VOLUME XVIII FEBRUARY, 1920. No. 4

THE CULTIVATION OF AEROBIC BACTERIA FROM SINGLE CELLS.

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(With 1 Text-figure.)

IN order to obtain cultures of aerobic bacteria from single cells it is hardly necessary to insist that a method of isolation should be employed which can be relied on to ensure that one organism, and one only, is the starting point for inoculation of the first subculture. Unfortunately, for technical reasons which will be explained, no such method is at present available.

The object of the present note is to show why the present methods of isolation are unsatisfactory, and at the same time to describe a simple and convenient method on which reliance can be placed. The methods at present in use include the fragmented glass method, the Indian ink method, the squared coverslip method, the capillary tube method, the Barber method, and the droplet method, of which the Malone method is a modification. It is assumed that the principles underlying the use of these methods are sufficiently well known to preclude the necessity for a detailed description of each. The fallacies and drawbacks incident to their employment will therefore mainly be dealt with, though a certain amount of description of the less well-known methods is unavoidable.

THE FRAGMENTED GLASS METHOD.

By this method of isolation, a description of which I am unable to find, an attempt is made to superimpose on selected single cells, scattered over agar or gelatin, small fragments of thin glass in the hope that successful inoculation of a suitable medium, such as broth, will result by placing a selected fragment with its adherent organism in such medium. The disadvantages of the method are as follows:

1. Only dry lenses can be employed.

2. The poor visibility of organisms lying under the prismatic edges of the fragments of glass often makes it impossible to be certain that only one organism is present under the fragment of glass selected.

3. When picking up the selected fragment it is difficult to be certain that organisms lying close to the fragment are not also unwittingly picked up by slight displacement of the fragment during the process of removal.

4. Once the selected fragment has been picked up it is difficult, or impossible, satisfactorily to re-examine it with a view to determining the presence or absence of organisms not previously noticed.

5. As usually employed no control observations of growth from single cell to single colony can be carried out to determine the presence or absence of more than one organism, because the selected fragment is at once placed in broth or other suitable liquid medium. It is of course possible to select a fragment of glass which appears to be lying on one organism only, to incubate the culture until colony formation is well established, and to examine again before removal. In practice, however, disadvantages 2 and 3 come into operation, and invalidate this method of control. Apart from disadvantages 1 to 5, which together prohibit the use of the fragmented glass method for scientific work, there are two minor drawbacks to the method. The first of these is the mechanical difficulty of superimposing small fragments of glass over selected organisms, there being often about 50 per cent. of failures even after considerable practice. And the second lies in the fact that frequently removal of the selected fragment fails to remove also the organism desired.

THE SQUARED COVERSLIP METHOD.

By this method, described by Hewlett, a large coverslip is employed on which are etched several numbered squares. On the coverslip is poured a thin layer of agar, or gelatin-agar, a minute quantity of a liquid culture of the desired dilution being spread with a glass rod over the agar when set. The coverslip is then inverted over a hollow moist cell, and examined with a dry lens. The position of an individual organism in a given square is then noted, and the culture is incubated. When colony formation is sufficiently marked to be visible to the naked eye the selected colony is removed, and dealt with in the ordinary way. In theory this method sounds promising, because it would appear to allow of control observations being made during development from single cell to single colony, and so of ensuring that one organism, and one only, is the starting point of the desired culture. In practice, however, the method is of little or no value for the isolation of bacteria, especially when small, because the visibility of organisms on an inverted culture is extremely poor, even under optimum conditions of illumination. It is therefore impossible to be certain that only one organism is present in a given square, and that a colony which appears to be derived from one cell only is not in reality derived from two or more coalescing colonies from two or more adjacent cells. And the necessity for waiting until colony formation is sufficiently far advanced to allow of removal under the naked eye greatly increases this danger.

THE INDIAN INK METHOD OF BURRI.

For accurate work this method, described in detail by Besson, is not satisfactory, owing to the tendency to concealment of minute organisms by the pigment, although theoretically they should stand out in bold relief even when near to the vanishing point of vision. Numerous experiments with Congo-red, and other pigments, have convinced me that these suffer from the same defects as does Indian Ink.

THE CAPILLARY TUBE METHOD.

In 1916 I experimented for several weeks with round, and with flat, capillary glass tubes filled with dilute broth or gelatin cultures. The best results were obtained with extremely fine flexible strands of tubular glass, attached in short lengths to microscope slides with plasticine, the desired section containing the selected organism being excised with a sterile knife, and dropped into broth. Prolonged examination of these sections at a temperature which precluded multiplication showed however that even under optimum conditions of illumination the method is a treacherous one, and it was therefore abandoned for the isolation of aerobic organisms, though the fact that it is possible to examine these fine tubular threads of glass with an oil-immersion lens was a strong temptation to continue the experiments. A further drawback to the method for aerobic organisms, unless freely motile, is the difficulty of ensuring passage of the contained organism into the surrounding broth, multiplication within the tube apparently not taking place. For anaerobic organisms, as recorded in 1918 by Holker, the method has obvious advantages, though the optical difficulties of ensuring the presence of single organisms still remain.

BARBER'S METHOD.

By this method an attempt is made to isolate single cells from liquid cultures with the aid of minute pipettes held in a mechanical finger operated by an ingenious adjustment device. This method is unreliable because

(*a*) Once the pipette has been removed re-examination to determine whether more than one organism is or is not present is impossible.

(*b*) Control observations of development from single cell to single colony cannot be carried out owing to the exclusive use of a liquid medium in the early stages.

(*c*) Of the optical difficulties attendant on the examination of droplets (*vide* "the droplet method").

THE DROPLET METHOD.

In theory this method is a good one, because it allows of the use of an oil-immersion lens. It may be used in one of three ways:

1. A sharp pointed spud of hard wood, sterilized by immersion in 40 per cent. formalin, and dried just before use in sterile wool, is dipped

into a broth culture of the desired dilution. Momentary contact is then made between the point of the spud and the centre of a minute ring cut on a sterile coverslip with a revolving diamond. The inoculated coverslip is then inverted onto the upper edge (moistened with Canada balsam) of a glass collar cemented to a microscope slide, and is examined with a dry lens. The minute droplet is easily recognized in the centre of the etched ring, and provided that its diameter is not greater than that of the microscopic field it can be thoroughly examined in a few seconds with the dry lens, and in a few minutes with an oil-immersion lens. A series of rings, each with its own droplet, can be examined in turn on each coverslip till one droplet is found, apparently containing only one organism. The coverslip is then removed and placed on its back in a sterile Petri dish. A small drop of broth is then delivered into the centre of the ring containing the selected organism, and is recovered with a fine sterile pipette, delivery being finally made into a tube of broth.

2. Delivery is effected by depositing minute droplets with a hair pipette attached with plasticine to a pipette of larger bore provided with a rubber teat, accurate delivery being effected by slight compression of the rubber teat at the exact moment of contact of the tip of the pipette with the centre of each etched ring. As in droplet method I, to ensure rapid examination, the diameter of the drop should not exceed that of the microscopic field afforded by the dry lens employed in the preliminary search.

3. Delivery is effected, on an inverted coverslip in a closed glass cell, of a series of minute droplets from a fine pipette by an ingenious arrangement devised by Malone, using two microscope stands for the purpose. Each droplet is examined in turn, and the selected droplet is finally collected with a fresh pipette, using the same mechanical device for collection as for distribution.

In practice the droplet method, whether delivery be effected by wooden spuds, by the direct pipette, or by the inverted pipette of Malone, is disappointing. This is because the minute size of the drop which must be employed to avoid grave error often involves a fatal retraction of the periphery of the drop, with the danger of leaving stranded in the shrinkage area—even in a moist cell—organisms which rapidly fade from view, but which nevertheless, as experiment shows, are not necessarily dead. This retraction difficulty can be to some extent avoided by using a 20 per cent. solution of glycerin in water as the bacterial vehicle, though the percentage of successful cultivations is in practice small owing to the lethal effect of the necessarily high concentration of glycerin. Even apart however from the retraction difficulty the visibility of bacteria in minute hanging droplets is always poor, and makes it impossible to be absolutely certain that one organism, and one only, is present in the droplet it is desired to use as inoculum. And finally, as in the

case of other methods involving the use of liquid media throughout, it is not possible with any modification of the droplet method to carry out control observations of development from single cell to single colony.

We learn, then, from this brief review of the methods of isolation at present available that for the reason just stated

1. Control observations from single cell to single colony are absolutely essential because of the optical difficulties which attend all known methods, making it impossible to rely upon any results from single observations.

2. Any method which aims at isolation from liquid media is inadmissible. This at once excludes Barber's method, all the droplet methods and the capillary tube methods.

3. For the same reason the fragmented glass method and the Indian Ink method must also be rejected.

We are thus left with only the squared coverslip method which has greater optical disadvantages than any other method, disadvantages which are inseparable from the use of an inverted medium inoculated on its distal surface.

It would seem therefore that the only chance of finding a reliable method of isolation is to employ direct examination of organisms on a solid medium, and to insist on a series of control observations in order to be certain that in the development from cell to colony one cell, and one cell only, is originally present.

At a time when it was believed that bacilli or cocci can only arise from pre-existent bacilli or cocci by equal binary fission the necessity, especially in the case of the larger organisms, for control observations during development from single cell to single colony was—naturally enough—not apparent. Now, however, that it is known that, for example, minute gonidial forms extruded from the mother-cell are often themselves highly fertile it is clear that complete control observations are essential in order to ensure that the unsuspected presence of these minute forms does not give rise to serious error.

Of the two methods now to be described one is suitable for oil-immersion work, and the second for dry lens work only.

FOR OIL-IMMERSION LENS.

A series of sterile coverslips is prepared, each with a small ring etched on one of its surfaces. At the same time is also prepared a dozen or more clean sterile microscope slides, over each of which is poured under cover of a Petri dish filtered peptone-agar in a thin layer. A dilute culture is now prepared, and in turn each coverslip is inoculated in the centre of the etched ring with the minutest possible droplet of the culture. Each inoculated coverslip is now placed face downwards on each agar slide, care being taken to ensure direct application of slip to agar without sliding of the former over the latter.

Great care must also be taken to ensure that the droplet of inoculum is sufficiently small not to run outside the ring when firm pressure is exerted over the slip when *in situ*. With a little practice this accident, which is of course fatal to the experiment, can be avoided, it being quite possible to effect delivery of a droplet so small in diameter as to be hardly visible to the naked eye, and well within the field of vision allowed by a dry lens of $\frac{1}{6}$-in., and the appropriate ocular. After careful search with the dry lens an oil-immersion lens is substituted, and the whole area within the etched circle is thoroughly examined. If only one organism can be found the immersion oil is carefully removed, and the slide is incubated at 37° C. for four to six hours, being examined at intervals of 20 minutes throughout this period.

When the observer is perfectly satisfied that the colony now in process of formation has started from the original cell, careful drawings being made throughout, and that only that particular cell was originally present within the etched ring, the slide is now replaced in the incubator. Twelve to eighteen hours after inoculation the coverslip is removed with sterile forceps, and a platinum loop charged with broth is rubbed over the area of glass enclosed by the etched ring, the second subculture being then carried out in the ordinary way.

This method, using an oil-immersion lens, is tedious on account of the necessity for removing the oil used at each examination, preparatory to each re-incubation. The brilliancy of outline produced by pressure of the coverslip on the agar surface and the increased amount of detail to be made out by using an oil-immersion lens, more than compensate however for this trifling drawback. And if it is desired, as was the case in most of my studies, to obtain careful drawings of warm-stage development of morphological changes in the passage from single cell to single colony it is incomparably the best method, though the relative loss of oxygen necessitated is certainly a drawback.

The accompanying figure illustrates what actually happens in such a case, and demonstrates how clearly the morphological changes can be followed by using this method, though in this particular case the experiment was not undertaken with a view to subsequent identification of the nascent colony shown.

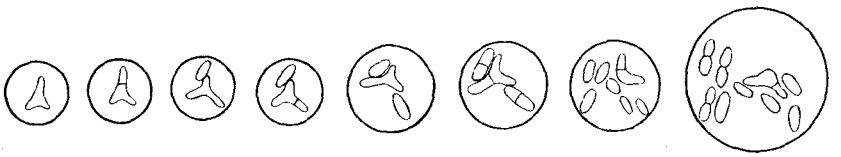

Fig. 1. *B. typhosus.* Record of development of a colony observed between 4.55 and 6.50 p.m. on + 10 agar, from 4 % glucose broth.

https://doi.org/10.1017/S0022172400007567 Published online by Cambridge University Press

THE PERFORATED PLATE METHOD—FOR DRY LENS ONLY.

For ordinary purposes of isolation of single cells, however, in which all that is required is a series of careful control observations to ensure that a culture has been started from one organism only—without any necessity, that is, for elaborate studies of morphological changes in individual cells as illustrated in the figure—the use of the dry lens alone is sufficient. An ordinary sterile glass slide is covered with a suitable medium, such as filtered peptone-agar or glucose peptone-agar, by pouring the latter at a temperature of as near 100° C. as possible under cover of the top of a sterile Petri dish. A liquid culture in appropriate dilution is then spread over the medium, as soon as set, with a glass rod, and a thin strip of perforated celluloid, or other suitable material, is placed over the slide. Sterile coverslips are now placed lightly over the celluloid, and the slide is placed within a sterile Petri dish, and is incubated at 28° C. for from 1 to 1½ hours. The object of placing coverslips over the celluloid is firstly to protect the culture medium from dust during subsequent examinations, and, secondly, to convert each of the tiny cells into a moist chamber, aqueous vapour arising from the surface of the moist medium becoming recondensed on the inverted coverslip.

At the end of 1 to 1½ hours the slide is examined cell by cell until the organism it is required to isolate is found, alone in a single cell, or until a cell is found in which a small number of suitable organisms occur so conveniently placed that there is no danger of the resulting colonies impinging on each other. When found the position of the selected cell is noted by the vernier attached to the microscope stage, and is recorded on a diagram with drawings of the organism or organisms it is desired to isolate. The slide is now replaced in its Petri dish and reincubated. It is further examined at short intervals until colony formation from single cells is established, careful search on each examination being made to ensure that organisms previously unrecognizable as bacteria are not now coming into view, and to make, if desired, such careful drawings of alterations in morphology of the organisms originally selected, or of their direct descendants, as is possible with a dry lens. When the colonies have reached a convenient size it will be found that they are still too small for removal under the naked eye, and if they are left until recognizable without the aid of a lens there is always the danger of coalescence of adjacent colonies—if a cell has been selected with more than one organism—and of the experiment being ruined.

If, however, the following simple technique is adopted there is no difficulty in picking off fragments of colonies—the growth of which from individual cells has been watched and recorded *de initio*—whilst yet too small to be detected under the naked eye. The procedure is as follows. The objective in use is replaced by a perforated metal stop, made for the purpose by Angus and Co., Wigmore Street, London, the perforation being conical in shape, with the apex at its lowest point. Into this conical space is dropped a No. 9 solid

steel needle, the point of which, when *in situ*, is blackened in the flame of a match. A minute drop of paraffin wax on a slide has, previously to removal of the objective, been exactly centred on the microscope stage. The blackened needle is now lowered till it touches the wax, the needle receding in its holder at the moment of contact. The position of the blackened point of contact on the white wax is now noted under the replaced objective, and is brought into the centre of the field, if not already there. The whole manoeuvre is repeated a second time in order to be sure that the point of contact is sufficiently approximate. The selected cell, from which the coverslip has been removed, is now examined, and the chosen colony is centred on a hooded stage. The point of the resterilized needle is now touched with broth in a small platinum loop, is brought into contact with the centre of the colony and, after racking up, is again touched with broth in a resterilized platinum loop, from which a tube of broth is inoculated in the ordinary way. Finally the objective is once more restored, and a control observation made that the desired colony has been touched, and no other.

The advantages of the perforated plate method are the following:

1. Extreme simplicity.
2. The expense of the outfit is negligible.
3. It is relatively rapid.
4. The method of cultivation ensures the maximum of oxygen and moisture.
5. Successful cultivation from fragments of the removed colonies takes place in my hands in 100 per cent. of the cases.
6. It affords an excellent control observation throughout against
   (*a*) contamination,
   (*b*) picking up fragments of a colony from more than one organism.

In conclusion it is necessary to point out that cultivation of bacteria from single cells is, even when employing a good method, a most tedious procedure, involving several hours' close work for each organism isolated, if the results are to be relied on. It cannot be too strongly insisted that all claims to have grown cultures with certainty from single cells must be accepted with reserve unless the whole process has been repeatedly controlled from selection of single cell to transference of the established colony, either in whole or in part.

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