

METHOD FOR THE ISOLATION OF BACTERIA IN PURE CULTURE FROM SINGLE CELLS AND PROCEDURE FOR THE DIRECT TRACING OF BACTERIAL GROWTH ON A SOLID MEDIUM

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By a pure culture we understand, as is well-known, a culture consisting of individuals of which we know with certainty that all are descended from one single cell, and from one only. As all bacteriologic work, of whatever kind it may be, depends on our working with such reliable pure cultures, many efforts have, of course, been made in the course of time in order to devise reliable methods of isolating a single bacterium.

The first investigator who solved the problem in a satisfactory way,—although not in regard to the bacteria proper—was Emil Chr. Hansen. The principle of his method was, briefly stated, to observe directly under the microscope the growth of the individual yeast-cell until it has formed a small colony in a gelatin droplet on the lower surface of a coverglass in a moist chamber. Yeast-cells are however far bigger than most bacteria, and there is no possibility of tracing with any certainty the growth of a bacterium, as for instance a colon bacillus, in a similar way in gelatin.

Of methods that have been proposed and employed for single cell cultivation of bacteria, the best known are those of Schouten, Barber and Malone, none of which have however attained any extensive application, no doubt partly owing to the intricate apparatus they require, and partly to the difficulty involved in picking up such minute objects as bacteria with such relatively coarse implements as pipettes and loops; and when Barber states that he is able to pick up successively each single one of four

bacteria, which he views in a small hanging-drop of broth, and to inoculate four broth test tubes with each of them separately, one feels predisposed to doubt the possibility of ever acquiring such practice.

The method most generally used at present is, no doubt, Burri's India ink method, which is the starting point for the procedure described in the following communication. As is well known, the principles of the India ink method are, briefly stated, these: the bacteria are emulsified in diluted India ink, of which emulsion minute droplets are deposited on a gelatin plate in a Petri dish by means of a mapping-pen. Those droplets which, by microscopical investigation with a high power dry lens, prove to contain only one single cell, are noted and allowed to stand until a small colony has developed, from which subcultures are prepared; or, the India ink droplet is removed, together with the bacterium, by means of a sterile coverslip that is superimposed on the black spot of the gelatin plate, removed again together with the India ink and the bacterium, and dropped into an appropriate fluid nutrient medium. This is, as has been said, an excellent method, by means of which, with some practice and patience, reliable single-cell cultures of most species of bacteria can fairly easily be produced. (It is, however, not all bacterial species that will stand the India ink.)

A drawback in Burri's method is the necessity of having the unhandy Petri dish standing on the microscope stage during the examination. Therefore, I devised a modification: by means of sterile Pasteur pipettes I poured liquid gelatin upon previously sterilized slides. On the gelatin surface three rows of India ink droplets were deposited, which could now be much more easily and rapidly examined by shifting the mechanical stage, the selected India ink droplets being subsequently removed as usual by means of sterile coverslips.

If it is desired to trace the development of the new formed elements on the gelatin, the slides are placed in a sterile Petri dish with a piece of moist filter paper at the bottom. In this way the India ink spot can be examined at intervals, and the development can be observed. The image however, will rapidly

become blurred under such conditions. The India ink will be broken up, the new-formed elements pushing in beneath it; even though the India ink be highly diluted, which facilitates observation, the image will rapidly lose its sharpness. Our objective being, in my case, partly to obtain single-cell cultures from some atypical bacterial elements, and partly to trace their development, it seemed natural, to attempt to do completely without the India ink, the multiplication of the bacteria being much easier to follow, the more dilute the India ink. When this was done I admit being surprised at seeing how well the organisms showed up as sharply defined, highly refractive elements, readily distinguishable from other chance particles or impurities on the surface.

Gelatin is however not a particularly suitable medium for most bacteria, and growth at 37°C. could not be observed in this manner, so I tried whether the bacteria were as clearly visible on an agar surface. In order to study this point, agar (common filtered broth agar) was melted, and by means of a coarse Pasteur pipette poured over a sterile slide. The bacteria were even more readily discernible on this surface than on the gelatin. It is difficult however to procure a perfectly level agar surface in this manner. This difficulty was overcome by abandoning the pouring of the agar on the slide and, instead, excising the medium out of an agar plate in a Petri dish by means of a knife, previously sterilized in a flame and cooled, lifting the excised cube of agar on the blade of the knife and depositing it on the sterilized slide to which it will immediately adhere.

The essential conditions were now provided for tracing bacterial growth on a solid medium, especially if certain difficulties could be overcome in regard to ensuring reliable and readily obtained single-cell cultures. At this point in my investigation a paper appeared in *The Journal of Hygiene* by Hort, in which he describes a method, the underlying principles of which are the same, namely, the observation of unstained bacteria without a contrast on an agar surface, partly by means of oil-immersion lenses, partly by a system of high-power dry lenses. Having given a review of the usually employed methods of isolation,

all of which, including Burri's method, he rejects, he suggests a new method of isolation having two modifications, one in which examination is undertaken with an oil-immersion lens, and one in which a dry lens is employed.

In bacteriologic literature, Hort is thus the first to point out that it is possible to see bacteria distinctly on the surface of an agar plate and to watch their growth by means of a high-power system of dry lenses without any staining or contrast. It is true that Hill, in his work on the morphology of the diphtheria bacillus, mentions that diphtheria bacilli can be seen distinctly on agar by means of a high-power dry lens, but he does not seem to realize the possibilities involved in this fact. It is no doubt possible, and I think, probable, that others too have been aware of this fact; Hort is however, as stated, the first who has defined it and understood how to make use of it.

The medium which Hort employs in his examination, he prepares in a manner similar to the one originally applied by me. He pours the hot agar over sterile slides in as level a layer as possible, taking care to keep the agar well within the edges of the slide. The mode of procedure now varies as to whether he employs the oil-immersion lens, or the dry lens system for further examination. In the first case a series of sterile coverslips have been previously prepared, each with a small circle etched on its surface by means of a diamond. In the center of this circle, a minute droplet of broth is now deposited, taken from a broth culture containing the bacteria under investigation in a suitable emulsion. The inoculated coverslip is now placed face downwards on the agar surface; the area within the small circle is thoroughly examined with an oil-immersion lens, and, in case only one single organism is found within the circle, the slide is placed in a Petri dish which is then placed in the incubator. The circle is examined at short intervals, until a small colony has formed from which subcultures can be prepared.

Hort himself remarks, in regard to this procedure, that great care must be taken to ensure that the droplet does not run outside the etched circle when the coverslip is applied to the agar, adding, however, that with some care this is easily avoided. It

is evident that we must feel perfectly sure on this point, considering that the area within the circle is the only one examined. However, it seems difficult to understand how one can be sure that the small droplet keeps within the etched circle, as a small liquid layer will form between the coverslip and the agar, the moment these two are directly applied to each other, which will immediately make the droplet invisible. The possibility will likewise always exist, of various currents arising between coverslip and agar, both when the slip is placed and removed; and, even though the growth of a colony from one single cell has been observed within the circle, this may become contaminated from a small colony immediately outside the circle, the moment the coverslip is removed. As a rule, there is little chance of this happening, but it does compromise the reliability of the method.

Moreover, there is the question of the power of certain motile bacteria to move on the uncovered agar plate. The possibility of such active motility on the part of the organisms is, of course, increased by the placing of the coverslip on the agar, by which a small liquid-filled space is formed.

For his second method Hort employs a medium prepared in a similar way. A highly diluted emulsion of bacteria is spread over the agar plate by means of a glass rod, the inoculated plate being now covered with a thin sterile strip of celluloid, which has been previously perforated. Small sterile coverslips are placed over the holes so as to form minute moist chambers. These are now searched with a high-power dry lens and the chambers containing one single cell only, are marked. The examination is now continued as described above. (It is not evident from Hort's treatise, whether the coverslips are removed during the examination under the microscope; if not, one would think that the dew on the coverslip would be obstructive.) The colonies having reached an appropriate size, subcultures are prepared by means of a special apparatus consisting of a tube with a needle adjusted in a special way and screwed on to the nose-piece like an objective. This method is reliable, but as Hort himself says in his final remarks: "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."

I believe the difficulties to have become considerably reduced in the procedure described in the following pages, and, the simpler a method is, the more reliable will it generally be. The principle upon which the method of isolation described below has been based is, as mentioned, the fact that a bacterium—possibly the minutest ones excepted—can readily be distinguished on the surface of a clear transparent medium, such as for instance agar, gelatin, or ascitic agar. The mode of procedure is, briefly stated, as follows: A young bacterial culture, such for instance as a twelve-hour old broth culture of colon bacilli, is inoculated on the agar plate in a Petri dish. The agar had better not be more than a few millimeters thick (bacteria can also be distinguished on very thick agar, but less sharply). The upper and lower surfaces of the agar should be parallel so as to ensure that the excised bits shall be of equal thickness everywhere, partly in order to obtain plane images, partly to avoid the risk of running down with the objective into the agar, which is in its immediate vicinity during examination.

It is important, in inoculating the culture, to be fairly clear at the outset as to the density of bacteria desired on the plate. In the diagram, figure 1, some dotted lines show how I am accustomed to proceed. A big droplet of the broth culture is deposited in the center of the circle, and, by means of a glass rod, bent at a right angle, the drop is pressed down between the parallel dotted lines. Now the glass rod is moved from side to side across the first inoculated area, and, finally, the remaining half of the dish is inoculated and it is placed in the incubator for about one hour at 37°C. (Inoculation can of course also be performed in a streak, which some will perhaps find more to the purpose, and in this way an appropriate difference in the density of the bacteria can likewise be obtained.) This measure is taken because the development in the case of the colon bacillus begins just after the expiration of one hour, and because bacteria are more readily discernible when in development, owing

to their increased refractive power. As previously described, a suitable square of the agar is now excised and placed upon the previously sterilized microscope slide, which is most conveniently sterilized by flaming (cf. fig. 2).

The microscope slide plus agar, which, for convenience sake, I shall term "slide" in the following discussion, is placed on the stage of the microscope, and an area is chosen where the organisms are placed at a convenient mutual distance, commencing

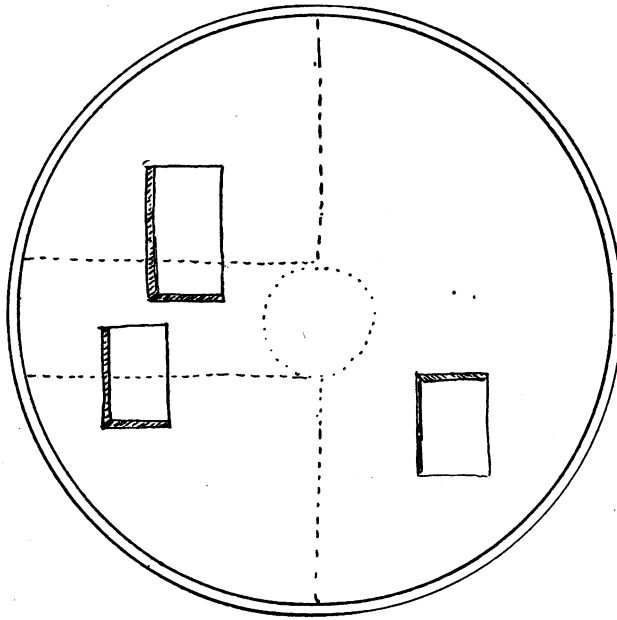


FIG. 1

the examination where they are lying close, and thence, by means of the mechanical stage, proceeding to where they are lying more scattered.¹

Having now come upon an area where there is one organism, only, within the field of vision, and this single bacterium having

¹ The objective employed by me was a Zeiss Apochromat; any sufficiently powerful objective will however do. The magnification, at which I usually worked, was 750 diameters. Illumination is a very important factor. The source of light must be uniform. I used a powerful metal filament lamp with frosted bulb, the light of which was considerably reduced by means of the diaphragm of the illuminating apparatus of the microscope.

been centered, the area is noted by means of the mutual relation of the regular scale and the vernier attached to the mechanical stage, and one should now be able to focus exactly in the same place again. At this point there is however a great difficulty to be overcome, as the least inaccuracy in the re-adjustment may have the effect of causing the selected organism to vanish from the field of vision, others being substituted, and,

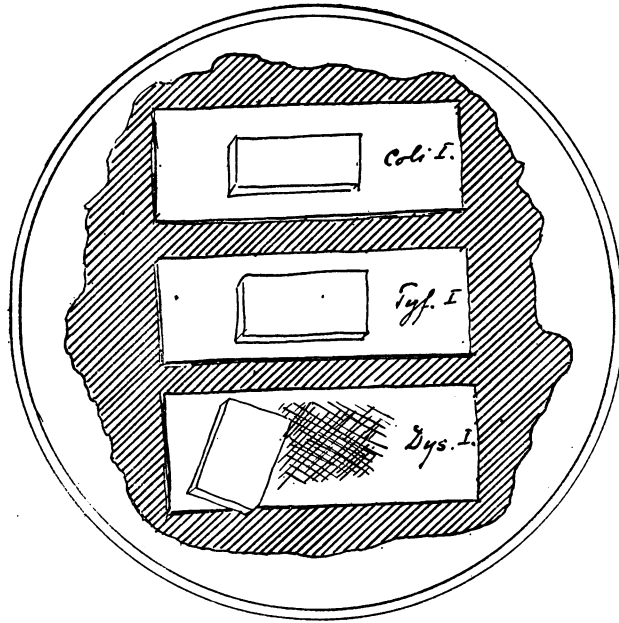


FIG. 2

even though the most painful care be taken, the same fatal accident may happen owing to quite negligible displacements of the scales, which it is often quite impossible to control.

In order to be able to find a particular bacterium again, I have proceeded in the following manner: Prior to placing the agar plate on the slide, a complex of fine lines are by means of a diamond scratched criss-cross, preferably on the lower surface of the slide, over the area to be covered by the agar (cf. fig. 2, above). The lines will become less frayed in the glass if the

scratching be performed in a drop of immersion-oil. In an objective (preferably a different one from that with which the growth is observed, as the micrometer lines will disturb observation) is placed a squared eyepiece micrometer, which is cemented on to prevent displacement.

If we now focus sharply on the scratches on the slide with the low power of the microscope, the lines in the eyepiece micrometer will be intersected by these scratches in a quite specific manner (cf. fig. 3), and thus we obtain two distinguishing marks instead of one. The course of procedure will now be as follows: the agar is placed on the scratched area of the slide, and we search for a place where there is only one organism within the field of vision. The spot is marked by means of the scales of the mechanical stage, the objective with the attached micrometer is placed in the tube, the microscope is adjusted to low magnification and focussed sharply on the scratches. Careful drawings are made on squared paper of the position of these scratches in relation to the eyepiece micrometer, the slide being now placed in a sterile Petri dish with a piece of moist filter paper at the bottom. (The filter paper must not be too wet as this may cause the development of so much aqueous vapour during incubation that the glasses become wet enough for the agar squares to slide, when the whole experiment is ruined.) By means of thus marking the position of the organism we have always succeeded in hitting upon exactly the same spot for repeated examination.

The growth is now watched at proper intervals, the adjustment being performed so that, firstly, the scales of the mechanical stage are placed in the proper mutual relation, which we have noted down, secondly, with the low power of the microscope we make the scratches on the slide correspond to the proper points in the eyepiece micrometer, and, finally, eyepiece and objective are changed, and we can now easily observe the alterations in the small colony in development. (The slides must of course previously be cooled to the same temperature as that of the objective, in case examination takes place at a lower temperature than that of incubation, as, otherwise, condensed moisture

will gather on the front lens and obstruct vision altogether.) In this way, the agar will preserve its shape for at least twenty-four hours, even though subjected to several examinations, provided these be not of too long duration.

Now, do not these repeated examinations involve a great danger of contamination from the air? A risk there is, of course, but it is apparently, insignificant. In the first place it can easily be ascertained that only very few "alien" colonies will be seen

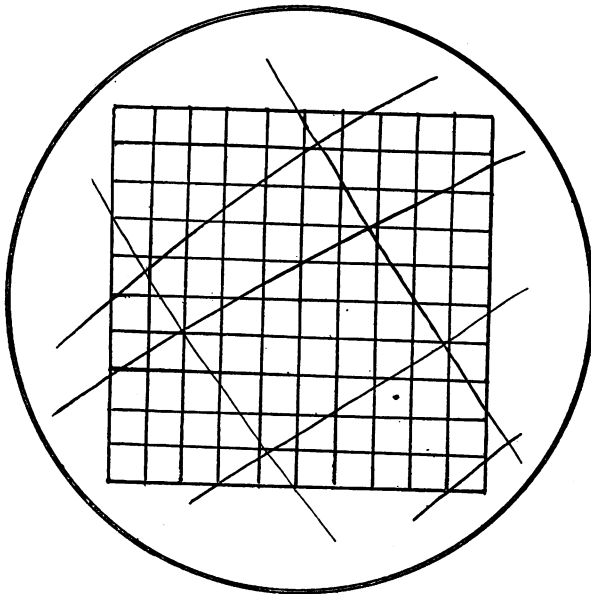


FIG. 3



FIG. 4

to develop in the Petri dish by contamination from the air, in spite of repeated examinations within forty-eight hours, and it would be a stroke of very bad fortune if such a germ from the air should settle just within the field of vision to be examined. If this should occur, it would soon be discovered; it could only escape detection in case a germ from the air dropped upon the selected colony immediately before inoculation was undertaken from it, and then it would most probably be disclosed in further investigations of the bacterial species in question, as it would

be almost inconceivable ill-luck if the "alien" microorganism should be one that was closely related to the isolated one.

Now, the new-formed colony having reached a convenient size, sub-cultures should be prepared from it. At this point the colonies have grown so large as to be distinctly visible with the low power of the microscope, the time and mode of re-inoculation depending on the relative situation of the colonies. In case the colony, the shape of which we recognize with the low power of the microscope, is placed in a sufficiently isolated position, we may defer inoculation until it has grown big enough to allow of our conveniently inoculating from it by means of a fine inoculation needle under the microscope at low magnification. If there is any danger of neighbouring colonies impinging upon it, we must undertake inoculation while it is yet small. As mentioned, Hort used a special apparatus for this purpose. A small harpoon, which one may prepare oneself, will however do.

On the front lens of an objective is placed a small lump of modelling wax to which is attached a fine thin platinum wire not thicker than 0.15 mm. with a blunt end (cf. fig. 4). Previous to "harpooning" the colony some preparatory practice is necessary. A small agar square is excised and fitted in the usual way and placed on a slide. A droplet of India ink or some other staining fluid is deposited on the agar with a mapping-pen. This spot is now centered in the field of vision at the low power of the microscope, and the objective with the attached platinum needle, which is screwed on to the nose-piece, is directed across the spot, the needle being adjusted by a pressure from the fingers so as to be mounted exactly above the spot, and gently depressed so as to touch the agar. The point of contact is readily discerned with the low power of the microscope and marked by means of the eyepiece micrometer. We know now exactly where the needle will hit, being able then to inoculate from the colony by adjusting it to the exact point in relation to the eyepiece micrometer at which the needle hit last. The needle is carefully lowered into the colony until it touches the agar plate. The point of contact is most readily noticed by

following the reflection of the needle in the agar; when the needle and its image meet, contact with the colony has been established. The point of the needle is now touched with broth in a small loop which is raised so as to encompass it several times, agar is inoculated from the loop, and finally the needle is washed in a tube of broth.

The "harpoon" is sterilized by flaming, and now it only remains to examine the area where the colony was previously situated. The bacteria from the colony will be seen to have been scattered somewhat, and we note whether the adjacent colonies are totally intact, both with the low and with the high power of the microscope. In case growth results from the inoculation we know that we have obtained a reliable pure culture. Compared with Hort's dry-lens method, the procedure described presents several advantages. Firstly, it is difficult to pour agar over the slides so as to obtain an even layer, and it takes a long time. Petri dishes with agar are always at hand in any bacteriologic laboratory; these should however be freshly poured to avoid the risk of obtaining pure cultures from chance microorganisms from the air which, being overlooked at inoculation, may have formed small colonies. Secondly, we avoid the perforated celluloid plate which is a hindrance to free operation and means a considerable limitation in the possibility of finding conveniently placed microorganisms. While Hort spends several hours on the pure cultivation of a bacterium by his method, I believe that the total work in my isolation method will, in most cases, only amount to a fraction of an hour for each single bacterium.

As has been shown, we are able to trace the growth from the single cell until a small colony has developed. Details can of course only be observed as long as the colonies are small and single-layered; so soon as the colonies are crowded in several layers, exact examination is of course out of the question. If, for instance, we desire to ascertain whether a morphologically atypical element is viable, and to follow its development, we isolate it as described above and observe its growth at proper intervals. If it is desired to get a survey of the way in which colony formation proceeds we need only, at proper intervals,

to excise small bits of the agar plate inoculated with a bacterial culture, and we obtain in this way a far better picture of the actual morphology of the bacterium than by producing preparations according to the usual methods, whether it be the milder procedure of emulsifying the bacteria in a drop of a staining fluid, or one of the various staining methods with previous fixation.

If, owing to the minuteness or too crowded placing of the microorganisms, we should fail in distinguishing what we desired to see, such as for instance the cell division lines, by means of the dry lens system, we need only place a coverglass on the surface of the agar. This will immediately adhere to the agar, and, by means of the oil-immersion lens we shall be able to detect the bacteria quite distinctly and also to trace, the growth of a single element.

Any one can readily be persuaded as to the facility with which bacteria are distinguished on an agar surface without staining or contrast, by examining an inoculated agar plate after a few hours' incubation.

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