USE OF THE SINGLE CELL METHOD IN OBTAINING PURE CULTURES OF ANAEROBES.

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The object of this work has been to determine to what extent the pipette method may be of service in the isolation of anaerobes. No question is raised as to the possibility of isolating anaerobes by plating or by any other of the methods ordinarily in use, but such methods are often time-consuming and sometimes leave the worker in doubt as to whether or not the culture is absolutely pure. Difficulties in isolation are especially great with mixtures of certain anaerobes, and doubt has been thrown on the results of some of the earlier workers because of the probability that their cultures were impure. The pipette method is relatively simple and rapid, and affords the worker the satisfaction of knowing that his culture is the progeny of a single cell. Further, it offers some advantages in the study of anaerobiosis, variability, motility, rate of growth, and behavior in a medium of small sowings exactly known.

Technique.

The technique followed in this work has been essentially that described for aerobes, except that the isolated organisms have been subsequently grown under anaerobic conditions. The pipette method has been fully described (1), and only such details will be given here as should be especially emphasized or are especially applicable to work with anaerobes.

The size of the isolation chamber may vary according to the needs of the investigator, but I have found most convenient one about 7 cm. long, 3.25 cm. broad, and 2 cm. high, which gives abundant working room and allows the use of a large cover-glass, 60 by 35 mm. The

preparation of the cover-glass is a matter of considerable importance, especially when colonies are to be grown on it. Carefully cleaned covers are smeared with vaseline and kept in stock ready for use. Just before use most of the vaseline is removed by holding the cover slantwise under the hot water tap. It is then washed with alcohol and again held under the tap. Finally, it is covered with alcohol and wiped clean with a cloth. It is then perfectly clear to the eye, but retains the very small film of vaseline so necessary to the preparation of small droplets. Prepared cover-glasses may be sterilized by dry heat or over the flame, care being taken to avoid heat sufficient to burn off the vaseline.

The point of the pipette should be made with an opening large enough to admit organisms readily, but not so large as to make it difficult to isolate the organisms in very small droplets. A diameter about equal to the average length of a spore-containing bacillus of *Bacillus tetani* is very satisfactory. It will be found most convenient to make very fine pointed closed pipettes and to break off portions of the point in a drop of broth under microscopic control until an opening of convenient size is obtained. A small quantity of broth should be allowed to enter the pipette before isolation is begun.

Certain doubts which have been raised as to the possibility of isolating a single organism in a hanging drop must arise from a misapprehension of the pipette method (2). The droplets containing the single organism may be made very small, and a mere film of liquid is present on the cover-glass, not a large rounded drop in the margins of which a second organism may lie concealed. The photomicrograph of a spore-bearing bacillus of *Bacillus tertius* (Fig. 1) illustrates the character of the droplet. This droplet has spread somewhat, owing to moisture condensed during the process of photography, and the small particles seen in it are probably due to the excess of vaseline used in the preparation of a specimen suitable for longer preservation during photography. In routine work the organism may be isolated in perfectly clear fluid and in droplets somewhat smaller than that shown in the illustration. After a series of organisms has been isolated, each droplet may be gone over with the oil immersion lens while the cover is on the isolation chamber, or, if the light there is insufficient, when temporarily removed to a shallow moist chamber. One cannot, of

course, exclude the possibility of the presence of any organism not visible to the oil immersion lens, but this limitation is common to all methods of isolating living organisms. If desired, the organism may be washed and rewashed in sterile broth, a procedure useful in removing any growth substances brought over from the original culture, but no guarantee against the inclusion of an invisible living substance. One may observe the first stages of development of an organism in a small drop of broth or at the margin of an agar droplet. In Fig. 2 a young colony of *Bacillus sporogenes* is shown growing out from the original position of a spore at the margin of an agar droplet.

The isolated organisms may be immediately transferred to test-tubes or grown on the cover-glass until colonies have formed and these then transferred. In transferring the organisms immediately after isolation the isolating pipette is discarded, and by means of new pipettes—a fresh one for each organism—the organisms are separately transferred to test-tubes. Each new pipette is supplied with a small amount of sterile broth before use, preferably from recently boiled broth or semifluid agar. In taking up an organism it is important to make sure that it enters the pipette and does not remain clinging to the margin of the opening. This precaution is not absolutely necessary in the case of aerobes or of anaerobes which are transferred to a liquid which is subsequently freed from air, but when a transfer to deep agar or to broth protected by a layer of vaseline or oil is intended, an organism not well within the pipette may remain on the vaseline or at the surface of the agar. In order to provide sufficient liquid for washing the organism well back a very small drop of broth may be made with the pipette in the immediate vicinity of the isolated organism and a small amount of broth added to the drop containing the isolated spore or bacillus just before it is taken up. The organism is then usually seen to enter the mouth of the pipette, and the additional droplet of broth is taken up in order to wash it well back. Sometimes a little manipulation is required to free an organism from substances which tend to make it cling to the film of surface tension of the droplet. Most bacilli and spores still enclosed in the mother cell give little trouble in this respect. When the tip of the pipette is brought to the desired depth in the medium in the test-tube, enough broth should be discharged to insure the

exit of the organism, and the discharge should be stopped before air is introduced.

With bacilli of some species it is desirable to minimize their exposure to the air during isolation. In this case it is well to introduce the isolating pipette directly into the culture from which isolations are to be made. The pipette is adjusted in the holder, a few isolations are made, and the organisms are transferred to an anaerobic environment as quickly as new pipettes can be prepared. Exposure to the air can be limited to 4 or 5 minutes, and the time of exposure can be fairly accurately measured. When pipettes for transferring the isolated organism are made in advance, or when two pipettes are used simultaneously, one for isolating and the other for taking up the organism, the time of exposure to air may be still further limited. No diluting fluid is needed in making isolations if bacilli are taken from a young culture just becoming cloudy.

Seeding Material.

In this work the aim has been to purify cultures isolated by the usual means. It might be possible to use the pipette method in isolating directly from the original source in cases in which the spores of anaerobes predominate and a considerable proportion of them are viable; but, in any case, it would be more convenient to make a preliminary isolation by one of the ordinary means. Spores for seeding were usually taken at maturity or within a few days afterwards, but in some instances spores from cultures 2 to 4 months old were sown and a fair proportion of positive results was obtained. In order to obtain proper bacilli for isolation, media were fairly heavily seeded, and the isolations made at the time when the first cloudiness appeared, usually within 4 or 5 hours. At this period nearly all the bacilli are living, and the longer elements or filaments which are then usually present are more easily isolated and possibly give a better chance of growth. In a few cases positive results were obtained with bacilli from cultures 24 hours old or more. In this paper the term "single bacillus" is made to include, in some cases, short filaments which may have been composed of several united bacilli.

Anaerobiosis.

In part of our series (Tables I and II, columns marked*) organisms were transferred to a fluid medium and the air was subsequently removed by means of an air pump. In the majority of cases boiling alone without vacuum was relied on to remove oxygen, and with

TABLE I.

Growth of Single Bacilli in Test-Tubes.

Species.	Medium and proportion of positives.													Total, all media.
	Glucose broth.	Serum glucose broth.	Semisolid glucose agar.	Semisolid serum glucose agar.	Semisolid serum agar.	Firm glucose agar.	Firm Veillon agar.	Liver peptone agar.	Minced brain.	Liver peptone water under vaseline.	Liver peptone water in vacuum.*	Milk.*	Plain broth.*	
B. sporogenes......	2/14					0/6								2/20
" *welchii*.........	10/25		0/3						0/1	0/7	3/6	1/3	0/1	14/46
" *tetani*..........	13/36		3/12				1/3				0/5			17/56
" *œdematis*.......	3/12													3/12
" *botulinus*.......	3/6						1/3				0/5			4/14
B. of Ghon Sachs..	0/16		1/7			0/1		0/6		1/6				2/36
B. aerofœtidus.....	1/3	0/1	1/2								0/23			2/29
" *putrificus*......	0/2	1/2		1/3										2/7
" *bellonensis*.....	0/3	0/3	1/2	0/3										1/11
" *tertius*.........	0/1	2/3	0/2	0/2	3/3									5/11
" *fallax*..........	0/8			0/2	1/2									1/12
" *œdematiens*.....	2/3	0/2	1/1											3/6
" *bifermentans*....	1/12	0/1	0/1	0/1	0/4					0/1				1/20
" *histolyticus*.....	1/1			1/3						2/2				4/6
Vibrion septique...	0/47	0/8	1/37	0/12	0/7					0/3				1/114
Total, all species.	36/189	3/20	8/67	2/26	4/16	0/7	2/6	0/6	0/1	3/19	3/39	1/3	0/1	62/400

* Air removed after inoculation by means of an air pump.

fluid media access of air was prevented by a layer of vaseline 1.5 or 2 cm. thick. Immediately before inoculation the upper part of the vaseline layer was liquefied by gentle heat. Both firm and semisolid agar was used without any layer of vaseline or other protecting substance, and the isolated organism was introduced deep into the medium (Fig. 3). Except with serum-containing media, tubes were, as a routine, boiled and quickly cooled before use.

A simple method of anaerobiosis was found adequate for growing spores in hanging drops. A shallow moist chamber about 45 mm. long, 25 mm. broad, and 2 mm. deep, inside measurement, is made by cementing strips of glass to a large slide. Organisms are isolated in an area of about 3 by 1 cm. in the center of a large cover-glass. The isolated spores are arranged about 1.5 or 2 mm. apart, and enough medium is added after isolation to provide each spore with a droplet

TABLE II.

Growth of Single Spores in Test-Tubes.

Species.	Medium and proportion of positives.													Total, all media.
	Glucose broth.	Serum glucose broth.	Semisolid glucose agar.	Semisolid serum glucose agar.	Semisolid serum agar.	Firm glucose agar.	Firm Veillon agar.	Liver peptone water under vaseline.	Liver peptone water in vacuum.*	Milk.*	Plain broth.*	Egg cube.*	Meat.*	
B. sporogenes.......	11/16	2/5		3/6		2/5		5/6		1/1	1/2	1/1		26/42
" *tetani*...........	7/17		3/6	2/6			0/5		0/14					12/48
" *botulinus*........							0/3		1/12					1/15
B. of Ghon Sachs...	3/3		1/3											4/6
B. putrificus.......				4/5						1/1	1/1	1/1	1/1	8/9
" *bellonensis*......		0/2	0/5	4/5	0/3				2/6					6/21
" *tertius*..........				10/12	1/1				1/7					12/20
" *œdematiens*......				4/5					3/7					7/12
" *bifermentans*.....			3/3	4/6					1/3					8/12
" *histolyticus*......									2/11		0/2			2/13
Vibrion septique....			1/1	3/3			0/4		3/5					7/13
Total, all species.	21/36	2/7	8/18	34/48	1/4	2/5	0/12	5/6	13/65	2/2	2/5	2/2	1/1	93/211

* Air removed after inoculation by means of an air pump.

about 1 mm. in diameter, although this quantity may be made to vary, as desired. Larger drops sown with many organisms are also made for comparison. After the organisms have been isolated and supplied with medium, the under surface of the cover-glass, with the exception of the area in the center occupied by the isolations, is covered with soft glucose agar taken from the surface of a culture of *Bacillus pyocyaneus* about 4 or 5 hours old. This culture should contain many actively growing organisms, and a little fresh medium

may be added to it just before spreading. The area in the center of the cover may be enclosed in a ring of soft paraffin in order to prevent any danger of the spread of the *pyocyaneus* to the isolated anaerobes. The bottom of the moist chamber is supplied with a similar layer of *pyocyaneus* culture. The entire surface of the bottom may be covered, but better light is afforded for observation and there is less danger of condensation of an excess of moisture on the cover-glass if an area at the center of the bottom is left free. The cover is immediately sealed on the moist chamber by means of vaseline, and the chamber is enclosed in a Petri dish before it is placed in the incubator.

Pyrogallic acid and potassium hydroxide, so arranged that they may be mixed in the bottom of the moist chamber after the cover has been sealed on, were used alone and in connection with the *pyocyaneus* culture, but appeared to offer no advantage.

Preparations growing on the cover may be examined at any stage of growth and returned to the incubator without disturbing the cover-glass. Moist chambers taken out of the incubator should not be exposed to room temperature while still warm, since the cover-glass cools more rapidly than the slide and may accumulate an oversupply of moisture of condensation. It is best, then, to allow the preparations to attain room temperature before removing them from the Petri dish.

Media.

The media employed are shown in Tables I and II. Glucose media contained for the most part 0.5 per cent of glucose and were adjusted to a pH of approximately 7.4. Serum media contained 3 to 5 per cent of unheated rabbit serum, or, in a few instances, horse serum. The liver peptone water consisted of Dunham's peptone without salt adjusted to a pH of 7.8. The media were tubed, and to each tube a small piece of liver was added. It was then autoclaved, and after autoclaving the reaction was found to be about pH 7.4. Semi-solid agar media were made soft enough to be easily drawn into a fine pointed pipette while cold As a routine, test-tubes of narrow diameter were used, and 10 cc. of media supplied to each tube.

Proportion of Positive Results Obtained from One-Cell Sowings in Test-Tubes.

Table I shows the number sown and proportion of positives of single bacilli sown into various media in test-tubes, and Table II gives similar data for spores. The denominators of the fractions in these and all subsequent tables give the number sown; the numerators, the number positive. Fifteen species, or possible species, are included. Questions as to the possible identity of certain species, as the bacillus of Ghon Sachs and *vibrion septique*, need not be discussed here. There were four strains of *Bacillus tetani*, one of them a Type III, two strains of *Bacillus botulinus*, and two of *Bacillus welchii*, one of which had been recently isolated from feces. For the most part the various strains had long been grown on nutrient media. In all strains of all species positive results were obtained either from single spores or from single bacilli, and in a majority of species from both. In the aggregate, 211 sowings of spores gave 44.1 per cent positive, while 400 sowings of bacilli gave only 15.5 per cent positive. The poorer showing of the sowings of bacilli is mainly due to the large number of negatives occurring in three species, *Bacillus aerofœtidus*, the bacillus of Ghon Sachs, and *vibrion septique*. The last two named gave positives with single spore sowings on the first trial.

As a routine, isolations were done in batches of five or six transfers. In a majority of species the first trial gave at least one positive among the first four tubes inoculated. In nearly 70 per cent of the thirty-six spore groups positives were obtained in at least one of the first four tubes inoculated. Fourteen spore groups, including nine species and ten strains, were sown into semisolid glucose agar, part with and part without serum. Of these, thirteen gave positives in at least one of the first four tubes inoculated. To isolate six spores or bacilli and transfer them to test-tubes requires, as a rule, less than 1 hour. These data give a better idea of the practicability of the method and the probabilities of success than do the percentages given in the tables, which include all experiments, tentative and otherwise.

Hardly enough experiments were made with all the different media to offer a fair basis for estimating their comparative value. The various glucose media served about equally well for bacilli, and semi-

solid serum glucose agar gave the best results for spores. Semi-solid agar media not only give a relatively high proportion of positives, but offer the most convenient form for one-cell inoculations, since they do not need to be liquefied for inoculation and require no protective layer of vaseline or oil. Further, if positives are examined early one may often observe the formation of a single colony at the point where the organism was discharged. Certain advantages of semisolid media for routine work with anaerobes have been described by Lignières (3).

Bacteria in positive cultures in all media were examined microscopically, gas formation and other characteristics noted, and transfers made to broth or other media under aerobic conditions, in order to detect possible contaminations with air organisms. Such contaminations during the manipulations occur so rarely and are so easily detected that they constitute no source of difficulty in the pipette method.

Growth of Isolated Organisms in Hanging Drops.

The method of anaerobiosis for preparations in hanging drops has been described. Single spores from eight species were successfully grown. The media used were chiefly semisolid glucose agar, with and without serum, and serum glucose broth. The species tested and the proportion positive were as follows: *Bacillus tetani*, 17/57; bacillus of Ghon Sachs, 1/7; *Bacillus sporogenes*, 42/50; *Bacillus putrificus*, 1/8; *Bacillus bellonensis*, 11/24; *Bacillus œdematiens*, 4/12; *Bacillus tertius*, 10/19; *Bacillus bifermentans*, 1/29. Total, 87/206, or 42.2 per cent.

In some batches of all species single spores from the same source were sown at the same time into about 10 cc. of a similar medium in test-tubes. The test-tubes gave a total of 55.4 per cent positives out of 65 sown, while the corresponding hanging drops gave 38.2 per cent positives out of 102 sown.

The hanging drop method has certain advantages; isolations are quickly made—$\frac{3}{4}$ hour or less suffices for a series of ten isolations—and only two pipettes are required for a whole series, one for isolation and one for supplying additional media to the droplets. Early growth, variability, motility, and spore formation may be conveniently observed.

With species which readily form spores, it would probably save time in isolation to sow spores in hanging drops in preference to the direct transfer to test-tubes. The strains which fail to grow on the cover-glass could be reserved for the other method. Lag occurs in the hanging drop as in test-tubes, and preparations should not be discarded if no growth occurs on the 1st day. When colonies have formed in the hanging drop they may be easily transferred to the test-tube. In most species spores formed readily in the hanging drop, and these spores have afforded convenient material for further isolations.

The hanging drop method has not proved successful for growing isolated bacilli, although a large number of bacilli sown in one drop will often grow. A number of attempts to grow young isolated bacilli of several different species gave uniformly negative results, possibly because of too long exposure to air before sufficient anaerobiosis was attained.

Growth of Spores of Bacillus tetani in Homologous Serum.

The growth in hanging drops of single tetanus spores in a medium containing tetanus antitoxin was compared with similar sowings in a control medium containing no antitoxin. Both media consisted of semisolid glucose agar containing 3 per cent horse serum. The antitoxin serum contained approximately 325 units per cc., and the tetanus spores were taken from the strain used in immunization. Growth occurred in three out of ten sowings in the antitoxin medium, and in two out of ten controls. In a series sown in serum glucose broth the antitoxin medium gave one out of four positive, and the control one out of five. Spore formation and the quantity and character of the growth were similar in the two media, except that in the antitoxin medium bacilli showed a greater tendency to adhere in chains. Sowings of single spores in quantities of 10 cc. of the antitoxin medium gave two positive out of three sown. One of these positives is shown in Fig. 3.

Effect of Exposure to Air on Isolated Bacilli and Spores.

In the course of routine work the period of exposure to air was noted in many groups of experiments, and the proportions of positives occurring after various periods of exposure are shown in Table III. Only batches in which at least one positive occurred and only those in which young bacilli were sown are included in this table. The bacteria were exposed to the air in the very small droplets of the

TABLE III.

Effect of Exposure to Air on Young Bacilli.

Species.	Length of time exposed to air and proportion of positives.									Total, all periods.
	4–6 min.	7–10 min.	11–17 min.	18–23 min.	24–30 min.	31–36 min.	37–43 min.	44–60 min.	61–72 min.	
B. welchii	3/3	1/2	3/5	1/3	0/4	0/2	0/2			8/21
" *tetani*	3/7	3/4	3/7	3/10	1/4	1/5	1/3	1/7	0/1	16/48
" *œdematis*	2/2	1/1		0/3	0/1	0/2	0/2	0/1		3/12
" *botulinus*	1/1	0/1	1/1	0/2	1/1					3/6
" *sporogenes*	0/1	1/1	0/1	0/1	1/1	0/1				2/6
B. of Ghon Sachs	2/2	0/1	0/1	0/1	0/2					2/7
B. aerofœtidus	1/3	1/2	0/1							2/6
" *putrificus*	2/3	0/3	0/1							2/7
" *bellonensis*	1/3	0/2								1/5
" *tertius*	2/2	3/3	0/1							5/6
" *fallax*	1/2	0/2	0/2							1/6
" *œdematiens*	1/2	1/2	1/2							3/6
" *bifermentans*	1/3	0/3	0/2							1/8
" *histolyticus*	2/2	2/2	0/2							4/6
Total, all species	22/36	13/29	8/26	4/20	3/13	1/10	1/7	1/8	0/1	53/150
Percentage positive	61.1	44.8	30.8	20.0	23.1	10.0	14.3	12.5	0	35.3

liquid, usually glucose broth, in which they had been grown, and the period of exposure is reckoned from the time when a bacillus issued from the isolating pipette until it was deposited in the test-tube under anaerobic conditions. To the time of full exposure on the cover-glass one should add, theoretically, the time necessary for the reduction of any free oxygen introduced with the organism into the test-tube. However, the time necessary to attain sufficient anaerobiosis for growth must be short, since a very tiny amount of liquid is brought into 10 cc. of medium.

It appears from Table III that, on the whole, the probability of growth of young bacilli decreases with the increase in the time of exposure to the air, and that different species vary somewhat in sensitiveness to oxygen, as far as can be judged from the comparatively small numbers under each.

The results obtained with *vibrion septique* are not included in this table, since a different method of isolation was used for the batch in which a positive was obtained. Nearly 100 trials with sowings of young bacilli of *vibrion septique* were made by the usual method without success. The organisms were living when isolated, since in many cases the bacillus was distinctly motile, although motility ceased after an exposure to the air of $\frac{1}{2}$ or $\frac{3}{4}$ minute. A great variety of media was employed. The addition to glucose broth of salicin, of unheated pieces of rabbit kidney, and of broth from filtered young cultures of *vibrion septique* and of *Bacillus welchii* gave no success. It hardly seems likely that the medium was at fault, since single spores of this species grew readily in semisolid serum glucose agar—in one series of four all were positive. Furthermore, larger sowings of young bacilli grew rapidly in every medium tested. The time of exposure to air was cut down to $1\frac{1}{4}$ to 2 minutes by the employment of transfer pipettes prepared in advance and by the simultaneous use of two pipettes, but no growth was obtained. Sowings of ten or fifteen bacilli after an exposure to the air of 4 to 6 minutes also gave negative results.

Finally, a series of isolations was made under sterile paraffin oil spread on the cover-glass. The first oil series gave one positive out of seven sown. The organism which grew was motile when transferred. A second paraffin oil series of eight transfers gave all negatives, and all of a series of fourteen isolated under vaseline or vaseline diluted with paraffin oil were also negative. Motility persisted somewhat longer under vaseline than in similar droplets exposed to the air. In small hanging drops made in an atmosphere from which oxygen had been removed by pyrogallic acid and potassium hydroxide motility persisted for an hour or more.

It seems probable that young bacilli of *vibrion septique* are especially sensitive to free oxygen, and that this sensitiveness explains in large part the negative results obtained after the transfer of single bacilli.

Our experiments tend to confirm those of Bachmann (4), who found the vegetative forms of certain anaerobes very sensitive to an exposure to air of only 10 minutes plus the time required to obtain full anaerobiosis subsequently. In Bachmann's experiments bacilli were exposed to the air in agar plates, and the plates were subsequently brought under anaerobic conditions in which organisms remaining viable might form colonies.

In our experiments spores showed no reduction in the proportion of positives as the result of exposure to air during periods as great as 85 minutes at least. In 61 one-spore sowings, in which the time of exposure to air was noted, the periods of exposure and the proportion of positives were as follows: 12 to 17 minutes, 2/3; 18 to 23 minutes, 2/6; 24 to 30 minutes, 6/9; 31 to 36 minutes, 6/8; 37 to 43 minutes, 5/9; 44 to 60 minutes, 6/10; 61 to 72 minutes, 4/10; 73 to 85 minutes, 3/6. Total for all periods, 34/61. These results represent the aggregate of six species and include only batches in which at least one positive occurred.

The effect of exposure to air on anaerobes was not made the subject of a special study in our experiments, but only as incidental to the technique of isolation. It would appear that in the isolation of bacilli one should minimize the exposure to air as far as possible, while the viability of spores is not appreciably affected by an exposure of an hour or more.

Lag.

In a great majority of the experiments the inoculated test-tubes were not enclosed in an anaerobic jar, and the time of first appearance of growth could be approximately noted. In all positives occurring in tubes in which bacilli were sown, growth appeared on the day following inoculation. The first appearance of cloudiness in broth or of a colony in agar varied approximately from 16 to 21 hours after sowing. Growth from single spore sowings showed a marked tendency to lag. The amount of lag by days is shown in Table IV. A day was reckoned to the time of the last observation, usually about 5 p.m. If growth appeared on the 1st day, it was usually later than in sowings of single bacilli of the same species. It is seen from Table IV that about 37 per cent of the positives appeared on the 2nd day or later,

thus showing a lag of a day or more. The condition of the spores sown is doubtless an important factor. This fact is illustrated by the results with the washed spores of *Bacillus tertius*. Spores were twice washed by centrifugation in normal salt solution, and two of the three positives lagged 3 days or more. Three grew out of a series of five sown. Unwashed spores from the same source gave all positives, and all grew without lag. In addition to the washing the spores were kept several hours in salt solution at refrigerator temperature.

TABLE IV.

Time of Appearance of Growth from Single Spores Sown in Various Media in Test-Tubes.

Species.	Total No. of positives.	Day following inoculation on which growth appeared and incidence of positives.						
		1	2	3	4	5	6	8
B. sporogenes..................	20	6	12	2				
" *tetani*......................	12	9	2		1			
" *putrificus*..................	6	4	2					
B. of Ghon Sachs..............	4	4						
B. bellonensis.................	4				2		1	1
" *œdematiens*..................	4	4						
" *tertius* (unwashed spores).....	8	8						
" " (washed ").....	3	1			1	1		
" *bifermentans*................	7	7						
Vibrion septique...............	4	2		1	1			
Total......................	72	45	16	3	5	1	1	1

The marked tendency to latency observed in the growth of the spores of some species illustrates one source of difficulty in separating these from other species by plate methods.

Variability in Morphology of Bacillus sporogenes.

The method employed in isolation is available for a study of variability in anaerobes as in aerobes, as is shown by the following experiment. Four spores from a culture of one-cell origin were isolated and sown in hanging drops. Of the four colonies formed, one showed a marked tendency to the formation of filaments, these appearing in the form of a fine network. Six spores from the filamentous colony

were isolated in a new series of hanging drops, and all showed the same filamentous tendency to a greater or less degree as compared with a series of controls from a non-filamentous source. In further series on a different medium the filamentous strain reverted to normal. A long filament isolated and transferred directly to a test-tube gave the same type of growth as controls.

DISCUSSION.

It is a matter of common knowledge that large sowings of bacteria into a new medium give a better chance of growth than small sowings. Large sowings offer a larger assortment of individuals, some of which may be more vigorous or otherwise better capable of adapting themselves to new conditions, and, furthermore, any growth products carried over with the bacteria may favorably modify the new environment. When a single cell is sown the change in environment is more complete, and unless the new environment is identical with the old a process of adaptation may be necessary. In the experience of the writer, single aerobes have often given 100 per cent of positives when sown into as much as 10 cc. or more of new medium favorable to the species sown.

The importance of employing a suitable medium is shown by experiments on Pneumococcus Type I. Two batches of plain broth made during successive weeks and apparently similar in constituents and reaction were compared. Six tubes of Batch 1 and six of Batch 2 were alternately sown with pneumococcus taken at the height of growth. Tubes of Batch 1 received each one pair of pneumococcus, and those of Batch 2 received each one to four pairs. All the tubes of Batch 1 showed abundant growth which proved to be pure culture of pneumococcus, while all the tubes of Batch 2 remained sterile. All the tubes contained 10 cc. of medium. A flask containing 75 cc. of Broth 1 also gave an abundant growth with a sowing of one pair. Large sowings in Broth 2 grew readily.

When single anaerobes are sown, the lack of proper conditions of anaerobiosis may offer more obstacles to growth than is the case when larger sowings are employed. Burri and Kürsteiner (5) have shown that the growth of *Bacillus putrificus* initiated under strict anaerobic

conditions may continue with increased activity under conditions of aerobiosis unfavorable to the beginning of growth.

In our experiments 100 per cent of positives were obtained with certain lots of five or six tubes each in which single spores of *Bacillus tertius*, *Bacillus sporogenes*, and *Bacillus bifermentans* were sown. Single spores from a 48 hour culture of *Bacillus subtilis* gave all positives in six tubes of glucose broth with a vaseline layer. The medium was the same as that used for anaerobes except that the free oxygen was not expelled by heat. These results would indicate that the technique of the transfer of the single organism is not at fault, and that failures to grow should be ascribed to a lack of viability of the organism or to its failure to adapt itself to a new environment. The viability of anaerobic bacilli is affected by exposure to air, a fact which explains in part the small proportion of positives as compared with single bacilli or cocci of aerobes. The negatives in spore sowings can hardly be due to lack of proper conditions of anaerobiosis, since spores are little sensitive to short exposures to air, and abundant time is available for the reduction in the new medium of the small quantity of free oxygen introduced with the spore. The quantity of medium to which the spore is transferred can hardly be an important factor in itself, since spores sown in small hanging drops give a smaller proportion of positives than when sown into test-tubes. The quality of the medium is probably more often the determining factor, and if viable spores and a medium exactly suited to each species are employed, all, or practically all the spores might be expected to grow. The process by which an organism adapts itself to a new and less favorable medium is imperfectly known. Our experiments indicate that organisms apparently similar vary greatly in their power of adaptation.

SUMMARY.

The pipette method has proved a feasible method of obtaining one-cell pure cultures of anaerobes.

Both bacilli and spores may be used as seeding material, but spores give a much higher percentage of positives.

Boiling alone affords a sufficient degree of anaerobiosis to the medium for initiating one-cell growths, and semisolid agar is the most convenient form of medium.

Exposure to air during isolation apparently has no effect on the viability of spores of anaerobes, but young bacilli of some species suffer from a comparatively short exposure to free oxygen.

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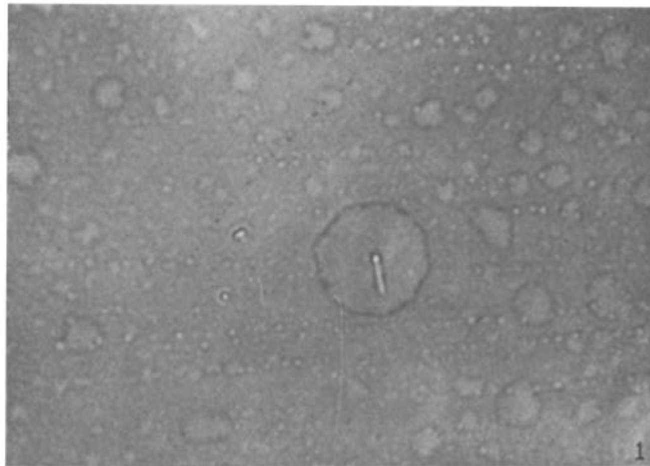
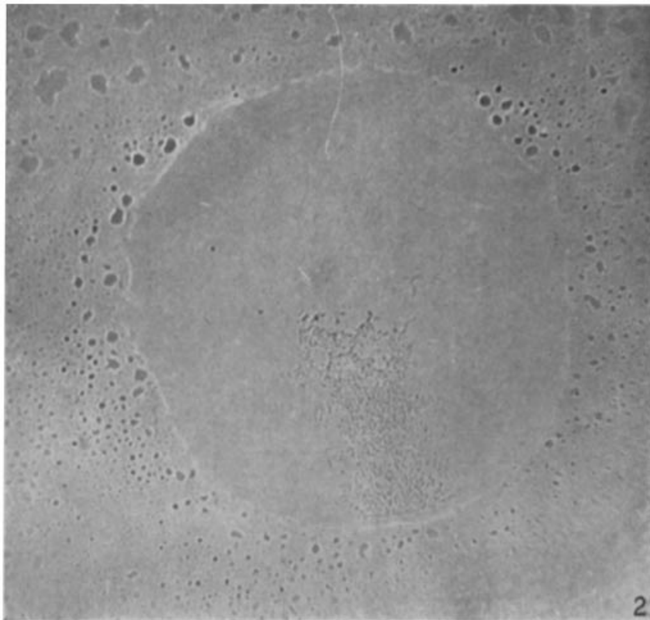
EXPLANATION OF PLATE 23.

FIG. 1. Spore-bearing bacillus of *B. tertius* isolated in a hanging drop of broth. × 1,000.

FIG. 2. Colony of *B. sporogenes* grown from a single spore in a hanging drop of semisolid glucose agar. × 120.

FIG. 3. Colony of *B. tetani* grown from a single spore in glucose agar containing tetanus antitoxin. About natural size.





(Barber: Anaerobes.)