THE GENUS SPIRILLUM EHBG. WITH SPECIAL REFERENCE TO CELL INCLUSIONS AND THE CHROMIDIAL THEORY

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In previous papers dealing with the structure and life history of *Bacillus mycoides*, *Azotobacter* and *Rhizobium*, the writer (1932, '34, '37, '38) has attempted to show that bacterial cell inclusions, especially fat bodies and volutin balls, have been a principal cause of error in interpretations of cell structure and methods of reproduction.

The present investigation on the genus *Spirillum* is concerned with the cell structure and, especially, with the part cell inclusions may have played in the origin and development of the chromidial theory of the nucleus. The literature contains many papers dealing with the structure of spiral bacteria which, because of their large size, have been favorite objects for cytological study. Since the literature concerning the finer structure of bacteria has been reviewed again and again, and since the several theories of cell and nuclear structure are well known, a further general review is not necessary.

Reviews by Arthur Meyer (1912), Dobell (1911), Guilliermond (1908), and Enderlein (1925) combine to include most of the literature from the earliest period of study through 1925. The more recent work is included in a bibliography by Lindegren (1935).

The theory that the bacterial nucleus consists of chromatin granules, chromidia, diffused throughout the cell plasm has had many advocates and is perhaps the most generally accepted theory at the present time. It is true, however, that this theory has received many adverse criticisms based on cytological evidence and theoretical considerations.

The writer has long believed that much of the support for the theory of a chromidial system is based on the study of species which are known to contain certain cell inclusions which might easily be confused with chromatin bodies.

Since the largest of all true bacteria occur in some species of *Spirillum*, and, since much of the support for the theory of diffused chromatin bodies has been obtained from a study of these forms, this group was chosen for a complete study of the problem.

MATERIAL AND METHODS

An attempt was made to obtain pure cultures of some of the large species from other laboratories and from the American Type Culture Collection, but no such cultures became available. *Spirillum rubrum* Esmarch and *Spirillum virginianum* Dimitroff were procured from the American Type Culture Collection. The cells in these species are rather small and not especially favorable for cytological study.

It is of interest to note that Giesberger (1936), commenting on the matter of cultivation and the lack of pure cultures wrote as follows: "Es will mir vorkommen, dass die Ursache davon zu suchen sei in dem Umstand, dass die Reinzüchtung von Spirillen noch immer eine ungenügend gelöste Aufgabe ist. Kennzeichnend für die unbefriedigende Lage ist wohl in genügenden Masse die Tatsache, dass eine vor wenigen Jahren angestellte Rundfrage an die bekanntesten bakteriologischen Laboratorien der Welt, Reinkulturen von *Spirillum*-Arten zur Verfügung zu stellen, einen fast negativen Erfolg hatte."

Since pure cultures could not be obtained, it became necessary to make original isolations or to proceed with the study from raw cultures in plant infusions. Isolation of pure cultures of spiral bacteria has generally been regarded as a rather difficult matter, although various workers, notably Kutscher (1895) and Giesberger (1936), were successful with relatively simple methods.

Raw cultures containing an abundance of spiral forms were readily obtained in plant infusions prepared from creek and pond waters. Hay or other plant material was added to some of the jars, while others contained only fresh water algae which soon

began to decay when brought into the laboratory and placed in darkness at room temperature. The incubation period required for development of raw cultures rich in spiral forms was found to vary somewhat in the several cultures from different waters. In the early stages of decomposition, spiral bacteria were rarely seen, but as the cultures aged, they became more and more abundant until, at times, the surface pellicle was literally a swarming mass of spiral cells. This condition was generally reached in a period of about one to three weeks. Microscopic examination showed that several distinct types of spiral bacteria were present.

Isolation of pure cultures of some species was found to offer no difficulties. This was the case with *Spirillum serpens* (Müller) Winter, *Spirillum undula* (Müller) Ehrenberg, *Spirillum tenui* (Müller) Ehbg. and a small form identified tentatively as *Spirillum itersonii* Giesberger. All of these species grow well on plain beef-extract peptone agar. In plates streaked with a loopful of inoculum from raw cultures, the colonies can be recognized after 24 hours incubation at room temperature. In some cases, isolation on agar slants was carried out from the 24 hour plates, but more generally after incubation for 36 to 48 hours.

While the isolation of the species listed above was accomplished by the simple routine method just described, it was found impossible to isolate pure cultures of *Spirillum volutans* Ehbg. by any of several methods employed. Even in raw cultures this species occurred but rarely and appears to be a relatively rare organism or one in which conditions for development are more exacting and not always suitable in infusions. It appeared in abundance in but two of the many infusions prepared. One of these was of decaying algae from a pool while the other was a hay infusion prepared from creek water. After three weeks the giant spiral cells were present in great abundance in the surface film of these two cultures which contained also *S. undula* and *S. serpens*. Photographs shown in figures 1, 3, 4 and 5 were prepared from the raw cultures. The organisms continued in vigorous growth for about 10 days when they began to diminish in numbers and eventually disappeared.

Isolation of pure cultures was attempted by means of streaked plates and poured dilution plates. The plating media included beef-extract peptone agar, infusion agar prepared according to Zettnow (1897), Vogt's (1899) pea infusion agar, and tryptose agar. Semi-solid agar was employed in the poured dilution plates. When a loopful of the material containing a great many cells was streaked out on agar and examined microscopically for evidence of growth and multiplication, the large spiral cells could be easily seen lying on the agar surface, but there was never any indication of growth or cell division. Colonies of *S. volutans* Ehbg. were not found in any of the poured dilution plates although other species of *Spirillum* were isolated by this method.

CYTOLOGICAL OBSERVATIONS

Since *S. volutans* Ehbg. is the largest of all true spirilla and a favorable subject for cytological study, my observations on this species will be recorded first. The raw cultures described above furnished an abundance of material and served the purpose almost as well as if pure.

In living unstained preparations, viewed either by bright or dark field illumination, the cell body presents a most striking appearance. The most notable features of the living cells are the enormous size, rapid, flashing rate of motility, and the more or less numerous granules contained in the cell plasm. The granules appear as highly refractive or as dark bodies depending on depth of focus. The cytoplasm is hyaline without any indication of pigment. The granules, as well as the bipolar flagella, are readily seen by dark-field illumination or in films prepared with nigrosin or by Benian's congo red method.

The internal structure is brought out clearly by vital staining methods. When mounted in dilute solutions of methylene blue, the organisms retain motility for some time after the stain has begun to act. As staining proceeds, some granules stain deeply while others are non-stainable. The cytoplasm stains much less intensely than the granules. The stainable granules are volutin, because they become red in staining, are not readily de-stained with dilute sulphuric acid, and dissolve quickly in

hot water. It was noted that volutin is more abundant in some cells than in others. In very young cells the content is less granular and stains more uniformly.

Non-stainable bodies were present in great abundance (figs. 1, 3, 4 and 5). These resisted the action not only of methylene blue but of other anilin dyes including fuchsin, safranin, and crystal violet. However, when treated by fat-staining methods the reaction was positive in all of the methods employed. The bodies were stained with Sudan IV, indophenol blue (Dietrich and Liebermeister method), and by the several methods of Eisenberg (1909). In addition to these well known methods for staining fat bodies in bacteria, the new method of Hartmann (1940) was tested and found highly satisfactory. The new compound, Sudan Black B, stains fat quickly and intensely. The method has certain advantages over the older methods since most of the trouble from precipitates is eliminated, and the slow evaporation rate of the solvent removes the necessity for sealing.

As may be seen from the accompanying photographs of fixed stained films, fat bodies are not always arranged in the same manner in different cells or even in the same cell. Two of the individuals in figure 1 show a single axial row while all the others contain additional bodies which constitute in part a second row. This mode of dispersal is seen best in figures 3 and 5. In figure 5 the cell to the right shows an arrangement which is frequently seen. There are two distinct rows, each lying contiguous to the outer membrane, and so arranged that the individuals in one row alternate with others in the opposite row, thus forming a spiral. The peripheral cytoplasm appears as regular blocks between the fat bodies while the remaining cytoplasm is compressed into thin plates which take the form of a spiral thread. The arrangement is not always perfect, nor does it generally occur throughout the full length of any one cell. Further reference will be made to this arrangement of fat bodies and cytoplasm in a later paragraph concerning the so-called spiral nuclear filament described in other species by some writers.

For the preparation of fixed stained films, suspensions in water were exposed for about two minutes to fumes of osmic acid which

kills the cells quickly. Films were then prepared and air-dried in the usual manner without heating. The air-dried films were then covered with 95 per cent ethyl alcohol which was allowed to act about 5 minutes, after which the films were redried. This method caused very little or no distortion.

Various staining methods were employed for differentiation. Stained by Gram's method, the cells gave up the crystal violet quickly and except for the non-stainable fat bodies took the red color with safranin as the counter stain. Although generally reported as gram-positive, the reaction was found to be negative. Control films of known gram-positive species when mixed in the same film with the spiral cells were strongly positive. There is, therefore, no hesitation in pronouncing this a gram-negative species. The same was found true for all other species of *Spirillum*.

Fixed films stained with Loeffler's methylene blue gave the same differentiation obtained with this dye by vital methods, namely, red-violet volutin balls, colorless fat bodies, and blue cytoplasm. Some films were overstained with methylene blue and then treated with Lugol's iodine solution followed by Vesuvin (figs. 4 and 5). Volutin and cytoplasm were black, while fat bodies remained colorless. The photograph shown in figure 3 was made from a film stained with methylene blue followed by safranin. The volutin balls were blue-violet, cytoplasm red, and fat bodies unstained. The cells in figure 1 were stained by the iron alum haematoxylin method. Volutin and cytoplasm were black, while fat bodies were unstained.

As implied in the descriptions above, none of the formed bodies in the cell could be regarded as chromidia nor as true vesicular nuclei. It appears that, if these large cells contain differentiated chromatin, the substance must be so finely divided and dispersed in the plasm that individual particles are invisible.

Other species of *Spirillum* including *S. undula* (Müller) Ehrenberg, *S. serpens* (Müller) Winters, *S. tenue* (Müller) Ehrenberg, *S. itersonii* Giesberger and *S. virginianum* Dimitroff were examined by the methods described above except that pure cultures were employed. Preservation of these cultures has been insured

by depositing them in the American Type Culture Collection. It may be noted here that pure cultures of the several species have been found quite useful in teaching bacteriology. They are especially suitable for flagella staining, the study of volutin and fat bodies, the rigid nature of the cell wall, and motility.

S. undula is similar to *S. volutans* but on a smaller scale. The typical spiral form is seen best when cultivation is carried out in liquid media. However, for the study of cell structure, agar cultures are preferable because there is less trouble from confusing artifacts. Cells from young cultures contain few or no granular bodies. The content stains uniformly with any of the usual anilin dyes. As the culture ages, characteristic unequal staining occurs, and the appearance which has been so frequently described as banded, barred, vacuolated, beaded, or alveolar is clearly shown. The cells in figure 2 were taken from a 72-hour agar slant culture, killed with fumes of osmic acid, fixed with alcohol, and stained with methylene blue, Lugol's iodine solution, and Vesuvin.

The volutin balls and cytoplasm stained by this method are deep black, while fat bodies remain colorless. It may be seen that some fat bodies equal the diameter of the cell body while others are relatively small. There is also a tendency toward arrangement in a spiral form and for the cytoplasm to appear as zig-zag lines and irregular masses. Since volutin balls are embedded in the cytoplasm, it sometimes happens that the latter is almost completely obscured by the more deeply stained volutin. That the clear gaps are actually due to fat bodies is easily proved by fat staining methods. Volutin was identified by the usual well known methods. The plasm between fat bodies appears to be a substance which is uniform in structure and in staining reaction. It must consist, either of cytoplasm alone, chromatin alone or a finely dispersed mixture of both.

S. serpens (Müller) Winters was found to be the most common species developing in raw cultures as well as the most easily isolated and identified. It grows vigorously on plain broth and agar. Two types of colonies occur on plating from old broth cultures. The typical colony form is striated and could be classed

as the rough type. Smooth colonies show no markings; they are smaller and more typically dome-shaped than rough colonies.

Cytologically the pattern simulates that of *S. volutans* and *S. undula*. Fat bodies are not abundant until the cultures have grown 24 hours or longer. Volutin is present in most cells within 18–24 hours when cultivated on plain beef-extract peptone agar plus 0.05 per cent potassium acid phosphate. It is true, however, that the number of volutin grains is quite variable in different cells. In the absence of fat and volutin, the plasm stains uniformly, although less densely than that of *S. undula* and *S. volutans* (fig. 6).

S. tenue, *S. itersonii* and *S. virginianum* are less satisfactory for cytological study because the cells are small. Each deposits both fat and volutin. The structure of *S. volutans* Ehbg. is typical for all the species studied, and because of the large size is more easily seen.

DISCUSSION OF CYTOLOGICAL OBSERVATIONS

The only previous cytological study of the true *S. volutans* Ehbg. was made by Bütschli (1902). He described the cell plasm as a honey-comb or alveolar structure differentiated into a peripheral layer and a more dense "central body." Because the central portion contained granular bodies which stained red with haematoxylin, he regarded this portion as homologous with a true nucleus. In view of the structure reported above and illustrated in the accompanying photographs, it seems obvious that Bütschli's red-staining granules were volutin; the alveolar structure was due to fat bodies; and that there is no differentiation into "central body" and peripheral cytoplasm.

Zettnow (1891, 1897, 1899) studied several species of bacteria including *S. undula* and *S. giganteum*. He confirmed the structure described by Bütschli and introduced the terms *ectoplasm* and *endoplasm*. He believed that bacteria consist principally, or in some cases entirely, of nuclear substance. After a long lapse of time, Zettnow (1918), returned to the subject to present new evidence, and to revise his former conclusions. All of his papers are well illustrated with excellent reproductions of photo-

graphs. In his final paper he concludes that the structures which he formerly designated as chromatin bodies were nothing but grains of volutin, and that chromatin granules, if present, must be so finely dispersed as to be invisible. Rejecting the true nuclei described by Meyer (1912) and others, he regards the theory of finely dispersed chromatin and the theory of a true nucleus too small to be seen as the only possibilities supported by cytological evidence.

Several workers reported on the structure of *S. giganteum* (Kutscher) Migula, and supported the chromidial theory in some manner. Swellengrebel (1907) described spiral or zig-zag "chromatin filaments" and grains of volutin distributed through alveolar, honey-combed cytoplasm. Hoelling (1911) denied the chromatin filaments reported by Swellengrebel, recognized volutin bodies as such, and described the cytoplasm as alveolar. Guilliermond (1908) described the cytoplasm as net-like, recognized volutin as such, and believed that chromidia were also present.

Nakanishi (1901), who reported true vesicular nuclei in many species of bacteria, studied *S. giganteum* and *S. serpens*. He recognized non-stainable granules and regarded them as the cause of the honey-comb structure; noted the presence of stainable granules in both species; failed to identify either type of granule, and regarded them as vacuoles filled with reserve substance. He could find no nucleus in *S. giganteum* but reported a nuclear thread divided into several pieces in *S. serpens*.

Dobell (1911) described nuclei in some unidentified species of *Spirillum* from intestinal contents of frogs and lizards. He thought that the nucleus could occur as a spherical body, several scattered granules, or as a chromatin thread depending on different stages of development. Two of his figures resemble *S. undula* and *S. itersonii*. His position is weakened by the fact that he made no tests to distinguish the so-called chromatin bodies and filaments from volutin, and it was impossible to distinguish sequence of development in the material he examined.

Dimitroff (1926) reported the structure of *S. virginianum* as follows: "Stained specimens show granular (barred) structure

and chromatin spirals in the cytoplasm." He seems not to have recognized cell inclusions, although both fat and volutin are deposited.

Evidence from these studies on spiral bacteria shows that no worker has supported the theory of a definite vesicular nucleus. Bütschli alone advocated the notion of a chromatin containing "central body" homologous in structure with a true nucleus. Zettnow in his early studies confirmed the work of Bütschli but eventually favored the idea of dispersed invisible granules of chromatin. All workers failed to recognize the part played by fat bodies in the so-called honey-comb structure, and with the exception of Swellengrebel, Hoelling, and Guilliermond, confused volutin with chromatin.

A diffuse or chromidial nucleus has been described for many bacillary species as well as for spiral bacteria. There seems no reason to doubt that most if not all of the so-called chromidia were volutin balls, or compressed cytoplasm conditioned by non-stainable fat bodies as shown above.

More recently investigators have sought to prove the presence of thymonucleic acid in bacterial cells by means of Feulgen's reaction. Some workers have reported stainable discrete particles; others saw only diffuse staining of the protoplasm, while still others reported the reaction as negative. The literature has been reviewed by Stille (1937).

CHROMIDIA AS A MECHANISM OF HEREDITY

The theory that chromatin granules variously dispersed in the plasm of bacterial cells perform the functions of a true nucleus as been accepted by many bacteriologists. It has been criticized by others who have maintained that the cytological evidence is at fault, and that the theory fails to provide a mechanism for an orderly transmission of hereditary units.

The advocates of a diffuse chromatin nucleus have held a chemical rather than a morphological conception of the nucleus. They have been satisfied to prove that the bacterial cell contains "nuclear material" in the form of particles of "chromatin" variously dispersed in the cytoplasm. It is obvious that much

of the cytological evidence for a diffuse nucleus is erroneous, and there seems to have been no very clear conception as to the precise manner in which diffused chromidia could function as a nucleus.

The true nucleus has long been known as a morphological unit which behaves in an orderly and uniform manner. It is an entity and a mechanism which performs definite functions, and its manner of functioning is well known. Although all true nuclei contain chromatin, it is now believed that this substance is inert. According to modern theories of cytology and genetics, the genes, not the chromatin, are the essential hereditary material.

The gene material is embedded in chromatin to form chromosomes, which in turn are housed in the nucleus. It is known that individual genes are connected together within a chromosome in a definite linear order, the gene string, and that any alteration in *position* of a given gene results in mutation. Division of chromosomes, nucleus, and cell occurs in such an orderly synchronized manner that each daughter cell receives its full complement of hereditary material.

Application of the conception of gene strings to bacteria has been made by Lindegren (1935) who appears to have pointed to a logical way out of the difficulty. According to his conclusions, the nucleus of bacteria might well enough consist of a single gene string with or without the usual encrustation of chromatin. A naked gene string, although wholly invisible, could perform all the functions of a true nucleus. If the gene string should become encased in chromatin, it might appear as a definite visible body. The smallness of bacteria and transverse division of the cell are not objections. Lindegren has shown, by diagrams drawn to scale, that a space 0.2μ in diameter is sufficient to accommodate a gene string of maximum theoretical size, and provide ample room for transmission of genes to the daughter cells in an orderly, definite manner.

This theory of the bacterial nucleus places emphasis on morphology and behavior but does not require that the nucleus must exist as a visible structure. It is in harmony with the view

long held by bacteriologists, that the bacterial cell must contain some functional equivalent of a true nucleus, but it is opposed to the theory of a diffuse nucleus since the gene string does not exist in a diffused form.

In the light of what we know concerning the cytology of bacteria and the laws of genetics, there seems to be no very good reasons to believe that the bacterial nucleus consists of diffused particles.

SUMMARY AND CONCLUSIONS

Various species of the genus *Spirillum* were isolated from plant infusions by simple plating methods. It was found impossible to isolate *Spirillum volutans* Ehbg. by any of several methods employed. Review of the literature affords convincing evidence that this species has not yet been cultivated in pure culture.

All the species investigated deposit fat bodies and volutin. The alveolar or honey-comb structure frequently described by previous workers is conditioned by non-stainable fat bodies. Chromidia and spiral nuclear filaments described by other workers are regarded as volutin and stained cytoplasm. Whether the plasm contains minute invisible particles of chromatin was not determined.

The theory that the bacterial nucleus may consist of a naked gene string, or a chromatin-encrusted gene string (chromosome) is preferable to the theory of a diffuse chromatin nucleus.

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PLATE I

FIG. 1. Cells of *S. volutans* Ehbg. from raw culture. The cells were killed by fumes of osmic acid, fixed with alcohol and stained with iron alum haematoxylin. The clear areas are fat bodies; cytoplasm and volutin balls are stained black. ×2250

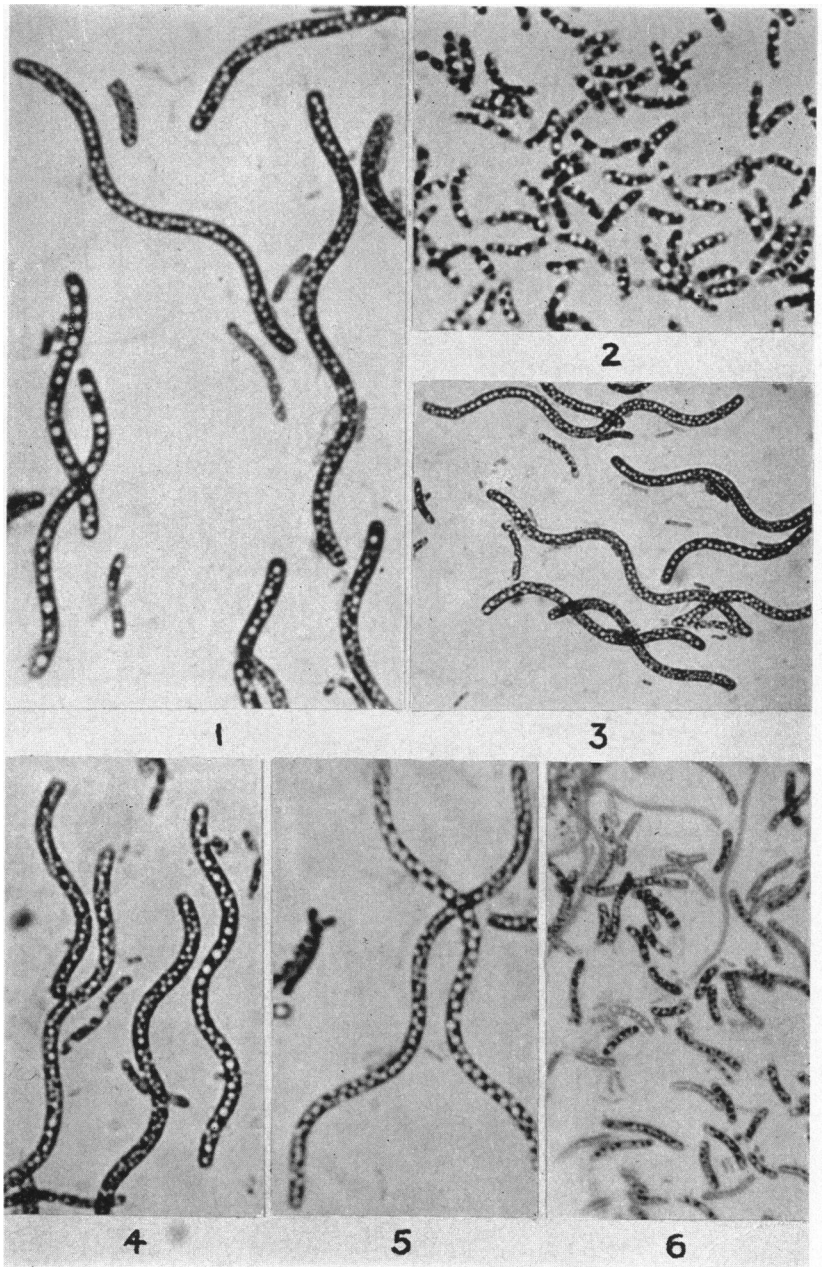
FIG. 2. Cells from pure culture of *S. undula* (Müller) Ehrenberg grown on beef extract peptone agar 72 hours, killed and fixed as above and stained with Loeffler's methylene blue, Lugol's iodine and vesuvin. Cytoplasm and volutin balls are stained black; fat bodies are not stained. ×2250

FIG. 3. *S. volutans* Ehbg. as in figure 1. Stained with Loeffler's methylene blue and safranin. Numerous unstained fat bodies throughout the cytoplasm and a few volutin balls are shown. There is no indication of a "central body." ×1500

FIG. 4. *S. volutans* Ehbg. Cells treated as in figure 1. Stained as in figure 2. Axial unstained fat bodies, volutin balls and cytoplasm are shown. ×2250

FIG. 5. *S. volutans* Ehbg. As in figure 4. The fat bodies are arranged in form of a spiral. The cytoplasm compressed by fat bodies appears as a nodose spiral thread. ×2250

FIG. 6. *S. serpens* (Müller) Winter. Cells treated as in figure 1, stained as in figure 2. Volutin balls, fat bodies and cytoplasm are shown. ×1500



(I. M. Lewis: The Genus Spirillum Ehbg.)