SOME PROBLEMS IN THE IDENTIFICATION AND
CLASSIFICATION OF SPECIES OF SPIRILLUM
II. LATER TAXONOMY OF THE GENUS SPIRILLUM

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Giesberger's Taxonomy

Giesberger (1936) was the first investigator of the genus Spirillum to define certain physiological characters useful in identification. He accomplished this by determining, in a mineral base medium, the utilization of various carbon compounds as the sole source of carbon and energy. Giesberger found in his strains good correlation between utilization patterns and distinctive morphology; each distinctive morphological group utilized a distinctive group of carbon compounds. This coupling of the distinctive morphology with the utilization of selected carbon compounds makes possible the identification of his species. However, the pattern of the utilization of the carbon compounds does not in all cases correlate with morphology. Williams and Rittenberg (1957) found that four morphologically distinct groups of strains of Spirillum utilized the same carbon compounds. In such cases with a choice between physiological and morphological criteria in distinguishing species, morphological characters were chosen as being the more distinctive. Giesberger (1936), himself, separated and tentatively identified his species on the basis of morphological criteria; the utilization of the carbon compounds was an additional but secondary character in the diagnosis.

Giesberger (1936) provided exact morphological descriptions (including measurements) as well as growth characteristics for the five species studied by him. The photographs of his species are reproduced as Figs. 7, 8, 9, and 10. His table, showing the utilization of the carbon compounds by the various morphological types, is reproduced as Table 1. Giesberger made a systematic survey of the species of Spirillum, described in the literature up to 1936, rejecting all inadequately described species with two exceptions. Giesberger (1936) considered the morphology of

Table 1. Utilization of Various Compounds as Sole Carbon Source
 (Reproduced from Giesberger's (1936) dissertation:
Beitrage zur Kenntnis der Gattung Spirillum Ehbq.)

	Cultures of morphological Group I (<i>S. serpens</i>)	Cultures of morphological Group II (<i>S. itersonii</i>)	Cultures of morphological Group III (<i>S. tenue</i>)	Cultures of morphological Group IV (<i>S. undul</i>)
SATURATED FATTY ACIDS				
Formic acid	-	-	-	-
Acetic acid	+	+	+	(+)
Propionic acid	-	(+)	(+)	-
n-butyric acid	+	+	+	-
OTHER ACIDS				
Oxalic acid	-	-	-	-
Succinic acid	+	+	+	+
Fumaric acid		+		
Malonic acid		-		
D-lactic acid	+	+	+	(+)
Citric acid	-	+	+	-
DL-malic acid	+	+	+	(+)
D-tartaric acid	-	-	-	-
Pyruvic acid (Pyruvic)	+	+	+	+
CARBOHYDRATES				
Xylose	-	(+)	(+)	-
Arabinose	-	(+)	(+)	-
Glucose	-	+	+	-
Levulose	-	+	+	-
Mannose	-	(+)	(+)	-
Galactose	-	(+)	(+)	-
Maltose	-	(+)	(+)	-
Lactose	-	(+)	(+)	-
Saccharose	-	(+)	(+)	-
ALCOHOLS				
Methyl alcohol	-	-	-	-
Ethyl alcohol	-	+	-	(+)
n-propyl alcohol	-	+	-	(+)
n-butyl alcohol	-	+	-	(+)
Glycerine	-	+	(+)	-
Mannitol	-	-	-	-
Ethylene glycol	-	-	-	-

+ = Growth

(+) = Weak growth

- = Absence of growth

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S. volutans and S. minus to be so distinctive that these organisms could be recognized on this basis alone. Giesberger suggested the transfer of some species to other genera, a recommendation which has been accepted for a number of these organisms by Bergey's Manual (7th ed., 1957). The details of Giesberger's reclassification of the species of Spirillum has been discussed elsewhere. (Williams and Rittenberg, 1957).

Giesberger (1936) was able to obtain very few cultures of species of Spirillum from other workers and laboratories. Two strains of S. tenue, isolated by den Dooren de Jong (1926), were obtained from the Laboratory of Microbiology in Delft and a culture of S. virginianum (Dimitroff, 1926) from the American Type Culture Collection. The physiological characters of these cultures, as originally described by de Jong and by Dimitroff, showed some changes and did not agree in all respects with those found by Giesberger (1936). That such changes occur in cultures of the bacteria maintained for long periods of time has been recognized by all who work with them. To prevent such changes from occurring in bacteria, it is customary to lyophilize stock cultures of many groups. As yet, it has not been possible to maintain viable lyophilized cultures of species of Spirillum by the customary methods. Dr. W.C. Haynes, ARS Culture Collection Investigation (Northern Regional Research Laboratories, U.S. Department of Agriculture, Peoria, Illinois) has been able to maintain lyophilized cultures of species of Spirillum by using bovine serum and reducing the period of desiccation (personal communication). Such lyophilized cultures (maintained for periods of time ranging from 2 to 15 years) are viable, and, in some instances, typical morphology has been maintained. In cultures maintained by routine transfer, "dissociation" is a common occurrence (Williams and Rittenberg, 1957). In view of the fact that such changes may and do occur in species of Spirillum, maintained as stock cultures, it is recommended that descriptions (where these have been adequately presented) be used in the identification of isolates of this genus until such time as reliable type or neotype cultures may be maintained.

Giesberger (1936), noting that differences occurred in the morphology of cells of a pure culture of species of Spirillum, discussed this problem as follows:

Before a bacteriologist starts on the description of the form of a given bacterium, he must realize that the bacterial cell is not to be considered as a static but rather as a more or less dynamic complex. As external conditions change, this complex may react with a change in cell form. . . .

Variation in cell form, occurring in an otherwise uniform culture, is likewise a point to which attention should be given, since, in the spirilla, especially, the spiral shape may be more pronounced in one cell than in another. One often finds rod-shaped and yet highly motile cells. In general, however, 80 to 90 per cent of the cells of the cultures isolated from water were completely uniform, so that a reliable basis for the description of cell form exists.

In order that such a reliable basis for the description of cell form might be established for species of Spirillum, Giesberger (1936) recommended that all measurements and descriptions of cell form be made under standard conditions. He used 24-hour cultures grown in peptone water at 30°C for this purpose.

Variation in cell form in species of Spirillum, while noted by Giesberger, were not described explicitly. Variation due to external conditions or to changes in the composition of the medium has been shown by van Niel (1944) in Rhodospirillum rubrum. Williams and Rittenberg (1957) noted minor differences in cell form of isolates of the same general morphological type even under standard conditions of the composition of the medium and cultures. In some instances, these differences were so slight that they could be considered to be the normal variation; in other cases, the differences were of such magnitude that the possibility that a subspecies or even a different species was involved had to be considered.

Forms intermediate between two distinct morphological types are not new and have plagued taxonomists in all fields. Bacteriologists, however, do not have the numerous so-called "nonessential" characters of higher forms nor ecological and sexual differences for use in deciding the status of such intermediate forms. The investigator, confronted

with the problem of correct taxonomic position of such intermediates could well echo Cohn's complaint (quoted earlier) about the difficulties involved in speciation in bacteria. As a rule, the decision as to the taxonomic position of such intermediate forms depends on the individual judgment of the taxonomist, the decision is a subjective one. The International Code of Nomenclature of the Bacteria and Viruses (1958) affords some assistance, particularly through recognition of subspecies and of infrasubspecific forms.

Variations in cell form within a species of Spirillum may be illustrated from one species. Among the many strains of the genus Spirillum isolated in the author's laboratory were many agreeing in morphology with Giesberger's S. serpens. Approximately 60 such cultures were obtained from natural sources; of these 18 were retained for further study on the basis of slight differences in morphology. When these isolates were tested by the use of several carbon compounds, it was found that the differences in morphology were also reflected in metabolic differences. None of the isolates agreed, in all details, with the description of S. serpens, as given by Giesberger (1936) which reads as follows:

"Spirillum serpens Giesberger

In peptone water highly motile, cells turns rather shallow; width of cells 0.8 to 1.0 μ ; length of turn 8.0 to 9.0 μ ; width of turn 1.5 to 1.8 μ .

Motile by means of bipolar flagella tufts. Volutin occurs as reserve material. Gram-negative. Catalase positive. Non-spore-forming. Rich growth on peptone agar. Colonies cream colored, typically folded. Liquefies gelatin slowly. Light orange-yellow growth on potato. Only acetic, n-butyric, succinic, lactic, malic, and pyroracemic (pyruvic) acids are suitable growth substrates. In addition to complex organic compounds, NH_4 compounds are utilized as nitrogen sources."

The dimensions of our laboratory isolates, while coming within the range cited by Giesberger for S. serpens, did

not agree exactly. None of the isolates liquefied gelatin and all of them utilized nitrate and urea as nitrogen sources, a characteristic not found by Giesberger for any of his isolates. A few of the organisms showed a transient growth on sugars and alcohols; some would not grow on unsupplemented peptone media; others showed a very restricted pattern in the utilization of the carbon compounds. The problem of which isolates were to be regarded as true representatives of the species S. serpens was resolved by selection of the strain which most nearly agreed in its characters with those given by Giesberger (1936) for S. serpens, designating this as a variety, and regarding the remaining cultures as infrasubspecific variants or forms. This procedure, however, did not provide a permanent solution to the problem of these apparently aberrant forms of S. serpens. Later other spiral organisms, morphologically similar to S. serpens, were isolated from widely varied geographical sources; none of the strains exactly fit Giesberger's description of S. serpens. Further, none of them are the same as the S. serpens var. azotum Williams and Rittenberg (1957).

The only other spiral organism, similar to S. serpens morphologically, is Vibrio rugula (Fig. 3, no. 16) described by Cohn (1872). Cohn distinguished V. rugula from V. serpens on the basis of fine cytoplasmic inclusions in V. rugula. He noted also differences in the character and number of spiral curves, differences in cell diameter, and the fact that in cells of V. rugula a distinct swelling may occur in the center. Migula's (1900) description of Spirillum rugula is essentially the same as that of Cohn's V. rugula in regard to the morphological characters except that he did not refer to a central swelling. The growth characteristics, given by Migula for S. rugula, are not sufficiently different from those given for S. serpens, to distinguish the two species. At the present time, S. rugula has been assigned to an appendix of the genus Spirillum Ehrenberg as being inadequately described. It is highly probable that this center swelling, described and illustrated for V. rugula by Cohn (1872), is the source of the mention of spore-formation in species of Spirillum by Migula (1900) and others. The swelling in the cells of V. rugula, as described and illustrated by Cohn is similar to microcyst formation by protuberances, described by Williams and Rittenberg (1956). Since no cultures of Vibrio rugula (Spirillum rugula) are

known to exist, it is not possible to determine if this species forms microcysts. Even if microcyst formation may occur in S. rugula, none of the morphological forms, grouped together as S. serpens, produce microcysts.

These aberrant forms (infrasubspecific forms, variant strains) were considered to be variants of Giesberger's S. serpens and his description was taken as a basis for fixing a "neotype" for this morphological type. Variations in the morphology of either Vibrio serpens or Spirillum serpens, as depicted by the various bacterial taxonomists discussed earlier, are shown in Fig. 11. With the exception of Müller's drawing, the morphology of all the organisms shown is similar. Organisms similar to the symmetrically curved organisms shown by Müller (1786) may be obtained by mixing certain of the variant strains in pairs in liquid media, allowing the organisms to fuse, and isolating them in symmetrical form. The only character mentioned by all individuals (Müller, 1786; Cohn, 1872; Migula, 1900; and Giesberger, 1936) for either V. or S. serpens is the apparent straightening of the cells during rapid motion. All the variant strains show this character which has been illustrated by Pijper et al. (1953) in cinematographic records of motility in this organism.

The answer to the problem of the variant strains of S. serpens is not solved by the creation of a large number of subspecies, differing only in minor details. A solution is provided, however, by Rule 8, Recommendation 8a of the International Code of Nomenclature of the Bacteria and Viruses (1958).

(2) Type is a term which has frequently been used to designate a subdivision of a species, particularly in cases where the distinguishing characters are regarded as insufficient to justify the erection of a subspecies. Types are often distinguished on the basis of antigenic characters. However, in view of the use of the word "type" in a different sense as defined in Principle 11 and Rule 9, it is recommended that the terms serotype (or serological type), biotype (or physiological type), phagotype (or phage type), and morphotype (or morphological type) be appropriately substituted for type as a designation of a subdivision of a species.

This recommendation proposes a method of naming or designating strains within a species showing differences in morphological characters. The significant term is "morphotype" which is defined as "a subdivision of a species distinguished by possession of some special or unusual morphological character." Note must be taken of the provision that for every species there should, wherever possible, be recognized a "nomenclatural type" as defined in Principle 11 and applied in Rule 9. Principle 11 states "A nomenclatural type is that constituent element of a taxon with which the name of the taxon is permanently associated." None of the earlier cultures of Spirillum serpens are known, hence no authentic type culture is available. Giesberger, however, reisolated an organism which he considered to be identical with his original isolate of this organism. This organism has been deposited with the American Type Culture Collection by Williams and Rittenberg (1957) and designated as the neotype culture for this species (ATCC No. 11130).

The variations in the morphology of S. serpens and its variant strains are shown in Fig. 12. Giesberger's S. serpens var. serpens, the variety S. serpens var. azotum (Williams and Rittenberg, 1957), and four morphotypes are shown. Such variation in both morphological and physiological characters are found in other species of Spirillum. A more detailed account will be presented elsewhere.

It is highly doubtful that the species, S. serpens, S. undula and S. tenue, as described by Giesberger (1936), are identical to the organism called by these names by earlier authors. Nevertheless these organisms are sufficiently well described and figured by Giesberger (1936) so that they can be reidentified with some confidence. The descriptions of these species by earlier authors, are inadequate, and about all that can be said in regard to an isolate is that it is "morphologically" similar to a particular drawing or photograph by Ehrenberg (1838), Cohn (1872) or Migula (1900). Some other problems connected with the taxonomy of Spirillum will be mentioned briefly. One such problem is that of the identification of the type species of the genus.

The only species described and named in Ehrenberg's (1830) first description of the genus Spirillum and S. volutans, which became the type species (selection by monotypy),

This designation of the type species was first indicated by Stiles (1905). This designation of Spirillum undula as type species by Vuillemin (1913) is incorrect because S. undula was not included at this time (1830). In 1838 Ehrenberg named a new species, S. tenue. He (1832) transferred to the genus Müller's V. undula as Spirillum undula and S. undula is incorrectly designated as the type species in Bergey's Manual, 1957.

Diagnoses of these species, however, have not remained uniform through the years. It is doubtful that the Vibrio undula of Müller (1773, 1786) is the same as the Spirillum undula of Ehrenberg (1838) or even of Cohn (1872). The description of S. undula as given by Giesberger (1936) is not the same as that by Migula (1900); as shown by a comparison of the photographs of S. undula by the two authors (Figs. 5 and 9). Nor is it the same (Fig. 9) as shown in the drawing of S. undula by Cohn (Fig. 3, no. 20). It is even doubtful that any two investigators of the genus Spirillum have given the name S. undula to identical organisms. Two subspecies of Spirillum undula were described by Kutscher (1895) and called S. undula majus and S. undula minus; the two organisms differed in size, type of spiral curves and origin. Giesberger (1936) believed his species to be the same as that described by Cohn (1872) and similar to Kutscher's S. undula minus.

Some difficulties arise as a result of the necessary acceptance of S. volutans as the type species due to the fact that it has never been obtained in pure culture, at least there is no such culture known. This objection has recently been removed by the isolation of this organism in pure culture, by Dr. S.C. Rittenberg (unpublished experiments) even though the satisfactory cultivation of the organism still presents some problems. There is little doubt that a suitable medium for the growth of S. volutans in pure culture will be found in the near future and all objections to this organism as the type species will be removed, making possible the recognition of a neotype culture. The history of the genus Spirillum Ehrenberg indicates that once a particular spiral organism has been shown to have a distinctive type of physiology, it is promptly removed to another genus, leaving only those spiral organisms whose chief physiological characteristics appear to be growth, provided the correct medium can be devised. The difficulties in the isolation

and cultivation of S. volutans indicated that this organism will have some distinctive physiological characters. In the event that the genus Spirillum is divided in the future, then the name of the genus, Spirillum Ehrenberg, would remain with Spirillum volutans.

The descriptions and number of species in the genus Spirillum Ehrenberg have not been changed from the 6th to the 7th editions of Bergey's Manual (1957). The diagnosis of the genus also remains the same. Recently Williams and Rittenberg (1957) have proposed a new diagnosis of the genus Spirillum to accommodate new species, isolated by them and described as having only a single flagellum. The key to the species of the genus contains all adequately described species with one exception, Spirillum lipoferum, described by Beijerinck (1926). This organism was omitted due to the recommendations of Giesberger (1936) who, following the procedures given by Beijerinck, isolated an organism which he (Giesberger) considered similar to S. lipoferum. Due to the fact that the organism which he isolated was only slightly curved and had only a single flagellum, Giesberger (1936) recommended that the organisms be placed in the genus Vibrio. Since Williams and Rittenberg (1957) emended the diagnosis of the genus Spirillum Ehrenberg to accommodate species showing only a single flagellum, the omission of S. lipoferum on this basis is unjustified and it should be included. The change in the diagnosis of the genus Spirillum to accommodate species showing only a single flagellum brings up a new problem; that of differentiating between species of the two genera, Vibrio and Spirillum.

The distinction made by Migula (1895, 1900) between the two genera on the basis of flagellation (one to three polar flagella for the genus Vibrio and tufts of flagella for the genus Spirillum) provided a definite criterion for the separation of the species of the two genera. The elimination of this criterion by Williams and Rittenberg (1957) makes it difficult to establish generic limits. The tendency of species of Spirillum to lose their typical spiral curvature, thus appearing as straight rod-like organisms, also contributes to the difficulty of distinguishing the genera Pseudomonas, Vibrio, and Spirillum. By substituting the genus Pseudomonas for the genus Bacterium, the complaint of Cohn (1872), quoted earlier, could be echoed for the difficulties in allocating the several species in the three genera. To

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paraphrase Cohn, there are definite rod-like, Gram-negative organisms, with a single polar flagellum which display fluorescence and have a number of physiological characteristics, which may be placed without hesitation in the genus Pseudomonas; there are other short, slightly curved or bean-shaped organisms, having only a short single polar flagellum, which may be placed in the genus Vibrio; there are also corkscrew-spiralled organisms with a tuft of polar flagella which can easily be placed in the genus Spirillum. Between the two limits of rod-shaped organisms with a long curved polar flagellum (the genus Pseudomonas) and corkscrew-spiralled organisms with tufts of polar flagella (the genus Spirillum) there are many intermediates. If only those organisms, characterized mainly by a lack of definite physiological characters were included, the similarity between these intermediate forms would be emphasized. In a recent article on Bacterial Classification, Sneath (1957), makes the following comment on the problem of intermediate forms:

"In higher organisms we also find intermediate forms, but this does not shake our faith in classification (that is to say degree of similarity). It is simply an expression of the fact that relationship is an infinite variable quantity. . . . There are 'points of rarity' in distributions of features where individuals with certain combinations of features are rare. . . . If there is no point of rarity, it is useless to make a division, since we say 'these cannot be separated'."

Sneath (1957) is also critical of "intuition" as a guide in the separation of species and genera of bacteria. Recently Shewan and Hodgkiss (1954) have proposed the use of certain "vibriostatic" agents as a means of differentiating species of Vibrio from those of Pseudomonas. It is possible that similar agents may be found which will differentiate species of Vibrio from those of Spirillum.

The difficulties of determining the type of flagellation in species of Spirillum by means of flagellar stain preparations has been discussed by Williams and Rittenberg (1957). If unfixed cells are used, many of the species show only a

single flagellum; if any type of fixation is used (including formalin and heat) tufts of flagella are often observed in the flagellar preparations on species which show only a single flagellum in preparations using unfixed cells. Even in flagellar stain preparations using fixed cells, organisms could be observed (in the same slide) which showed a single flagellum at both poles; one flagellum at one pole and two or more at the other pole; or cells with tufts of flagella. Flagella stains are either inadequate for determining the true state of flagellation in species of Spirillum or more than one type of flagellation may exist in cells of the same species. Electron microscope studies of flagellation have not been used for species of bacteria for purposes of taxonomy; most of such work has been done on the flagella of the bacteria in connection with the controversy over the function of flagella—whether they are the cause or the result of motility in bacteria. Some of the work done with the electron microscope on species of the Spirochaetales may have taxonomic significance but was not carried out primarily for this purpose. On the other hand, flagellar stains have been used for taxonomic purposes; i. e., as used by Leifson on speciation in Chromobacterium.

Electron micrographs of spiral organisms by various individuals indicate that real differences may exist in the type of flagellation to be found. Three main types have been shown in the flagella of these organisms: (1) true tufts of flagella, which appear to originate from the entire end of the cell, as shown by van Iterson (1947) and Houwink (1953) (2) small tufts arising from a circumscribed area at the end of the cell, as shown in photographs by van Iterson (1953) and Grace (1954); (3) a single flagellum in species of Vibrio in photographs by van Iterson (1953) and Grace (1954). A single flagellum has been shown by Pease (1956) for small cells of Spirillum undula, which she (Pease) has called "gonidia". These "gonidia" develop into larger spiral cells showing tufts of flagella. Until more data are obtained on the type of flagellation in species of both Vibrio and Spirillum, this particular character will have little significance in the taxonomy of these organisms.

The format and arrangement of the genera and species in the 7th edition of Bergey's Manual has undergone considerable change from that found in the 6th edition. The principal change has been an increase in the number of or-

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ders and the arrangement of the genera and species in these orders. The Gram-negative, polar flagellated organisms have all been placed in the order Pseudomonadales, which contains two suborders and eight families. Five of these families contain spiral organisms but the majority of the species are found in the family Spirillaceae, which has ten genera. A perusal of the keys to the genera made by Skerman (1957) [to be found in the back of Bergey's Manual (1947)] will show four other orders as well as an addendum to the Class Schizomycetes von Naegeli which contain spiral organisms. The majority of these spiral organisms occur in sheaths or in trichomes and are thus well differentiated from the free-living spiral cells found in the order Pseudomonadales. Any relationship between spiral cells which occur in trichomes or sheaths and the free-living spiral cells must await comparative studies of both types of organisms. It is entirely possible that the spiral shape is simply fortuitous.

SUMMARY

The following recommendations are made in regard to the identification and classification of species of the genus Spirillum Ehrenberg.

1. The diagnoses of the five species of Spirillum—S. serpens, S. undula, S. tenue, S. itersonii and S. kutscheri—should be emended to conform to those given by Giesberger (1936).

2. Neotype cultures of adequately described species of the genus Spirillum should be used as aids in the identification of isolates. In the event that the characteristics of the strain isolated do not agree, in all details, with those of the neotype, it may be designated and named as a morphotype with the distinguishing characters listed.

3. The distinctive and unique morphologies of the species of Spirillum should be the primary basis for species separation with physiological characters used where needed in the identification of the species of adequately described organisms.

4. The type species of the genus Spirillum Ehrenberg is Spirillum volutans Ehrenberg 1830, not S. undula Ehrenberg 1830.

The need for further investigations in connection with

all characters of species of Spirillum has been emphasized. Until more data are obtained, the differentiation between the various spiral bacteria, as found in Bergey's Manual (1957, 7th ed.) will serve, provided it is realized that much of this differentiation is based on incomplete and inadequate knowledge.

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PLATE V

Reproduction of the photographs of G. Giesberger's species of Spirillum from Beitrage zur Kenntnis der Gattung Spirillum Ehbg., 1936. By permission of Dr. G. Giesberger.

Figure 7

1. Spirillum serpens (Müller) Winter.
2. Spirillum serpens Flagella.
3. Spirillum serpens Volutin.

Figure 8

4. Spirillum serpens Colony on agar.
5. Spirillum itersonii Giesberger.
6. Spirillum itersonii Colony.

Figure 9

7. Spirillum tenue (Müller) Ehrenberg.
8. Spirillum undula (Müller) Ehrenberg.

Figure 10

9. Spirillum kutscheri Migula.
10. Spirillum kutscheri Migula.

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PLATE VI

Figure 11

Reproductions of various drawings and photographs of Vibrio serpens and Spirillum serpens.

- A. Vibrio serpens after Muller, 1786.
- B. Vibrio serpens after Cohn, 1872.
- C. Spirillum serpens after Migula, 1897.
- D. Spirillum serpens after Giesberger, 1936.

Figure 12

Flagellar stain photographs. (After Leifson)

- A. Spirillum serpens Giesberger, 1936.
S. serpens var. serpens Williams and Rittenberg,
1957.
- B. Spirillum serpens var. azotum Williams and Rittenberg, 1957.
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