The Life-history of *Bacillus hirtus*.

(Synonyms *Bacterium hirtum*, Henrici ; *Pseudomonas hirtum*, Ellis.)

BY

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With Plate XVI.

INTRODUCTION.

THIS investigation was commenced on the assumption that *Bacillus hirtus* belonged to the genus *Pseudomonas*, because in a former work which I published (1) I found that the ciliation was polar (vide Migula's classification, 11, p. 46). Since then, however, I found that under cultivation, this species became more motile, and when fresh cilia preparations were made the ciliation became peritrich. This was somewhat disappointing because, so far as I am aware, the life-history of the genus *Pseudomonas*, from spore-formation to spore-formation in the next generation, has not been completely investigated. I carried on the work, however, because the only other investigator of this species, Henrici (7), has not given us a full account of its characteristics, and its developmental history is entirely wanting. It was placed by him in the genus *Bacterium* under the name *Bacterium hirtum*, because it appeared to him to be perfectly immotile. But as the following results will show, its motility under cultivation is as great as that of any *Bacillus*, and as its cilia are arranged all round the cell, it belongs to the genus *Bacillus*. As in my earlier investigation of this species (1) cilia were found only at the poles, the question still remains unsolved as to whether under cultivation a cylindrical form of cell may not change its ciliation from polar, to peritrich. The increase in its motility may be caused by that very fact. I propose relegating the solution of this problem to a later investigation. It is important to consider this question for, if true, it would mean that the two genera *Bacillus* and *Pseudomonas* would have to be merged into one genus embracing all the forms at present classed under the two genera. I do not know of a single species at present classed under the genus *Pseudomonas* which has been so long cultivated and in which cilia preparations have been so extensively made, that it can be stated with certainty that its ciliation is polar. I have already shown (2) that non-motile cylindrical forms of Bacteria under cultivation become

motile, and I think it not improbable that this distinction between *Bacillus* and *Pseudomonas* will, on further investigation, also prove to be non-existent. Against this probability may be set the fact that all spiral forms of Bacteria are undoubtedly polar in their ciliation, and I have satisfied myself in the case of *Spirillum volutans* (*Spirillum giganteum*?) that increased motility on cultivation does not make its ciliation other than polar. It must, however, be remembered that in this genus, the body of the cell continually undulates when it moves, and is not stiff as stated by Migula (11, p. 47), so that this may render the formation of cilia at any place other than the poles impracticable. I may say that, in this investigation, I have taken every precaution to ensure that the form I have investigated was that discovered by Henrici (7) and named by him *Bacterium hirtum*.

THE SPORES.

The method of examination was as follows. The material was placed in a drop of water, mounted on a coverslip as a very thin layer, and after it had dried, passed through the flame somewhat rapidly in order to fix the spores on to the glass. At the same time, by comparison with unfixed spores mounted in water, care was taken that the size was not materially altered by this process. The dried material was then subjected to the action of stains. In order to ascertain the size of the spores, treatment with stains is necessary, as otherwise the exine cannot be determined with certainty. It is seen at once that the spores are somewhat larger than the average. As the size of the spores of any particular species varies within comparatively narrow limits this should be taken into account in the diagnosis of a species. For *Bacillus hirtus*, the length varies from $1.5\ \mu$ to $2.5\ \mu$, and the breadth from $1.0\ \mu$ to $1.8\ \mu$.

The size of the spores as compared with that of other species can be seen from the following table, taken from the accurate investigations of Gotheil (6).

	Length.	Breadth.
<i>Bacillus ruminatus</i> . . .	$1.5-1.7\ \mu$	$0.8-1.0\ \mu$
„ <i>tumescens</i> . . .	$2.5-3.0\ \mu$	$1.7-2.0\ \mu$
„ <i>graveolens</i> . . .	$1.9-2.5\ \mu$	$1.39-1.7\ \mu$
„ <i>Petasites</i> . . .	$1.7-2.2\ \mu$	$0.83-1.11\ \mu$
„ <i>ellenbachensis</i> . . .	$1.7-2.2\ \mu$	$0.83\ \mu$
„ <i>mycoides</i> . . .	$1.4-2.2\ \mu$	$0.83\ \mu$
„ <i>subtilis</i> . . .	$1.7-1.9\ \mu$	$0.83-0.94\ \mu$
„ <i>pumilis</i> . . .	$0.94-1.52\ \mu$	$0.55\ \mu$
„ <i>simplex</i> . . .	$1.39-1.7\ \mu$	$0.83\ \mu$
„ <i>cohaerens</i> . . .	$1.7-2.2\ \mu$	$0.83-1\ \mu$
„ <i>carotarum</i> . . .	$1.31-2.2\ \mu$	$1\ \mu$
„ <i>fusiformis</i> . . .	diameter, $1.3-1.8\ \mu$	

It will be seen that the size of the spore can only be utilized for diagnostic purposes, when it approaches either the maximum or the minimum given in the above table. As the length of the spores of *Bacillus hirtus* is $1.5-2.5\ \mu$ and the breadth $1.0-1.8\ \mu$, they rank as very large spores, being surpassed in the above list only by *Bacillus tumescens*.

With regard to the appearance of the spores, this can only be satisfactorily examined by staining, as without any treatment we see nothing except a very strongly refractive oval mass. Fig. 1 shows the spores treated with an Iodine stain. The intine is very clearly marked as a brown peripheral layer forming the boundary of the very strongly refractive contents. The exine, by this method of staining, is somewhat difficult to see, usually only a slight shimmer, radiating on all sides from the spore, is seen. By allowing the stain to act for a somewhat longer period, it becomes visible at last as a faint line surrounding the intine. The intine can be seen more clearly by slightly raising the tube above the focus-point. When the adjustment is very slightly below the focus-point, the whole spore is coloured uniformly brown. The best stains for this purpose are Methylene-blue or Fuchsin. Stained with Methylene-blue (1 c.c. alcoholic sat. solution of Methylene-blue to 40 c.c. H_2O) the spores are more rapidly and more distinctly coloured, and it is not difficult to investigate the structure of the exine. It is seen to be a slightly irregular coat, which at both poles has a somewhat blunt point (Fig. 2). The intine is more distinct, and can be seen still more clearly by very slightly raising the tube above the focus. When the stain has acted for some time the spore becomes permeated throughout. In this condition the intine and its contents contract somewhat, probably due to the effect of the alcohol used in the making of the stain. This brings into prominence the structure of the exine, which does not take part in the contraction (Fig. 3). This stain is to be recommended as the best for this purpose. When stained with Fuchsin (2 c.c. sat. alc. solution, 10 c.c. abs. alc. 10 c.c. H_2O), it was found that after about ten minutes, the exine was sharply brought into prominence. The intine, however, was only seen as a dark line, limiting the strongly refractive contents, but was not coloured. With this stain, as with Methylene-blue, the bluntly pointed ends of the exine are plainly visible (Fig. 4). In Fig. 5 is shown a spore much more highly magnified, which illustrates more clearly its structure when treated with reagents. The spore is thus seen to be an oval structure, with two coats, the inner, delicate and closely wrapped round the contents, the outer a looser somewhat irregular band, to the presence of which is due the resistance of the spore to heat and other injurious influences. When seen 'end on' the spore appears round (Fig. 6). In some cases, especially in unstained preparations that have not been dried on the coverslip, the spores are sometimes seen obliquely. In such cases the spore varies in shape between the circular and

the oblong, but as all parts are not in focus, it appears somewhat distorted. The structure of the spore is therefore normal, and the occurrence of the bluntly pointed ends is common enough to make it not of much value for purposes of diagnosis.

GERMINATION OF THE SPORE.

To observe the germination, the spore-material was sown very thickly over the surface of the Agar in an Agar-tube, which was then placed in an incubator (32° C.) and examined at various times. Even after three hours some spores had germinated, but the most favourable period for their examination is after 6–7 hours, when a very large number of germinated spores can be observed. This facilitates the task of observing the various stages in the germination. As in all observed cases, the first sign of germination is a swelling up of the whole spore, doubtless due to the absorption of water, with a consequent loss of its very strong refractive power. By observation of Figs. 7–9, it will be seen that the vegetative cell issues from the equator of the spore. I could not observe a single case of polar germination, and believe that equatorial germination is constant for this species. When observed after three hours, in several cases cell-division had already taken place (Figs. 8–9), but in no case were any vegetative cells present, unattended by the spore-membrane. The stages of germination can be seen by comparing Figs. 7–9. First a swelling of the spore, then very soon afterwards a splitting of the exine at the equator take place. Next the young vegetative cell protrudes through the split, and in many cases cell-division takes place (Fig. 8) when the young cell is still attached to the spore-membrane. To observe the further development, the culture was examined after five hours, when a comparatively thick though transparent layer is seen on the surface of the Agar. Cell-division has now proceeded much further in the advanced stages, the spore-membrane has been discarded, and examples similar to those represented in Fig. 10 are comparatively common. In this culture these threads of two-celled individuals were almost exclusively present (an *individual* is distinguished from a *cell* in that no account is taken of any transverse cell-walls that may be present, regard is paid merely to the entity, thus Fig. 9 is an *individual* consisting of two *cells*, Fig. 15 *a* is an *individual* consisting of four *cells*). Even at this stage there is a trembling movement which infers the possibility of movement apart from molecular movement. This subject is discussed more fully under the heading 'Motility.' It is sufficient to mention here that as there are indications of motility even at this early stage, the young individuals soon free themselves from the trammels of the spore-membrane and life in the next generation has begun.

In connexion with the germination of the spores, it would be interesting to know the fate of the intine. Is it cast off with the exine or does

it function as the wall of the newly formed vegetative cell? I have never been able to see any traces of it in germination, and as it forms such a compact covering to the contents of the spore, I am inclined to think that it envelops the young vegetative cell, when it leaves the spore.

GELATINE-PLATE CULTURES.

The appearance on gelatine-plates has been very well described by Henrici (7). A synopsis of this given in Migula's *System der Bakterien*, Vol. II, is as follows:—'Auf Gelatineplatten sind die Kolonien schon nach 15 Stunden sichtbar: die eingeschlossenen werden zu kleinen, weissen Körnern: die oberflächlichen vergrössern sich zu kreisrunden, flächig angeordneten Scheiben: in der Mitte ist ein dichter, weisser Kern, um diesen eine blassere Zone, dann kommt ein milchweisser Ring, dann eine wasserhelle Zone, und zum Schluss eine trübe Randzone: diese Scheibe, die um 3 Tage einen Durchmesser von 4 mm. besitzt, ist von einem Verflüssigungsringe umgeben. Bei 80 facher Vergrösserung bilden die eingeschlossenen Kolonien unregelmässig runde graue Scheiben, die durch abwechselnd hellere und dunklere Stellen ein fein zerklüftetes Aussehen erhalten; allmählich werden sie dunkel und undurchsichtig und der anfangs glatte und scharfe Rand bildet ein Gewirr kurzer Fäden. Die oberflächlichen Kolonien erscheinen zunächst als unregelmässig geformte, graue Scheiben, die von verworrenen dunklen Linien durchzogen sind; vom Rande laufen breitere oder schmälere, bald längere, bald kürzere, zickzackförmige, blitzähnliche Auswüchse aus; allmählich wird das Bild regelmässiger; entsprechend der schon mit freiem Auge wahrnehmbaren zonigen Anordnung sieht man eine kreisrunde, graue, aus einem bald dichteren, bald lockeren Fadengewirre bestehende Scheibe, von deren Rande ein Gewirr kurzer Fäden borstig absteht. Die am Boden flach ausgebreiteten Kolonien zeigen ein den oberflächlichen ähnliches Bild.' This description agrees in the main with the appearances presented by this form, but as the growth on gelatine-plates is notoriously inconstant, and as in the identification of species much stress is laid on growth on gelatine, we have to be certain that the characters that are laid down as distinguishing marks are really constant. Thus the exact composition of the gelatine, its mode of preparation, and more especially the amount of water present influence the appearance of colonies to a very considerable degree, and it is probable that this inconstancy in the shape and form of these colonies is the reason why there is such an apparent multitude of species. Over a thousand species have been described belonging to the genus *Bacillus* alone. It seems to me that a somewhat more elaborate and painstaking investigation would considerably reduce the number, as a very widely distributed

organism like *Bacillus subtilis* must have appeared, I have no doubt, under a dozen different names.

In all cases, growth on gelatine-plates begins to be apparent by the appearance of minute greyish-white specks, both on the surface of the gelatine as well as beneath the surface. The latter colonies do not proceed much further. The superficial colonies, however, are round, have sharp contours, and each colony appears denser in the middle than at the sides, because owing to abundant growth the middle part is raised more above the surface than is the peripheral growth. It often results in the appearance of a 'nucleus' at the centre of a colony. Again, as a peptonizing ferment is present, the appearance of the edge of the colony begins to alter, and as described by Henrici, the beginning of liquefaction is the cause of the various strata which the colonies exhibit, the one furthest from the centre being of course the most transparent. Again, the colonies have at first sharply defined edges, but later become irregular and often exhibit 'blitzähnliche Auswüchse.' It must not be expected that these outgrowths invariably occur, and as a matter of fact in about 50% of the gelatine-plates they are absent. It depends on the constitution, density, dryness, &c., of the gelatine whether they occur or not. Again it must be borne in mind that many other Bacteria possess the same characteristics. Another point which should be noticed is that the relation between time and the size of the colonies is by no means constant. The growth on Henrici's gelatine-plates was extremely rapid: in all those which I made I did not obtain a quarter of the growth mentioned by him, though ultimately as large and larger growth was obtained when the colonies were not too crowded. The following results show the relation between size and time in a few instances. They were not all made at the same time of year, hence as the temperatures were necessarily different, another factor is introduced. It suffices to show that for any given form no constancy in this respect can be expected.

- I. Minute specks after eight days, which after fourteen days showed a diameter of 1.5–2.0 mm.
- II. Minute grey colonies after three days, which became very thick and granulated and completely liquefied the gelatine in thirteen days.
- III. Minute grey colonies after two days. Gelatine completely liquefied after nine days. In this experiment the plates were placed on top of an incubator (32°C.), and were therefore slightly warmer than the surrounding atmosphere.

Other experiments, not recorded, showed the same variations in the growth and liquefaction of the gelatine.

The following are not to be depended upon, in diagnosing a species by means of gelatine-plates.

1. Any relation between size of colonies and time of growth.
2. Any relation between time and liquefaction of the gelatine.
3. Any diagnostic characters based on irregularity of growth of the colonies.

With regard to No. 3, the round or oval form of the colony is often departed from, and even in the same plate there is often considerable diversity of structure. The chief external factors which influence the growth on the gelatine are the following :—

1. Temperature.
2. Crowding or otherwise of the colonies.
3. Percentage of water in the nutrient gelatine.

With regard to *Bacillus hirtus*, under all circumstances, the colonies are first noticeable as minute grey specks, which in course of time liquefy the gelatine. In addition it may be stated, that usually the colonies become extremely irregular, and that very often the superficial colonies show a granulated appearance and vary in thickness from the centre to the periphery, the thickness being greatest at the centre and least at the periphery. In most cases this results in the formation of a 'nucleus' at the centre, leading down to the periphery by means of stratified layers. Less often growth is characterized by the formation of irregular threads or bands stretching out from the surface, which is well described by Henrici as 'blitzähnliche Auswüchse.'

AGAR-PLATE CULTURES.

A culture was made from an inoculation taken from a growth in an Agar-tube which was one day old. After fifteen hours at 32° C. there was a splendid growth. The superficial colonies ranged from mere specks up to the diameter of 1 mm. The larger colonies were well raised from the surface and white in colour. Under the microscope ($\times 80$) each appeared as if made up of threads densely packed together. At the edge a clear transparent line bounded the colony. Under higher magnification, the individuals are seen to be motile, and almost invariably consisted of two cells joined together as in Fig. 11, evidently indicating that all the cells were rapidly dividing, and that separation took place as soon as the wall had been formed. I take this to be the surest sign, that the conditions of growth are very favourable. In this culture every individual was motile. The demonstration of the cilia is given in the paragraph on the motility of this form. The colonies under the surface were much smaller, uniformly opaque with a ragged edge, sometimes thrown out into jagged promontories. After twenty-four hours, some of the colonies were from 2–2½ mm. in diameter, and very much raised from the surface. As they get bigger they often lose their round outline, becoming somewhat wavy. If the growth on the plate has been very

rapid, or if the colonies were planted too close together, the whole plate becomes suffused with a uniform homogeneous layer, and often this homogeneous layer is the first indication of growth that is obtained. In this particular culture I saw the most prolific formation of spores that I have ever witnessed. Every available spot had been used up for this purpose. In some cases, some of the individuals had lengthened without dividing: in these, the spores were arranged very close together, often as many as 6-9, so closely packed together that the cell would have found it impossible to find room even for another half spore (Fig. 12). It thus seems as if, in the case of *Bacillus hirtus*, a plentiful supply of oxygen influences favourably the formation of spores. The formation of spores, however, must be due to more than one factor, for in another Agar-plate which I set, under approximately the same conditions, there were no spores, but all the individuals were extremely motile. After forty-eight hours, however, the motility had been lost, and spore-formation had commenced. It was not, however, as profuse as in the former culture. It will be seen later, that in an Agar-tube growth the formation of spores usually takes place after three days, so that the conditions under which growth takes place in an Agar-plate expedite the formation of spores. I consider that spore-formation is the result of the sum total of at present unknown external circumstances. In order to test the effect of the absence of oxygen on spore-formation, cultures were grown in Buchner's anaerobic tubes. Apparently the formation of spores was not influenced by the absence of air. This subject was not pursued any further, as it was beyond the scope of this investigation.

There are two characteristics of *Bacillus hirtus* Agar-plates, which, though not invariable, yet may help in diagnosing the species. The first is that, as was pointed out to me by one of my students, Mr. Pettigrew, the Agar often appears partially liquefied. It never extended so far as to liquefy completely the whole of the medium, but a distinct liquid film was formed on the surface, after which growth and also liquefaction appeared to cease. It is probable that a ferment was secreted which dissolved the Agar, but that later other secretions acted as a deterrent to further action of the ferment.

The second characteristic is that occasionally the Agar-plate cultures take on a deep bronze colour. When the colonies are first formed, they possess the normal white colour, but occasionally the deep bronze colour appears. The conditions of the formation of this development were also not further investigated, but it is probable that slight peculiarities in the constitution of the Agar may have contributed to this peculiar growth. When I inoculated from a bronze colony on to an Agar-tube, I found that at first a fleshy crinkled white growth appeared on the surface. This, after three days, assumed a bronze colour at the top, which gradually

encroached on the white, till after ten days the lower half was white and the upper half bronze-coloured. This fleshy crinkled growth in this species is not normal for growth in Agar-tubes. Microscopically there was nothing remarkable except a diminished formation of spores. As a matter of experience I have found that a fleshy growth is almost always associated with a diminution or absence of spore-formation in those forms which habitually form spores. With regard to the bronze-coloured colonies and bronze-coloured Agar-tube cultures, they retain their colour for at least a year, but when inoculated into a fresh tube, the colour of the new generation is again normal.

GELATINE STAB-CULTURES.

It is as useless giving any particulars of the rate of growth of a gelatine stab-culture for diagnostic purposes as for a gelatine plate-culture, for having made them at different times of the year, using tubes which necessarily did not belong to the same preparation, I have never obtained uniform results. Henrici records a rapid growth. In my cultures growth was oftener extremely slight. In Fig. 13 is shown a drawing of a gelatine tube after three months. Fig. 14 shows another gelatine stab-culture only three days after inoculation. The liquid part had a yellowish-brown colour, was turbid, and had a thick deposit at the bottom of the liquid. Lower down, the continuation of the stab showed a few small round colonies each with fine radiating branches. In a few days after this the gelatine became completely liquefied. In a third experiment, about half the gelatine had become liquefied only after two months. In all cultures a constant feature was the appearance on the surface of a light grey speck. Sometimes it increased rapidly in size, but oftener growth was exceedingly small. Along the stab there was a very scanty growth, showing its strong aerob tendency. The surface growth extends into a roughly circular greyish-white covering on the top of the gelatine. At some time or other liquefaction always sets in, provided that the organisms are still alive. Usually the liquefied part has, in suspension, a very fine flocculent greyish-white precipitate. If growth proceeds filter-fashion as in Fig. 13, there is a thick sediment at the bottom of the filter-tube. The same happens when the whole of the gelatine has been liquefied. I examined the individuals of a gelatine tube, in which the gelatine had taken four months to liquefy completely, and found them perfectly healthy, but there was no spore-formation.

GROWTH IN VARIOUS LIQUID MEDIA.

As a further help in diagnosing this species various liquid media were prepared, because Bacteria in the assimilation of N and C differ in the mode of combination of these substances which best suits them. The various

media were taken from Meyer's list, mentioned in Gottheil's work (6). The media were inoculated from spore-material and incubated at 31°–32° C. For the sake of uniformity I have numbered these liquid media in the same manner as Gottheil (6). No. II is not easily available, and I have therefore discarded it.

O. Lemco	1.0 gram.	I. Lemco	1.0 gram.
Peptone	1.0 „	Peptone	1.0 „
Cane-sugar	0.5 „	Cane-sugar	1.0 „
Dextrose	0.5 „	Water	100 c.c.
Milk-sugar	0.5 „	III. Peptone	1.0 gram.
Seignet salt	0.1 „	Mineral soln.	100 c.c.
Water	120 c.c.	IV. Asparagin	1.0 gram.
V. Asparagin	1.0 gram.	Mineral soln.	100 c.c.
Glycerine	1.0 „	V. Asparagin	1.0 gram.
Cane-sugar	0.5 „	Cane-sugar	3.0 „
Mineral soln.	100 c.c.	Mineral soln.	100 c.c.
V. Asparagin	1.0 gram.	Vβ. Asparagin	1.0 gram.
Milk-sugar	3.0 „	Galactose	3.0 „
Mineral soln.	100 c.c.	Mineral soln.	100 c.c.
VI. Ammonium tartrate	1.0 gram.	V. Asparagin	1.0 gram.
Glycerine	1.0 „	Glycerine	1.0 „
Cane-sugar	0.5 „	Mineral soln.	100 c.c.
Mineral soln.	100 c.c.	VII. Pot. nitrite	1.0 gram.
VIII. Pot. nitrite	0.05 gram.	Cane-sugar	0.5 „
Soda	0.5 „	Glycerine	1.0 „
Mineral soln.	100 c.c.	Mineral soln.	100 c.c.
X. Asparagin	1.0 gram.	IX. Dextrose	0.5 gram.
Dextrose	3.0 „	Cane-sugar	0.5 „
Mineral soln.	100 c.c.	Mineral soln.	100 c.c.
		XI. Ammon. sulphate	0.25 gram.
		Sodium carbonate	0.5 „
		Mineral soln.	1000 c.c.

MINERAL SOLUTION.

Potassium phosphate	1.0 gram.
Calc. chloride	0.1 „
Magn. sulphate	0.3 „
Sod. chloride	0.1 „
Distilled water	1000 c.c. + a trace of iron.

In the appended table + + + indicates strong growth. + + weaker growth. + still weaker. o no growth.

The following table shows the results of inoculations in these media.

	O.	I.	II.	III.	IV.	V.	V.	Vβ.	V.	V.	VI.	VII.	VIII.	IX.	X.	XI.
	+++	+++	not tried	++	o	o	o	o	o	o	++	o	o	o	o	o
Reaction	acid	acid	—	alkaline	—	—	—	—	—	—	acid	—	—	—	—	—

Fischer (4, p. 55) in speaking of the N and C requirements of Bacteria, says :—‘Nicht brauchbar als Kohlenstoffquelle sind Harnstoff, Oxalsäure, d.h. diejenigen, deren Kohlenstoff unmittelbar mit Sauerstoff verkettet ist, und ebenso Cyan, die Stickstoffverbindung. So hat es den Anschein, als ob der Kohlenstoff am brauchbarsten sei, wenn er nur mit Wasserstoff verbunden ist, also als CH_2 , weniger gut als CH , noch minderwertiger als CH.OH und gar nicht als CO und CN . Eine ganz glatte Skala hat man hier freilich nicht vor sich.’

So far as O, I, and III are concerned it is impossible to say how the C atom is arranged. In the others the C atom is attached in all the ways mentioned by Fischer, but capacity to grow in any particular medium is not, it seems to me, such a simple affair that it depends upon the mode of attachment of the C atom. The sum total of conditions must be favourable, and what these are we at present do not know.

The growth on No. I affords a diagnostic property which I have never known to fail, though I have made a large number of cultures in this medium. About the third day (sometimes sooner) a white pellicle forms on the surface. It is composed of non-motile individuals, and when first formed is devoid of spores. The spore-formation, however, is only postponed. After six days there are still usually no spores present. In a four weeks old culture, however, it will be found as a general rule that spores are quite abundant. I have always used this medium for accelerating the motility of sluggish cultures, and for initiating motility in cultures which, owing to their previous habits (in the laboratory, not in nature), had not when examined a trace of motility. The cells are of normal breadth and have dense contents. The short threads are very actively motile, the longer ones less so, doubtless owing to the fact that there is more inertia to overcome. The appearance of some of the longer threads is shown in Fig. 15, though it must not be inferred that this formation of threads is a constant feature; the only constant characteristics are (1) formation of pellicle, (2) acid reaction.

The growth in Nutrient Medium O is naturally much similar to that in Nutrient Medium I. In this case there always appeared an extremely thick sediment at the bottom of the culture and a pellicle similar to that on No. I on the top. The formation of spores was very scant. After thirteen

days the threads had somewhat lengthened, thus showing signs of a want of health, but a very large number were still actively motile. It is probable that an abundance of food-supply has acted as a deterrent to spore-formation within the first six to seven days, and that later it has become impossible owing to the abnormal amount of excreted matter consequent on the postponement of spore-formation beyond the normal three days. The constant characteristics are (1) formation of pellicle, (2) strong acid reaction, the reaction in this culture being stronger than that in No. I.

The growth in No. III is weaker than in Nos. O and I, evidently because the organism has to obtain its carbonaceous as well as its nitrogenous supply from the peptone, so that it evidently suffers from a disadvantage. When examined after eight days, the colourless nutrient medium had become turbid with a large number of suspended particles in solution. As was the case with the others, a pellicle was formed on the top. Spore-formation was wanting and there was a precipitate at the bottom, only in this case the precipitate was somewhat gelatinous in appearance. The culture showed, as was to be expected, a tendency to form long filaments, apparently as before, due to the fact that whereas the organism has sufficient energy to extend in length, it had not sufficient to carry out the process of division. When, however, short cells had been formed, these were invariably motile. The reaction was strongly *alkaline*, which should be a good diagnostic for the identification of this species. The constant characteristics are (1) formation of pellicle; (2) gelatinous precipitate; (3) strong alkaline reaction.

The growth in No. VI is similar to that in Nos. O and I. Here also we see a tendency to form filaments, an absence of spores, a pellicle on the surface, a slight sediment at the bottom and a turbidity in the liquid. The reaction is acid. All these which have just been mentioned are constant. It will be observed that the source of N in this case is ammonium

tartrate $\left(\begin{array}{c} \text{CH.OH.COONH}_4 \\ | \\ \text{CH.OH.COONH}_4 \end{array} \right)$. It will also be observed that this is the

only successful medium, of those tried by me, in which the chemical composition is known. The availability of the C cannot depend on the combination CH.OH, for the same combination is obtained in several of the other media. In others, e.g. those in which asparagin is used, we have the combination CH, which is supposed to be still better, and yet no growth has taken place in Nos. IV, V, V α , V β , V γ , V δ , in all of which asparagin is present. As mentioned above, I do not think that the question lends itself to an easy explanation, and the sum total of the conditions in each case must be considered.

GROWTH ON DEXTROSE-AGAR.

I have examined in detail the growth of this organism on Dextrose-Agar, from spore-germination to spore-formation in the next generation, in order to obtain a general idea of its life-history. The Dextrose-Agar was made up of the following materials: Agar, 1.6 grams; Dextrose, 1.0 gram; Na, 0.2 gram; Lemco, 1.8 grams; Peptone (Witte), 1.2 grams; Water, 100 c.c. In all cases growth commenced from the spore. This was effected by heating the spore-material for two minutes in boiling water, which thus killed off the vegetative cells but would not appreciably diminish the germinating power of the spores. The spore-material was placed in a drop of sterile water, contained in a small sterile test-tube. The latter was then immersed in the boiling water. After heating the spores were sown over the surface of the nutrient Agar and then incubated at 32°C.

Germination.—The details of germination are given above.

After fifteen hours.—The appearance of the culture after fifteen hours varies considerably. Sometimes rapid growth has taken place, which is marked by the appearance of a light grey, somewhat transparent layer. Sometimes, however, the growth has been slow, and it is only by holding the culture up to the light that it is seen that growth has taken place; a filmy covering is seen which is very transparent. At this stage almost all the individuals are either 1-celled (Fig. 16) or 2-celled (Fig. 17) and very motile. The motility is of five kinds.

1. A forward movement accompanied by a violent wriggling of the whole body.
2. A forward movement accompanied by a fairly rapid oscillation in which the centre of the cell is the fulcrum.
3. Rotation.
4. A forward followed by a backward movement along the same direction, or else a sharp turn in another direction.
5. Violent trembling of the whole body unaccompanied by a forward movement.

In fact all the varieties of motion which can be observed in a member of the genus *Bacillus* can be seen in a fifteen hours old culture of this species, provided that growth has been favourable. *After twenty-four hours* no great change has taken place in the appearance of the individuals as seen under the microscope, although there is usually a far thicker growth on the surface of the Agar. This takes the form of a somewhat light grey layer, the commonest of all colours on Agar cultures. The individuals were examined by means of the stains, Iodine, Methylene-blue, Bismarck-brown, and Fuchsin, all of which serve their purpose very well. The intimate structure of the cell is discussed in the section dealing with the cell-contents.

In a two days old culture the light grey covering is continued so as to form a pellicle on the surface of the condensed water. This formation is

never wanting. Microscopically the presentation is almost the same as in a one-day-old culture, except that there is a slight increase in the number of 2-celled as opposed to 1-celled individuals. This is to be expected in an older culture for, as stated above, there is present a larger amount of excreted matter which cannot but be unfavourable to growth, and in addition the amount of available food which can be drawn upon must be far less than it was twenty-four hours previously. In most cultures of this age there is no sign of spores, though sometimes cultures pass through the stages of growth very quickly and some individuals have begun to form spores even after two days. The motility is unabated, and as is seen in Fig. 18 the cilia have the peritrich arrangement characteristic of the genus *Bacillus*. There is no appearance of degeneration in the cells, as the cytoplasm always presents the same dense appearance in all the cells, when stained with the usual colour-reagents. I find Methylene-blue to be the best for this purpose, as when a dead cell is stained with it, not only is its cytoplasm very much less densely stained than that of a cell which was alive when treated with Methylene-blue, but the colour alters appreciably, changing to a more purple hue. At this stage the number of dead cells is extremely small. *In a three days old culture*, a great change has usually taken place. If the culture be normal, almost all the individuals are beginning to form spores, and very many of these spores have the glistening appearance peculiar to mature or nearly mature spores. The motility is unchanged in those individuals in which spore-formation has not taken place, and very often the individuals showing spores are also motile. Usually, however, the latter have come to rest indicating that a loss of ciliation has occurred due to the withdrawal of cytoplasm, in order to supply the spore, but motility remains in those individuals in which the demands of the spore have not exhausted the cytoplasm of the cell, for it is evident that an independent thread of cytoplasm like a cilium cannot remain functional apart from the cytoplasm of the interior of the cell, and if the latter be used up to form a spore, the detachment and consequent falling off of the cilia must inevitably take place. I have demonstrated in a former paper (3) the manner in which the cilia penetrate the cell-walls and establish continuity with the cytoplasm inside the cell. In Fig. 19 is shown a case in which spore-formation has not completely exhausted the cytoplasm, and these must be the individuals which, whilst forming spores, do not lose for the present their motility. As regards the non-sporing individuals, their structure does not differ from that of individuals taken from one-day and two-day-old cultures. At this stage a certain number of individuals have been cut off in the struggle for existence, and when stained with Methylene-blue, or any of the other stains, indicate their condition by the weakness of the colouring, and with Methylene-blue, as was mentioned above, by a change in colour as well.

In the case of individuals in which young spores are present, the cytoplasm still stains deeply, but in most cases when the spores are fully ripe, as explained later, the cytoplasm stains only very lightly. In normal development, the number of spores entirely free from the vegetative cells which formed them is very small, though if the culture be well advanced the number may be considerable.

In a four days old culture there will usually be seen free swimming vegetative cells without spores, spore-containing individuals which may or may not be at rest, and free spores. If the culture be well advanced the number of free spores will greatly preponderate, and in many cultures of this age scarcely anything except free spores is to be seen. In some cultures, however, there may be very few spores formed. It is difficult to account for this, for there is no apparent difference between these cultures and those in which spore-formation has been very active either in appearance or in the conditions of growth, but probably an important factor is that the amount of excreted matter is less in the latter case, thus not preventing the normal development of spores. In non-sporing cultures it is noticeable that the vegetative growth is much greater, which naturally infers a greater quantity of excreted matter. On the other hand it is remarkable that by withdrawing a culture from its own excretion products by reinoculating into another tube it is possible to postpone the formation of spores, so that it seems as if both the absence of excretion matter and the excess of it are detrimental to spore-formation. It seems as if unfavourable conditions, e.g. the presence of much excreted matter, induced a tendency to form spores in normal cultures, but if the unfavourable conditions had gone too far, that the organism was not able to do this. There are doubtless other factors which influence the course of the life-history, but at present they are very insufficiently worked out. The same remarks apply to the motility of four-day-old, as to three-day-old cultures, i.e. the motility is dependent on the spore-formation. In Fig. 20, which was taken from such a culture, it will be seen that some individuals are still in the same condition as those in one-day-old cultures.

From now on, no change takes place in the appearance of the growth on the surface of the Agar, except that due to the gradual evaporation of the water and the consequent drying up of the nutrient medium which slightly alters the appearance of the surface growth. The course is now complete and no further change takes place till the spores, finding the necessary conditions of temperature and moisture, begin the cycle once more by germinating.

CELL-DIVISION.

Migula, in his *System der Bakterien*, p. 140, makes the following assertion with reference to the mode of division of the cells of Bacteriaceae: 'Gegenüber den Coccaceen ist diese Teilung dadurch charakterisiert, dass ihr eine zur Teilungswand senkrechte Streckung der Zelle vorhergeht. Es ist hier also ein entwicklungsgeschichtliches Merkmal gegeben, durch welches die Coccaceen scharf von den übrigen Bakterien getrennt werden.' My observations confirm this statement so far as the majority are concerned. In most cases, the individual showing two cells, in which the division wall is not fully formed, is longer than any one-celled individual. This, however, is not universal, as is seen in Fig. 23 where division has taken place without previous elongation, and in Fig. 24 is shown a case where very slight elongation has preceded the act of division. Hence, though Migula's statement is true for the majority, its application does not appear to me, from my observations, to be universal. All we are entitled to assert is that elongation in length preceding division is much more common in the Bacteriaceae than the converse process, and we may also assert that this precedence of elongation is much more common than is increase of volume preceding the division of the Coccaceae. The two orders cannot be absolutely distinguished by this characteristic; all we are entitled to say is, that in one order one kind of division is much more common than it is in the other.

The second point to determine is whether a division-wall is formed before or after the constriction at the point where the wall appears. Meyer (9) figures a case (Fig. 9 *d*) in which there appears to be a very slight constriction preceding the formation of the division-wall. I have not noticed this stage in this *Bacillus*, and in another form of *Bacillus*, in which I was able to follow the division from beginning to end, no constriction was to be seen preceding the appearance of the division-wall. I was able to observe a single cell dividing, and then the daughter-cells repeating the process, until the genesis of more than a hundred individuals from a single cell had been directly observed: in this case the daughter-cells remained attached, so that I was able to observe the process of division several times over. In no case did a constriction precede the formation of a wall. If this were the usual mode of procedure, it should not be difficult to find stained specimens exhibiting this stage in the division, but I have never been able to see this stage in any of my stained preparations. On the other hand, by careful staining, numerous cases are seen in which individuals without the slightest constriction possess division-walls (Figs. 9, 11, 17, 30). The method of staining, which gave the best results with me, consisted in treating the cells with about 50% Formalin for 3–4 minutes, then adding to the liquid by means of a platinum-loop, two or three loopfuls of a somewhat dilute

solution of Fuchsin or Methylene-blue. The exact amount to be added can easily be ascertained after a little experience. The division-wall is seen as a very narrow band stretching across the cell, perpendicular to the long axis of the cell (Fig. 24). It appears to stain less intensely than the mature wall, when it first makes its appearance, but this may be due to an optical effect, the result of its narrowness. The next stage in the process is the formation of the constriction (Fig. 25). Two processes now seem to go on concurrently, the extension of the constriction and the partition of the division-wall. In some cases the constriction proceeds so quickly that the cell is already half cut off before the division of the wall has taken place (Fig. 26). In other cases division of the wall has taken place before the constriction has advanced a third of the way across (Fig. 27). The new transverse wall is divided by a clear space running longitudinally across the division-wall (Figs. 26, 27). The latter at this stage is seen as two very thin lines, one on each side of the clear line, and by their narrowness easily distinguishable from the wider, more deeply staining walls of the rest of the cell. This clear space is due to a mucilaginous change in the composition of the division-wall, at the place where a clear space is seen. There is no doubt that for some time there is at this stage protoplasmic continuity between the separating cells, though it cannot be demonstrated at this stage of the division. The next stage in development after the appearance of the thin clear line consists in the gradual strengthening of the new wall, with a concurrent rounding off of the end, until ultimately the constitution of the division-wall is not distinguishable either in density or in breadth from the remainder of the wall (Fig. 28). When two dividing cells have reached this stage they gradually draw away from each other, though they are often still connected. Sometimes two cells which have separated and are half a length apart move and wriggle about as if a rope connected the two. And such in fact is the case. In Fig. 29, stained by Grain's method, the union between the cells by means of a protoplasmic cord is evident. In Figs. 30-34 are shown on a larger scale the stages in the division of the cell, showing the two different ways in which the final stages in the division are completed.

With regard to the time required for the whole process of division to take place, Meyer (9) asserts that in one of his observations an hour elapsed between the appearance of the division-wall and the formation of a definite constriction. It is natural to expect that there will be great variation in the rate, but in nature the process must, under favourable conditions, take place at a much faster rate. In one experiment which I made in connexion with this point a one-cell individual divided up until, roughly speaking, about 128-140 cells had been formed. This represents the seventh order of division. To form these cells about three hours were required in this particular instance. I could not follow it further because

the whole mass suddenly separated into its component parts, and each individual began a separate existence. This naturally caused them to disappear from the microscope-field. From the data here supplied, the time necessary for a single complete division from beginning to end was

$$\frac{3 \times 60}{7} = 25 \text{ minutes.}$$

If we bear in mind how short a time it takes for an Agar surface to be completely covered with a thick growth when it has only been inoculated at a single point, it must often happen that a complete division takes place in less time even than twenty-five minutes.

DEVELOPMENT OF SPORES.

To observe the formation of spores, material was taken from a three days old culture, in which, as a general rule, a number of spores of all ages is seen in the microscope-field, and there will usually be a large number of individuals showing the early stages of development. Meyer's stain (10) given for nuclear coloration does very well for the first stages of spore-development, but it is necessary to modify the method, so far as the quantity of stain to be used is concerned. The method which I employed consists in fixing the material for three or four minutes in a drop of Formol, then staining by adding to the Formol a few loopfuls of a solution made up as follows:—

2 c.c. alc. sat. solution Fuchsin.
10 c.c. abs. alc.
10 c.c. H₂O.

Sometimes it will be found advisable to dilute the Formol with water. With regard to the number of loopfuls of the stain which will be required, that must be tested in each individual case: sometimes good results are obtained by using four or five loopfuls to the drop, and examining after four minutes. At other times, especially when the drop is comparatively small, we can use less of the stain, and examine after a longer interval. Again, results quite as good can be obtained by using other stains, e.g. Methylene-blue. A good stain can be obtained by mixing 1 c.c. of a saturated alcoholic solution of Methylene-blue and 40 c.c. of water. Care must be taken that the drop containing the material does not dry up, otherwise accurate observation of young spores is impossible. The first stage in the development is seen in the formation of a clear oval area in the interior of the cell (Fig. 35). This space is of the same size as the mature spore, and represents, as it were, the site that will be occupied by it. This space is clearly marked off from the surrounding cytoplasm, but this depth of staining does not differentiate the cytoplasm from the wall. In one instance I observed 12-15 cells attached together, all of which were at this stage of development. In each

clear space a round spot was visible, from which in one or two cases slight radiations were observable (Fig. 36). This I take to be the nucleus, surrounded by cytoplasm, which thus makes it a fairly prominent object. I did not see a single case in which more than one of these round objects was visible, and the method of preparation of the slide precludes the idea that they are artificial products. They are not always found at the centre (Fig. 37). In Fig. 38 we see two examples of this stage in the same individual. It seems, therefore, that the first stage of development in the formation of the spore consists in the withdrawal of the bulk of the cytoplasm from a clear space. In this space is found a nucleus, which is surrounded by a thin film of cytoplasm, and connected to the cytoplasm surrounding the clear space by thin strands of cytoplasm. Fig. 39 represents the probable construction of the cell at this stage. We have therefore before us an instance of free cell-formation. The next stage is somewhat difficult to obtain. A change has taken place in the staining capacity of the young spore. As is seen in Fig. 40, the young spore stains more deeply than the surrounding cytoplasm, having evidently absorbed unto itself those materials, cytoplasmic and reserve, which are necessary for its construction. It is not possible now to see the nucleus. In a still later stage the staining capacity of the young spore is still greater, as evidenced by its deeper colour, and by careful observation and the proper amount of staining, the coats of the spore become marked off at the periphery by their denser colour (Fig. 41). In the fourth stage we see a change in the staining capacity of the spore, for the spore-coats being now dense, they prevent the access of the stain. This results, therefore, in the spore again becoming less deeply stained than the surrounding cytoplasm, and it gradually assumes the strongly refractive appearance characteristic of mature spores, giving each spore the appearance of a bright oval speck of light. To examine this stage a more concentrated solution of the stain must be used than the one given above, in order that the walls of the spore may be stained. This stage is seen in Fig. 42. The cytoplasm outside the spore stains only very slightly at this stage, because doubtless most of it has been absorbed by the young spore. This stage is best observed in a three days old culture. In such a culture it is usual to see actively motile individuals in which mature spores are enclosed. The cytoplasm, therefore, cannot all have been absorbed into the spore in such cases, for there must be some left to maintain communication with the cilia, and it is obvious that when all the cytoplasm has been absorbed into the spore, the cilia must drop off and motion cease. This fifth stage is represented in Fig. 43, which shows a mature spore resting inside a perfectly empty sac. Nothing remains except the bare cell-wall. How long the cell-wall can remain intact without disruption must depend on the medium, and more especially on the excreted products. If the latter were acid in reaction, the cell-wall would soon be dissolved. Whatever the dissolving

agent or agents may be, their effect is seen in a slightly older culture where free spores vastly preponderate. Fig. 44 thus represents the final stage. In the last two stages a differentiation of the spore-coat into exine and intine can be observed. This differentiation is first observed in Fig. 42, but the markings of the exine cannot be ascertained till examined either at the Fig. 43 or the Fig. 44 stage.

This description agrees with that given by Meyer for the development of the spore in *Bac. Asterosporus* (9), and by myself for the development of the spore in the genus *Sarcina* (3). There is one point, however, to be observed. Meyer introduces the term 'Sporenvakuole' as indicating the clear space in which the spore is formed, and in my former work I have followed the nomenclature. It seems to me, however, that we are not dealing with a 'vacuole,' but rather with a 'cell,' and this clear space, containing a nucleus and cytoplasm, is of the nature of a cell, and the process is that of free cell-formation, so that the clear space should be referred to as a 'Spore-cell' rather than a 'Spore-vacuole,' for in the ordinary acceptation of the term a 'Vacuole' means a space filled with cell-sap. There are still several gaps connected with the development of the spore which are not yet bridged over. The history of the nucleus which serves as the nucleus of the spore is the most important. Does it migrate into the clear space, being cut off by division from one of the outside nuclei, or is it a nucleus of the cell, around which a clear space has been formed, marking the boundary of the new spore?

This and other problems will not be settled until higher magnifications than we possess at present have been placed at our disposal, or until more ingenious methods of observation have been arrived at.

MOTILITY AND CILIATION.

The name *Bacterium hirtum* was given to this form by Henrici (7), because when isolated it was found to be absolutely immotile. In an earlier paper I have shown that any non-motile form can be made motile by continual inoculation, and that *immotile forms of Bacteria* (excluding the thread forms) can be *made motile by the same method* (2). I applied this method to this form (1) and found that it became motile. On making cilia-preparations, the cilia were found to be situated at the poles, and the name of *Bacterium hirtum*, in accordance with Migula's classification, was changed to *Pseudomonas hirtum*. Since then, however (1903), I have kept this form under cultivation for nearly three years and have made a special study of its motility and ciliation. Under continual inoculation the motility once gained has never been lost, but the ciliation is undoubtedly peritrich and not polar, so in accordance with this fact, this form must be allocated to the genus *Bacillus*.

The first indication of motion is seen in a seven hours old culture. As

the material before inoculation was invariably boiled for two minutes at 100° C., every individual was the product of the germination of a spore. The motion exhibited at this stage is of the nature of an undulating and pulling movement. When observed in 2-5-celled individuals (Fig. 11) it is very distinct, more so in fact than in single-celled individuals, probably because they are slightly older, as evidenced by the fact that in them division has occurred once or twice since germination. In longer threads the inertia is too great to produce actual movement, but it is evident that there are cilia even at this stage by the manifest strains that are exerted, which is different from the slight trembling due to molecular movement. I was not successful, however, in obtaining cilia preparations from cultures of this age.

In a fifteen hours old culture the motility is very pronounced, the individuals being as active as any bacillus at its most active stage of motility. Fig. 45 is a micro-photograph and shows two individuals covered with cilia. As seen the cilia are undoubtedly peritrich, so that it is necessary to allocate this species to the genus *Bacillus*. The stain which I employed for cilia preparations was Night-blue, which is made up as follows:—

- A. { 1 gram Tannin to 20 c.c. Water.
 1 gram Alum to 20 c.c. Water.
B. ½ gram Night-blue to 20 c.c. Absolute Alcohol.

Add A to B, not B to A.

The method of preparation of cilia is as follows:—

A coverslip is washed very clean with alcohol, being finally passed through the flame. A glass slide is washed in the same way. A drop of water is placed on three places on the glass slide. Into one of them the material is inoculated. After passing the inoculating platinum loop through the flame and cooling it, a portion from the inoculated drop is placed into the second drop. In the same way a portion from the second drop is placed into the third drop. In this way the third drop though it contains fewer bacteria is almost clean, with scarcely any stainable matter except bacteria in it, so that clean preparations can be obtained. A portion from the third drop is now smeared over the clean coverslip. Care must be taken that the coverslip is so clean, that when the smearing takes place, the liquid does not roll itself up, but tends to spread out over the surface of the coverslip. The smear is usually performed with a platinum wire. To facilitate the process of smearing about 3 m.m. of the end of the wire is bent at right angles to the rest of the wire, the smearing being performed by this bent end. After the smear dries up, the stain is passed through a filter, allowed to drop on the coverslip and allowed to act for two minutes. The coverslip is then washed, dried, mounted in Xylol and examined.

This method of procedure is simpler and more expeditious than Löffler's method, and in cases where the individuals are actively motile is always to be recommended. This is a good stage for examining the kinds

of motion which are to be observed in this genus. It seems doubtful that the cilia can account for all the different varieties of motion. Recently it has been shown (8) that very probably the motility of the diatoms is due to the evolution of oxygen gas, which sufficiently accounts for the vagaries of these individuals. It is possible that the motion of any particular microbe is the resultant of these two forces, the vital activity of the cilia, and the movement caused by the evolution of gas. It would be interesting to compare the movements of a form of bacteria in which gas is known to be evolved, e. g. *Bacillus coli-communis*, with the movements of a non-gas-producing form, in order to ascertain whether there would be any differences due to the evolution of gas affecting the nature of the motility.

When twenty-four hours old cultures are examined, there is never a lack of active motion in normally developed growths. This age is usually best suited for cilia preparations. In Fig. 46 is shown one made from a culture of this age. In a three days old culture, unless spore-formation is very far advanced, which, as stated above, is not usually the case, the majority of the individuals are still motile. Usually at this stage a small number have either proceeded too far in the formation of spores or else, as is seen in the reduced intensity with which they take up stains, are either dead or in an extremely unhealthy condition. In both these cases there is no motility. In individuals, however, in which no spores are being formed or in which spore-formation is not far advanced, the motility is as great as in younger cultures. In the further growth of the culture the motility ceases owing to the extensive formation of spores, but even in 10–15 days old cultures there can always be seen a few motile individuals. As in reinoculation these asporogenous individuals divide and multiply, it is advisable before reinoculation to heat the material sufficiently to kill these forms but not the spores, if it be desired to obtain an abundance of spores. So far as motility of bacteria in general is concerned, as previously stated I do not think that a single cylindrical or round form of bacteria exists which cannot be made motile by suitable cultivation. In all forms which I have examined persistency in continual inoculation has invariably produced this result, so that motility in bacteria is a question of degree and not a question as to its existence or non-existence. It is important to emphasize this point, because the two latest and best classifications, viz. by Fischer (5) and by Migula (11), adopt the presence or absence of motility as a mark of ordinal importance, whereas, as a matter of fact, it is not even a specific value.

CONTENTS OF THE CELL.

To secure the material in the most favourable condition, the material must be fixed in a drop of 50% Formol for about three or four minutes. Then the stain (preferably Fuchsin) is added by means of a platinum-loop,

until the requisite intensity is obtained. The exact time and amount can only be decided by repeated trials, and determining, by examination of samples under the microscope, whether the necessary intensity has been obtained. In my own experiments three loopfuls of Fuchsin, prepared as for nuclear staining, allowed to act on the material contained in 50% Formalin for about eight minutes, gave the best results. The cell wall is shown distinctly (Fig. 47) as a uniform deep-red covering to the cell (if Fuchsin be used), whereas the cytoplasm is much more faintly stained. If the proper intensity has been obtained it will be noticed that the cytoplasm is not uniformly stained: patches of much fainter colour appear (Fig. 49). These are not of equal size and are evidently the vacuoles characteristic of mature cells. Throughout my investigation on this form I have not once seen any other differentiation of the cytoplasm, so that it would appear as if all the substances used for metabolic or reserve purposes were stored in the semi-liquid condition inside these vacuoles. I was also not able to distinguish the nucleus. This was rather surprising, inasmuch as I was able to see this structure in *Bac. Asterosporus*, and also in the first stage in the development of the spore. In the latter case, however, it must be remembered that the nucleus is encased by a film of cytoplasm, is attached by strands of cytoplasm to the remainder of the protoplasm of the cell, and is inserted in a faintly staining area, so that it becomes a much more conspicuous object (Fig. 36). The internal structure thus accords with that of the majority of Bacteria, and is in sharp contrast to that of such forms as *Spirillum volutans* (*Spir. giganteum*?), in which the cell is always full of fat-globules and 'volutans-spheres.' The vacuoles are very difficult to observe, but once the proper intensity has been obtained it will be found that they are universally present in all mature cells. I have not been able to demonstrate their presence in newly germinated cells. It is probable that, as in newly formed vegetative cells in other plants, the vacuoles do not appear until the cells reach their maturity. They have the varieties of size and shape characteristic of vacuoles, and their presence accounts for the absence of other indications of the presence of reserve or excreted matters, for these are doubtless to be found inside the vacuoles. The cell wall is seen more distinctly when plasmolysis takes place, for the cytoplasm, owing to the withdrawal of water, contracts, leaving a clear space between the cytoplasm and the cell wall (Fig. 48).

MOST CONSTANT CHARACTERISTICS OF *BACILLUS HIRTUS*.

The following characteristics may be accepted as the most constant.

Spore 1.0–1.8 μ broad, 1.5–2.5 μ long. Slightly oval, differentiated into exine and intine. Exine is bluntly pointed at both ends.

Germination follows by means of an equatorial split. The young

vegetative cell soon protrudes and divides to form a two-celled individual. Immediately after germination one-celled and two-celled individuals are almost exclusively found.

Motility commences very soon after germination and persists till the cytoplasm is removed by the act of forming spores.

On *Nutrient-Agar* after fifteen hours is found a uniform lightish-grey growth, extending either after this period or at furthest after twenty-four hours over the surface of the condensed water. In the healthy condition, when consisting only of one-celled and two-celled individuals, the length varies from 2.5μ to 4μ and the breadth is almost uniformly about 1.25μ (Fig. 21), but later the cells usually become somewhat longer, up to about 8μ (Fig. 22). The breadth remains the same in all stages of growth. Normally spore-formation commences after three days.

Gelatine plate cultures. Colonies first make their appearance as minute grey specks. Sooner or later the gelatine is liquefied. The colonies, however, often show stratification with a 'nucleus' at the centre, and sometimes exhibit zig-zag outgrowths from the surface.

Agar-plate cultures. Whitish colonies appear on the surface after twenty-four hours, sometimes in less time. The colonies may reach a diameter of $2-2\frac{1}{2}$ m.m. in twenty-four hours, or may have spread over the whole surface of the Agar-plate. Sometimes the Agar gives the appearance as if it were partially liquefied. Occasionally the culture takes on a deep bronze colour. Formation of spores very plentiful.

Gelatine-stab cultures. Growth appears as a light grey fleck on the surface of the gelatine. This grows larger, may or may not cover the whole surface of the gelatine. Gelatine is liquefied. When liquefaction takes place there is present a whitish-grey sediment at the bottom of the liquefied part. The liquid is yellowish-brown in colour and turbid.

Growth in liquid media. Grows in the following liquid nutrient media. (See text.)

O. Strong growth. Thick pellicle on surface. Strong acid reaction. Spore-formation postponed.

I. Strong growth. Thick pellicle on surface. Strong acid reaction. Spore-formation postponed.

III. Slightly weaker growth. Thick pellicle on surface. Gelatinous or mucinous precipitate. Strong alkaline reaction.

VI. Weaker growth. Thin pellicle on surface. Acid reaction.

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EXPLANATION OF PLATE.

Figs. 1-4, 6, 10, 15-20, 23-29, 48-49 drawn with drawing apparatus. Magnification 1650.

Fig. 1. Spore stained with Iodine, showing intine strongly and exine faintly coloured.

Figs. 2-3. Spores stained with Methylene-blue.

Fig. 4. Spore stained with Fuchsin, exine coloured, intine untouched by stain.

Fig. 5. Spore very highly magnified to show exine and intine.

Fig. 6. Spore stained with Methylene-blue seen 'end on.'

Figs. 7-9. Stages in germination of the spore.

Fig. 10. Appearance of individuals in a five hours old culture. Chains of two-celled individuals.

Fig. 11. A two-celled individual. Appearance of individuals when growing under optimum conditions of growth.

Fig. 12. Portion of a filament in which very prolific formation of spores has taken place.

Figs. 13-14. Gelatine stab-cultures. See text for explanation.

Fig. 15. Individuals from Nutrient medium No. 1 culture.

Figs. 16-17. Individuals from Agar culture. Fifteen hours old.

Fig. 18. Cilia preparation from a two days old Agar culture.

Fig. 19. Spore containing individual stained with Fuchsin, in which abundant cytoplasm is found in the vegetative cell, even after the spore is completely ripe.

Fig. 20. Cilia-preparation from a four days old Agar culture.

Fig. 21. Schematic representation of length of individuals from a twenty-four hours old Agar culture. The transverse line indicates cell-division at that point.

Fig. 22. Same from a two days old culture.

Fig. 23. Two-celled individual in which a division wall is formed, before elongation has taken place.

Fig. 24. Two-celled individual in which a division wall is formed after very slight elongation.

Figs. 25-34. Stages in cell-division. For explanation see text.

Figs. 35-44. Stages in spore development. For explanation see text.

Figs. 45-46. Micro-photographs taken with 1/12 oil-immersion lens to show distribution of cilia. Cilia stained with Night-blue. Kindly photographed by Mr. Garry.

Fig. 47. Cell stained with Fuchsin to show cellular membrane.

Fig. 48. Plasmolysed cell which separates the cell-wall from the protoplasm.

Fig. 49. Cell stained with Fuchsin to show cellular membrane.

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