APPLICATION OF 'AGAR FILM METHOD' ON SINGLE CELL CULTURE OF TUBERCLE BACILLI

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Single cell cultivation of bacteria used to require a highly advanced technique and much experience. Recently, however, technical difficulties have been overcome to a large extent by the appearance of the de Fonbrune pneumatic micromanipulator attached with a specially designed microforge and the phase contrast microscope. Our previous paper (Katsuyama *et al.*, 1957) reported single cell cultivation of *E. coli* with such apparatus for the study of streptomycin resistance. The cultivation was conducted by so-called "agar film method" originally developed by Kyo (1935) and Minomo (1942). The technique was described in detail in that paper (Katsuyama *et al.*, 1957). Beside of it, the authors have been endeavoring also to devise a single cell culture method for tubercle bacilli. Because, despite of our repeated trials, the original "agar film method" never succeeded in making a single cell of the tubercle bacillus develop to form a visible colony in concave space of a depression slide. Such cultivation environment was considered unsuitable for tubercle bacilli which require a prolonged period for visible colony formation. To the best of our knowledge, there has never been a report of satisfactory method for single cell culture of tubercle bacilli as yet. However, the present authors have finally reached a new device applicable to the above purpose. The present paper is principally describing the technical aspects together with examples of its application to drug sensitivity test.

PRINCIPLE OF THE NEW DEVICE

Bacterial cells are drawn into a micropipette directly from a dispersed culture of tubercle bacilli in Tween-albumin medium, then they are placed one by one onto twelve agar film sections mounted on a coverslip according to the method described in the previous paper (Katsuyama *et al.*, 1957). After that, each section is stripped off from the coverslip and transferred onto the surface of Kirchner agar slant so that the section face attached with a single bacterial cell may be upside. The slant tubes thus inoculated are incubated at 37°C for 4 to 8 weeks. The detail will be described below.

METHODS AND MATERIALS

1) *Preparation of seed suspension:* For single cell cultivation of tubercle bacilli, it is of the utmost importance to use a bacillary suspension of high viability and of good dispersion. The reasons are obvious. First of all, if viability is not sufficient, it may be possible that the bacterial cells taken are not alive or do not develop into colonies. Secondly, if bacillary suspension is not well dispersed and consists of many small and large clumps, then single cell cultivation would be impossible from the beginning and a capillary micropipette would not work.

Accordingly, the authors first employed a well-ground bacillary suspension prepared by rotating a round flask containing many crystal beads and a semi-dried mass of tubercle bacilli. However, this material was soon found unsuitable for the present purpose. Under the observation by the phase contrast method of microscopy, the bacilli were not morphologically intact and there were many bacillary debris, and still many clumps of various size. This state of the bacterial suspension made the later mani-

Fig. 1. Colony formation from a single cell of tubercle bacilli, H37Rv strain. (5 week growth).

pulation process extremely difficult, particularly at the stage when a single intact cell is to be selected and left on an agar film section. After this unsatisfactory result, the authors conceived of the utilization of a Tween-albumin medium culture of tubercle bacilli for this purpose. Usually, the grown organisms were not completely dispersed but some were in minute clumps. In this case, however, it was possible by our technique to fish up monodispersed cells selectively into a micropipette. On an agar film section, the bacilli were perceived bright with yellow-greenish light in the microscopical field of vision. In this way, this type of culture was found available for the present purpose. Here, it must be added that the Tween-albumin medium culture of tubercle bacilli should be subcultured at least three times with short intervals (one week or so) before its presentation to such experiment. Thereby, the viability of the culture would be increased to a sufficient degree. At the same time, daily shaking of the culture tubes would be necessary to promote the bacillary dispersion.

2) *Kirchner agar film medium:* Any microscopically-visible particles must be removed from agar. Because, it is sometimes difficult to distinguish the bacilli from

those particles through the microscope. For this purpose, methanolextracted agar is recommended. Kirchner basal medium added with this agar in 3 per cent was prepared and delivered into test tubes and sterilized. Just before use, the medium was melted and added with horse serum in 10 per cent, and then poured into a Petri dish warmed beforehand. Coverslips were floated on the surface of the melted agar medium one after another, then soon removed from it with tweezers. Holding the coverslip in the vertical direction for a while, excessive agar was removed and a thin agar film was left on its surface. When the agar was completely coagulated, margins at all sides were cut off with an operating knife and the remaining part was divided into twelve small sections. The coverslips mounted with agar medium as above were preserved in a Petri dish containing absorbent filter paper saturated with distilled water.

3) *Kirchner agar slant*: The same kind of medium as above was delivered into test tubes in the amount of 6 cc, and coagulated in a slant position.

4) *Technique to isolate a single bacterial cell*: See the previous paper (Katsuyama *et al.*, 1957).

5) *The phase contrast method of microscopy*: The microscope was equipped with a long-working distance condenser (Chiyoda) suitable for observation of objects mounted on a moist chamber placed on the specimen plane of the microscope. Observation was made in an enlargement of $\times 200$ or $\times 400$ using an object lens, the B.M. type of $\times 20$.

SOME EXAMPLES OF SINGLE CELL CULTIVATION

Example 1: Being isolated from a 9 day growth culture of H37Rv tubercle bacilli in Tween-albumin medium, a single cell was cultivated in the original method just described above. After 3 week incubation at 37°C, colony formation was observed in 52 Kirchner agar slant tubes out of the total 56 tubes inoculated.

Example 2: Using a 7 day growth culture of H2 strain of tubercle bacilli, single cell cultivation was made. An agar film section attached with a single

Table 1. Results of single cell culture of tubercle bacilli by the present method

Strain	No. of isolated single cells	No. of colony formation	Percentage of positive colony formation
H37Rv	9	5	55.6(%)
H2	21	15	71.4
H37Rv	19	9	47.4
H2	18	7	38.9
Aoyama B	24	14	58.3
H2	47	27	57.4
H2	46	21	45.7
Aoyama B	70	51	73.0
H2	31	19	64.5
H2	64	38	59.4
H2	57	46	80.7
H2	53	28	52.8
			(Average 61.0%)

Note: All trials were made after the given strains were passaged three times through Tween-albumin medium.

cell was placed on an egg medium slant (Ogawa medium) instead of a Kirchner agar slant. After 4 week incubation at 37°C, colony formation was observed in 7 cases out of the total 20.

Example 3: Using the same culture as *Example 2*, single cell cultivation was made. This time, however, an inoculated agar film section was put into a tube of Tween-albumin medium. In this way, submerged growth was observed in 13 tubes out of the total 20 ones.

In this series of experiment, it appeared that Kirchner agar slants are most efficient to receive inoculated agar sections. Besides, there is an advantage in such solid medium that a bacterial population developed from a single cell would present a colony appearance peculiar to each ancestor cell. This would be helpful in the case of the study of bacterial dissociation. In view of these facts, it was decided to use Kirchner agar slants in all the experiments thereafter. Table 1 indicates a summarized data of the single cell culture so far made in this method.

DRUG SENSITIVITY TEST BY SINGLE CELL CULTIVATION

Mycobacterial strains used: H37Rv, H37RvR-SM, H37RvR-INH and a streptomycin-dependent strain of tubercle bacilli (18b strain) were employed. The first three strains were cultured in Tween-albumin medium containing no drug and the last was cultured in the medium containing streptomycin at 200 µg per cc. A 10 day growth culture tube of each strain was offered to the present experiment.

Drug concentration: Streptomycin or isoniazid was added to both Kirchner agar film sections and Kirchner agar slants. These drugs were added to a melted state of Kirchner agar basal medium at 50°C together with horse serum to make drug concentrations as desired.

Principle of the test: The test was conducted in the following way. For instance, a single bacterial cell was placed on an agar film section of 10 µg streptomycin per cc, and then the section was stripped off and transferred onto the surface of a Kirchner agar slant of the same streptomycin concentration (10 µg per cc). In this way, single cells of each strain were cultivated at various concentrations of streptomycin or isoniazid, including drug-free media.

Results: The data in this experiment were presented as Fig. 2. As can be seen from this figure, single cell cultivation of H37RvR-INH was successful in all the 5 cases of drug-free environment. This observation indicates that the Tween-albumin culture of H37RvR-INH was considerably high in viability. Nevertheless, the percentage of colony formation in 5 or 10 µg isoniazid per cc environment was under 50 per cent. Therefore, it would be concluded that this isoniazid-resistant strain has not a pure bacterial population concerning the resistance against 5 to 10 µg per cc. As for H37Rv strain, colony formation was poor even in drug-free environment indicating that the viability of the Tween-albumin culture was not satisfactory. Accordingly, at 0.1 to 0.2 µg streptomycin per cc, colony formation was considerably rare, and no colony

Strain tested	Drug concentration (μg per cc)					
	Streptomycin					
	0	0.1	0.2	0.4	0.8	1.6
H37Rv	●○ ●○ ●○ ●○ ●○ ○	●○ ○ ○ ○ ○ ○ ○	●○ ●○ ○ ○ ○ ○	○ ○ ○ ○ ○ ○	○ ○ ○ ○ ○ ○	○ ○ ○ ○ ○ ○
	Streptomycin					
	0	10	50	100	1000	
H37RvR-SM	●● ●○ ●○ ●○ ●○ ●○ ●	●● ●● ●● ●● ●○ ●○ ●	●● ●● ●● ●● ●○ ●○ ●○	●● ●● ●● ●● ●○ ●○ ●○	●● ●● ●● ●● ●● ●● ●	
	Streptomycin					
	0	10	50	100	1000	
A SM-dependent strain named 18b	○ ○ ○ ○	○ ○ ○ ○ ○ ○ ○	●○ ●○ ○ ○ ○ ○ ○	●○ ●○ ●○ ○ ○ ○ ○	●○ ●○ ●○ ○ ○ ○ ○	
	Isoniazid					
	0	1	5	10		
H37RvR-INH	● ● ● ● ●	●● ●● ●○ ●○ ●○ ●	●○ ●○ ●○ ○ ○ ○ ○	●○ ●○ ●○ ○ ○ ○ ○		

Fig. 2. Drug sensitivity test of various strains of tubercle bacilli by single cell cultivation

Note: ● Successful colony formation } from a single cell.
○ Failed colony formation }

appeared at over $0.4 \mu\text{g}$ per cc. Concerning H37RvR-SM strain, it was found that colony formation occurred in 100 per cent at $100 \mu\text{g}$ streptomycin per cc but approximately in 64 per cent in drug-free environment. However, further analysis was not made to examine whether or not this phenomenon was due to the mixed presence of streptomycin-dependent cells in this strain. In the case of the streptomycin-dependent strain (18b strain), colony formation did not occur at less than $50 \mu\text{g}$ streptomycin per cc, and the viability of the original Tween-albumin culture was presumed considerably poor.

In the above experiment, it must be noted that test was made concerning drug sensitivity of each single bacterial cells and not of the bacterial population. However, it must be also emphasized that failed colony formation does not necessarily indicate the drug sensitiveness of an isolated single cell. Because, there is another possibility that the isolated single cell might be a dead cell.

STREPTOMYCIN SENSITIVITY OF 15 CLONAL DESCENDANTS EACH DEVELOPED
FROM A SINGLE BACTERIAL CELL ISOLATED FROM H37Rv STRAIN

As stated in *Example 1* of the part "Some Examples of Single Cell Cultivation", 52 colonies were obtained by single cell cultivation of H37Rv tubercle bacilli. At the time of 5 week growth, 15 colonies were selected at random out of them. From each of these selected colonies, a bacillary suspension was prepared in the following way. A colony to be tested was scraped off from the slant and put into a round flask of 300 cc capacity containing 60 crystal beads. The flask was rotated manually with occasional adding of distilled water. The density of bacillary suspension was adjusted to show the same turbidity as the standard 1 mg per cc suspension. This suspension was inoculated in the amount of 0.1 cc to each Ogawa egg medium slants without or with streptomycin in the concentration of 1, 5, 10 and 50 μg per cc. At the same time, the viability of the suspension was examined by serial tenfold diluting and planting procedure. In this way, streptomycin sensitivity of the selected 15 colonies was compared with that of the parent H37Rv strain. The inoculated slants were incubated at 37°C for 4 weeks. The result was summarized in Table 2. Brief survey of this table

Table 2. Streptomycin sensitivity of 15 daughter strains obtained by
single cell cultivation from H37Rv strain

Strain tested		Size of inoculum to each tube (Viable cells)	Streptomycin concentration ($\mu\text{g}/\text{cc}$)				
			0	1	5	10	50
The original H37Rv strain		560×10^3	##	++	10*	4*	0
Daughter strains	No.						
	1	100×10^3	##	++	0	0	0
	2	70×10^3	##	+	0	0	0
	3	320×10^3	##	+	0	0	0
	4	10^3	##	+	0	0	0
	5	1640×10^3	##	+	0	0	0
	6	20×10^3	##	$\pm$	0	0	0
	7	7×10^3	##	0	0	0	0
	8	8×10^3	##	0	0	0	0
	9	850×10^3	##	$\pm$	0	0	0
	10	160×10^3	##	$\pm$	0	0	0
	11	560×10^3	##	+	0	0	0
	12	1850×10^3	##	0	0	0	0
	13	8×10^3	##	0	0	0	0
	14	3×10^3	##	3*	0	0	0
	15	80×10^3	##	$\pm$	0	0	0

Note: One tube (slant) was employed for each drug concentration.

* Number of solitary colonies. (0) indicates no growth and other symbols (##, ++, +, and $\pm$) indicates roughly the grade of confluent growth

reveals some interesting facts. Namely, a small number of colonies appeared at 5 and 10 μg per cc in the case of the original H37Rv strain, but no colony occurred on the slants of those drug concentrations when inoculated with any of the 15 substrains obtained from the former by single cell cultivation. This observation seems to indicate the fact that the colonies obtained by single cell cultivation has a purer bacterial population regarding streptomycin sensitivity than the parent strain. No. 12 colony appears particularly sensitive to streptomycin, because it did not grow at all even at 1.0 μg per cc despite the largest size of inoculum in this table. On the contrary, No. 4 colony grew at 1.0 μg per cc, although the size of inoculum was the minimum. In other words, the table shows that H37Rv strain consists of bacterial cells which are not the same in streptomycin sensitivity, and that a colony developed from a single cell would be a purer population than the parent strain in the genetical sense.

DISCUSSION

As described above, it was made clear that a single cell of the tubercle bacillus placed on a suitable environment can multiply to form a visible colony. This is of course nothing particular in view of our bacteriological common sense. In practice, however, it has been extremely difficult to confirm this fact under our microscopical observation. And, the present author's success is simply due to the technique that a single cell placed on a thin film-like agar section is transferred on the surface of a Kirchner agar slant instead of being cultured in the sealed concave space of a depression slide as in the case of *E. coli*. The greatest reason why the authors have been endeavouring to devise a method of single cell cultivation of the tubercle bacillus lies in the hope that an analytical study on the virulence of the tubercle bacillus might be possible by using that technique. The modern microbial genetics indicates that a clone does not always mean a completely pure bacterial population concerning any bacterial characteristic. However, in view of the last experiment in this paper, it could not be denied that a daughter strain obtained by single cell cultivation would be a purer population than the parent strain in the genetical sense. Being encouraged by this observation, the authors are now conducting a large scale of experiment to dissociate a strain of virulent tubercle bacilli by single cell cultivation searching for an avirulent H37Ra-type variant.

SUMMARY

A method was devised for single cell cultivation of tubercle bacilli. The principle is as follows. Bacillary cells are fished up into a micropipette directly from a dispersed culture of tubercle bacilli in Tween-albumin medium, then they are placed one by one onto thin film-like Kirchner agar sections mounted on a coverslips. After that, each section is stripped off from the coverslip and transferred onto the Kirchner agar slants so that the section face attached with a single cell may be upside. The slant tubes thus inoculated are incubated at

37°C for 4 to 8 weeks. By this method, single cell cultivation can be successful in a rate of average 60 per cent. Examples of drug sensitivity test of single cells were demonstrated concerning various variant strains of the tubercle bacillus. An evidence was presented that a daughter strain obtained by single cell cultivation would be a purer population than the parent strain concerning a given bacterial characteristic.

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REFERENCES

- KATSUYAMA, S., KANAI, K. & YANAGISAWA, K. (1957): Use of single cell cultivation technique in the studies on streptomycin resistance of *E. coli*. *Jap. J. M. & Biol.*, 10, 157-169.
- KYO, K. (1935): Studies on the bacterial growth by single cell culture method using film agar. "Zikken Igaku Zasshi", 19, 1346-1373 (text in Japanese).
- MINOMO, T. (1942): Significance of so-called "Pettenkoferien"; Studies by single cell culture method using film agar. "Zikken Igaku Zasshi" 26, 406-432 (text in Japanese).
- SHIBATA, I. (1957): Studies on drug-resistant and susceptible tubercle bacilli applying the single cell culture method. 1. Single cell culture of drug resistant tubercle bacilli and standard laboratory strains. "Kekkaku", 32, 17-22 (text in Japanese).