+	4+
8.6	−	1+	−	2+	−	2+	−	2+	−	1+
9.0	−	−	−	−	−	−	−	−	−	−
9.8	−	−	−	−	−	−	−	−	−	−

[1] 2D indicates that the observation was made on the second day.

[2] − = no growth; ± = very slight growth; 1+ to 4+ = increasing amount of growth.

in the range of pH 5.0—7.2 and the optimum was pH 5.8—6.8. MULDER and VAN VEEN maintained their *Leptothrix* cultures at pH 7.0.

There is general agreement, therefore, that *S. discophorus* cannot tolerate very acid or alkaline conditions and grows best at about neutrality. In this respect it resembles closely *S. natans*.

Additional properties

All of our strains grew only when oxygen was present. They failed to grow in cultures maintained anaerobic with pyrogallol. *S. discophorus* like *S. natans* is a strict aerobe.

All strains reduced nitrates to nitrites and did not form indole from tryptophane. CATALDI obtained the same results with her strains.

None of our strains hydrolyzed starch, dextrin or cellulose or liquefied gelatin. All of them contained catalase.

It had been found previously that the sudanophilic granules of *S. natans* are composed of poly-β-hydroxybutyric acid and that this compound may account for as much as 22.5% of the dry weight of the cells (ROUF and STOKES 1962). The sudanophilic granules of *S. discophorus* also are composed of poly-β-hydroxybutyric acid and this compound may represent as much as 40% of the dry weight of the cells. This is among the largest amounts so far found in bacteria.

Deposition of iron and manganese

When grown in peptone-glucose-mineral salts medium without added iron or manganese compounds, the filaments of *S. discophorus* are thin, smooth and colorless. When $FeCl_3$ and ferric ammonium citrate are added

to the medium the filaments are thicker and have a pale yellow deposit of ferric oxide on the sheaths. When $MnSO_4$ or a combination of $MnSO_4$ and ferric salts are added to the medium, the filaments are wider than those grown with the ferric salts alone and exhibit a golden yellow, irregular deposit which thickens and turns dark brown as the culture ages. For example, filaments from a 2 days old culture of strain 31 were 0.86 μ wide when grown without added iron or manganese as measured in wet mounts, 1.27 μ when grown with ferric salts, 2.38 μ with $MnSO_4$, and 2.48 μ with both metals. The filaments from the latter culture were 6—10 μ wide after 7 days of incubation. The secondary deposit of metallic oxides can be much wider, therefore, than the primary sheath.

The amounts of iron and manganese deposited on the primary sheaths of *S. discophorus* were determined. Two strains, *21* and *36*, were used. For comparative purposes, *S. natans*, strain *4*, was included.

The peptone-glucose-mineral salts-vitamin medium, with and without $MnSO_4$ was used. The specific composition is given in the footnote to Table 3. The medium was distributed in 200 ml amounts into 2 l Erlenmeyer flasks. After inoculation, the cultures were incubated for 60 hrs at 30° C. The *Sphaerotilus* growths were harvested by centrifugation, and washed twice with distilled water. The cellular material was dried to constant weight at 90—95° C and analyzed quantitatively for iron and manganese. Dried cell material from 400 ml of culture of each organism was used for the assays. Iron was determined by the method described by Diehl and Smith (1952) and manganese by the method given in "Official Methods of the Association of Official Agricultural Chemists" (1955). These analyses were made for us by our Institute of Technology.

Table 3. *Iron and manganese content of Sphaerotilus*

Organism	*Medium*[1]	Per cent dry wt of filaments and cells	
		Iron	*Manganese*
S. discophorus, strain *21*	with Mn[2]	5.68	1.43
	without Mn	4.25	0.0
S. discophorus, strain *36*	with Mn	5.44	1.12
	without Mn	7.10	0.0
S. natans, strain *4*	with Mn	0.71	0.0

[1] It contained 0.4% peptone, 0.3% glucose, 0.2 μg/ml thiamin, 0.02 μg/ml biotin, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2$, 0.001% $FeCl_3 \cdot 6H_2O$, 0.05% ferric ammonium citrate and distilled water; pH 7.1.

[2] 0.005% $MnSO_4 \cdot H_2O$.

The analytical data are presented in Table 3. The iron content of *S. discophorus* ranged from 4.25—7.10% of the dry weight of the filaments and cells and was virtually independent of the presence or absence of manganese in the medium. These values are much higher, 6—10 fold, than the 0.71% iron in *S. natans*. The manganese content of *S. disco-*

phorus ranged from 1.12—1.43% and was completely dependent, of course, on the presence of $MnSO_4$ in the growth medium. In contrast, *S. natans* did not accumulate manganese even when grown with $MnSO_4$. This could be predicted from the colorless appearance of *S. natans* when grown with manganese salts.

The finding that *S. discophorus* accumulates about four times as much iron as manganese when grown with both metals was somewhat surprising, perhaps, since the filaments are dark brown which is the color of manganic oxide rather than light yellow, the color of ferric oxide. A relatively small amount of manganic oxide is sufficient, therefore, to dominate and to give *S. discophorus* its dark brown coloration.

Kinetics of growth

In our previous experiments growth of *S. discophorus* was determined visually. To establish more quantitatively the amount of growth and the course of the growth cycle, dry weight determinations of the cellular material produced in cultures were made as a function of time of incubation.

The medium contained 0.5% peptone, 0.2% glucose, the usual mineral salts with and without $MnSO_4$, and 0.2 µg/ml of thiamin and 0.02 µg/ml of biotin. The pH was adjusted to 7.1. The medium was distributed in 100 ml amounts into 1 l Erlenmeyer flasks in order to provide a large surface area. The flasks were autoclaved, inoculated and incubated at 30° C. At daily intervals, two flask cultures were removed and the cellular material was collected by centrifugation, washed twice with distilled water and dried at 95—100° C to constant weight. Dry weights from duplicate cultures agreed closely and were averaged.

The results obtained with stationary cultures of *S. discophorus*, strain *39*, are shown in Fig. 9 and the results with cultures aerated by incubation on a rotary shaker are shown in Fig. 10.

In stationary cultures, maximal growth is reached in 2 days, remains stationary during the 3rd day and then the dry weights gradually decrease on continued incubation as the cells die and lyse. $MnSO_4$ reduced growth somewhat. The maximal dry weights indicate a yield of about 1 g of cellular material per liter of culture. This amount of growth is comparable to that obtainable with common bacteria under similar growth conditions. The techniques used did not permit us to establish the exact shape of the curves during the first 24 hrs. Undoubtedly a lag phase exists.

Similar results were obtained in aerated cultures except that a sharp decrease in cellular dry weights occurred soon after maximal growth and continued more precipitously than in the stationary cultures. Aeration did not increase significantly the rate or extent of growth but favored lysis.

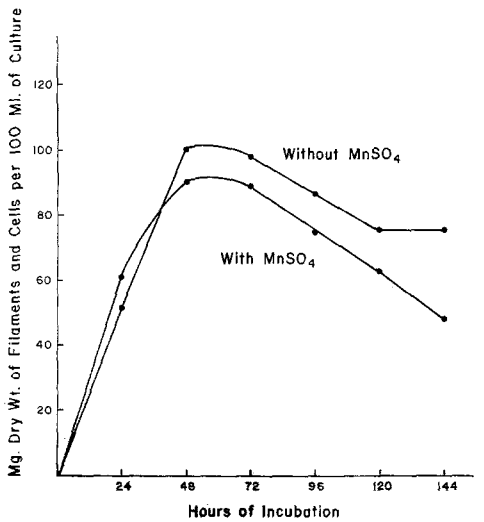

Fig. 9. Growth curves of *Sphaerotilus discophorus*, strain *39*, with and without $MnSO_4$, under stationary conditions at 30° C

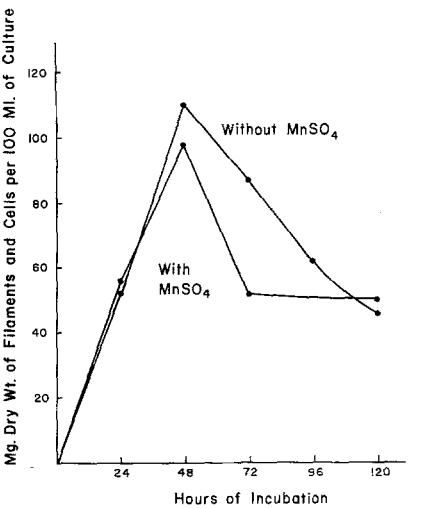

Fig. 10. Growth curves of *Sphaerotilus discophorus*, strain *39*, with and without $MnSO_4$ and shaken at 30° C

Oxidative assimilation

Previous experiments with *S. natans* (Stokes 1954) had shown that cell suspensions oxidized a variety of sugars, amino acids and related compounds. Noteworthy features of these oxidations were the high degree of oxidative assimilation of some sugars, to the extent of 70—80%, and the resistance of the assimilation process to inhibition by sodium acid and 2,4-dinitrophenol—compounds which usually repress assimilation in microorganisms. Similar experiments were made with *S. discophorus*.

Manometric techniques were used. The cells were grown in a medium which contained 0.4% peptone, 0.3% glucose (separately sterilized), the usual mineral salts except $MnSO_4$, thiamin and biotin. Strains *32* and *35* were used in most of the experiments and strains *36*, *39* and *42* in some of them. The cultures were grown at 30° C on a rotatory shaker for 1 or 2 days. The cell material was harvested by centrifugation, washed twice with 0.02 M potassium phosphate buffer and resuspended in the same buffer to a density of 380 units in the Klett-Summerson photometer (red filter). The relatively low concentration of buffer was used because it had been found that the cells lysed rapidly in 0.1 M buffer.

Table 4 contains some of the data, in a composite form, obtained with the various strains. Glucose, fructose, galactose and other sugars were oxidized. Also, glycerol, succinate, pyruvate, acetate, serine and glutamate were oxidized. However, with all of these compounds, only a fraction of the substrate present was oxidized, about 10% or less as calculated from the total amount of O_2 consumed compared to the theoretical amount of O_2 required for complete oxidation of all of the substrate. It

must be assumed that the remaining approximately 90% of the substrate was assimilated by the cells. The latter was confirmed in the case of glucose oxidation by the finding that virtually no residual glucose could be detected in the cell suspension after O_2 consumption had ceased. This is an unusually high degree of oxidative assimilation (STOKES 1952) and exceeds even that observed with *S. natans*. As with the latter organism, assimilation by *S. discophorus* could not be repressed by sodium azide and 2,4-dinitrophenol. The kinetics of oxidation of several substrates are shown in Fig. 11. The rate of endogenous oxidation is high.

Table 4. *The rate and extent of oxidation of various compounds by Sphaerotilus discophorus*

Substrate[1]	O_2 consumed µl/hr/vessel[2]	% Oxidation
Glucose	56	11
Fructose	60	12
Galactose	30	5
Mannitol	23	4
Sucrose	83	8
Maltose	29	5
Glycerol	18	4
Succinate, Na	40	11
Pyruvate, Na	30	8
Acetate, Na	20	8
Serine	28	8
Glutamate, Na	30	12

[1] The Warburg vessels contained 2 ml of cell suspension in 0.02 M potassium phosphate buffer, pH 7.1 (Klett density, 380 units), 4 or 8 µmoles of substrate in a volume of 0.1 or 0.2 ml, and 0.2 ml of 10% KOH in the center well to absorb CO_2. The gas phase was air and the bath temperature 30° C.

[2] Endogenous oxidation subtracted.

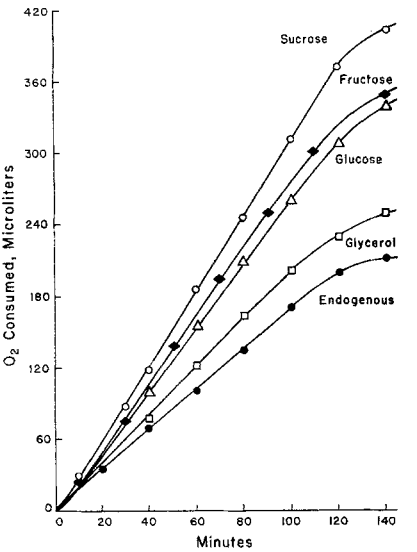

Fig. 11. Rates of oxidation of several compounds by *Sphaerotilus discophorus*

Compounds which were not oxidized, or perhaps slightly, included lactose, arabinose, xylose, butyrate, citrate, α-ketoglutarate, malate, oxalacetate, glycine, alanine and aspartate.

Discussion

S. discophorus is similar to *S. natans* in many of its properties and also strikingly different in some respects. Both species grow on solid media as distinctive, filamentous colonies and may dissociate to give rise to S or smooth colonies. Both species consist of rod shaped cells enclosed in tightly-fitting, transparent sheaths. The sheath of *S. natans* is a protein-polysaccharide-lipid complex (ROMANO and PELOQUIN 1963). The

chemical nature of the sheath of *S. discophorus* has not been reported. Both species tend to accumulate morphologically distinct deposits of iron oxide on the primary sheath. The cells of both species are Gram negative and motile by means of a single, long, polar flagellum.

Both species grow well in dilute organic media, and best at pH 7—8, and at about 30° C. The cells of both species accumulate large amounts of poly-β-hydroxybutyric acid internally as sudanophilic granules. Both oxidize a similar variety of sugars, sugar alcohols, amino acids and other compounds. These oxidations proceed with a high degree of assimilation of the substrates.

These similarities in the two organisms lend support to Pringsheim's system of classification which considers them to be species of the same genus, *Sphaerotilus*, and argue against the establishment of a separate genus, *Leptothrix*, for the *discophorus* type.

S. discophorus, however, differs from *S. natans* in one important property. It can oxidize manganous salts whereas *S. natans* cannot. As a result, cultures of *S. discophorus* grown with manganous compounds are distinctively brown and the filaments are covered with a dark brown deposit which contains manganic oxide. If autotrophy should be established in *Sphaerotilus*, then the difference between *S. discophorus* and *S. natans* will be that *S. discophorus* can derive energy from the oxidation of both manganous and ferrous ions whereas *S. natans* can obtain energy only from the oxidation of ferrous ions.

There are other, perhaps more minor, differences between the two organisms. The filaments (and cells) of *S. discophorus* are narrower than those of *S. natans* when consideration is given only to the primary filament and not to the secondary layer of inorganic oxides. Also, *S. discophorus* requires thiamin and biotin for growth and also vitamin B_{12} or methionine whereas *S. natans* does not require these growth factors or is only stimulated by vitamin B_{12}.

Summary

By means of hay infusion-$Fe(OH)_3$-$MnCO_3$ enrichment cultures, 11 pure strains of the filamentous, sheathed bacterium, *Sphaerotilus discophorus* were isolated from streams, rivers and lakes. The morphology of the colonies, filaments and cells are shown in a series of photographs. The strains grew well in dilute organic media but not in many of the common bacteriological media. All strains required thiamin and biotin for growth. Glucose, mannitol, salicin, raffinose, glycerol and other compounds were suitable energy sources and peptone and casein hydrolysate were satisfactory nitrogen sources for growth. The temperature range for growth was 5—35° C and the pH range 6.0—8.6.

The sudanophilic granules of *S. discophorus* are composed of poly-β-hydroxybutyric acid which may be as much as 40% of the dry weight of the cells. Growth with iron and manganese salts results in extensive

deposition of oxides on these metals on the filaments. Chemical analyses of 60 hr cultures showed that the iron content of the filaments ranged from 4.25—7.10% and manganese from 1.12—1.43%, calculated on a dry weight basis. Resting cell suspensions oxidized a variety of sugars, sugar alcohols, amino acids and other compounds and concomitantly assimilated about 90% of these substrates. The properties of *S. discophorus* are compared with those of *S. natans*.

Acknowledgement. These investigations have been supported, in part, by a research grant (WP-00239) from the Division of Water Supply and Pollution Control, U.S. Public Health Service.

We are glad to have this opportunity to express our appreciation to Mrs. MARGARET POWERS for her capable assistance.

Literature

CATALDI, M. S.: Estudio fisiológico y sistemático de algunas *Chlamydobacteriales*. Thesis. Buenos Aires 1939.

CHOLODNY, N.: Die Eisenbakterien. Beiträge zu einer Monographie. Pflanzenforsch. **4**, 69—133 (1926).

DIEHL, H., and F. SMITH: Quantitative Analysis, p. 370. New York: John Wiley & Sons Inc. 1952.

DONDERO, N. C.: *Sphaerotilus*, its nature and economic significance. (W. W. UMBREIT, editor.) Advanc. appl. Microbiol. **3**, 77—107 (1961).

GANTER, I. R., u. W. SCHWARTZ: Beiträge zur Biologie der Eisenmikroben. II. *Leptothrix crassa* Chol. Schweiz. Z. Hydrol. **18**, 171—192 (1956).

HÖHNL, G.: Ernährung und stoffwechselphysiologische Untersuchungen an *Sphaerotilus natans*. Arch. Mikrobiol. **23**, 207—250 (1955).

LIESKE, R.: Zur Ernährungsphysiologie der Eisenbakterien. Zbl. Bakt., II. Abt. **42**, 413—425 (1919).

MOLISCH, H.: Die Eisenbakterien. Jena: G. Fischer 1910.

MULDER, E. G., and W. L. VAN VEEN: Investigations on the *Sphaerotilus-Leptothrix* group. Antonie v. Leeuwenhoek **29**, 121—153 (1963).

Official methods of the association of official agricultural chemists. 1955. 7th ed. paragraph 6.15. Association of Official Agricultural Chemists. Washington D.C.

PRÄVE, P.: Untersuchungen über die Stoffwechselphysiologie des Eisenbakteriums *Leptothrix ochracea* Kützing. Arch. Mikrobiol. **27**, 33—62 (1957).

PRINGSHEIM, E. G.: The filamentous bacteria *Sphaerotilus, Leptothrix, Cladothrix* and their relation to iron and manganese. Trans. roy. Soc. B **233**, 453—482 (1949a).

— Iron bacteria. Biol. Rev. **24**, 200—245 (1949b).

ROMANO, A. H., and J. P. PELOQUIN: Composition of the sheath of *Sphaerotilus natans*. J. Bact. **86**, 252—258 (1963).

ROUF, M. A., and J. L. STOKES: Isolation and identification of the sudanophilic granules of *Sphaerotilus natans*. J. Bact. **83**, 343—347 (1962a).

— — Isolation and properties of *Sphaerotilus discophorus*. Bact. Proc. **41**, (1962b).

STOKES, J. L.: Inhibition of microbial oxidation, assimilation and adaptive enzyme formation by methylene blue. Antonie v. Leeuwenhoek. **18**, 63—81 (1952).

— Studies on the filamentous sheathed iron bacterium *Sphaerotilus natans*. J. Bact. **67**, 278—291 (1954).

WINOGRADSKY, S.: Über Eisenbakterien. Bot. Z. **46**, 261—270 (1888).

— Eisenbakterien als Anorgoxydanten. Zbl. Bakt., II. Abt. **57**, 1—21 (1922).

Prof. Dr. J. L. STOKES, Washington State University,
Department of Bacteriology and Public Health, Pullman, Washington, U.S.A.