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Studies on the Isolation of Bacteria Capable of Lysing the Cell Walls of Various Lysozyme-resistant, Pathogenic Bacteria *1, *2

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SUMMARY

For the isolation of bacteria capable of lysing the cell walls of lysozyme-resistant, pathogenic bacteria, cell wall agar plates prepared with walls of BCG were inoculated with soil samples, incubated at 24–30°C and examined at intervals for the appearance of the colonies surrounded by clear lysed zones. Three strains, the colonies of which exhibited distinct zones of lysis on these opaque media, were isolated in a pure culture and tentatively classified as a *Micromonospora* sp., a *Streptomyces* sp. and a *Flavobacterium* sp. respectively.

All of these strains exhibited definite lytic activities on a cell wall agar medium containing walls of *Corynebacterium diphtheriae*, *Staphylococcus aureus* and *Streptococcus pyogenes* (Lancefield group A) as well as on the BCG cell wall agar plates.

INTRODUCTION

As stated in a previous report (Kotani *et al.*, 1959), within recent years striking advances have been made in the field of bacterial anatomy and it has become possible to study various activities of bacteria at a subcellular level, by isolating each of the structural units constituting bacterial cells and examining its properties or activities from various points of view. Thus a number of reports have appeared on the isolation or characterization of subcellular structural units, such as cell walls (Salton, 1956), protoplasts (Weibull, 1956; McQuillen, 1956), the cytoplasmic membrane (Weibull and Bergström, 1958; Gilby *et al.*, 1958), intracellular particles (Alexander, 1956) and nuclears bodies (Spiegelman *et al.*, 1958).

Such a rapid advance is much due to utilization of the cell wall lytic activity of lysozyme as well as on technical devices such as the Mickle tissue disintegrator, sonic oscillator and ultracentrifuge.

It should be pointed out, however, that the majority of lysozyme-sensitive bacteria are non-pathogenic and most part of pathogenic bacteria are lysozyme-

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*2 A preliminary report was presented at the 31st Annual Meeting of the Society of Japanese Bacteriologist held in Okayama, April 1958.

resistant, so that cell wall lytic activity of lysozyme has been almost exclusively used for studies on the biology of bacteria, but is of little or no use for the analysis of their pathogenicity. Therefore it is most desirable to find cell wall lytic agents active on the cell walls of pathogenic bacteria.

The present report describes the results of experiments, which have succeeded in isolating bacteria exhibiting a distinct cell wall lytic activity on lysozyme-resistant, pathogenic bacteria; BCG, *Corynebacterium diphtheriae*, *Staphylococcus aureus* and *Streptococcus pyogenes* (Lancefield group A).

MATERIALS AND METHODS

1. *Organisms* and growth conditions:* Bacteria used as test organisms for the assay of cell wall lytic activities and their growth conditions are as follows.

Mycobacterium tuberculosis (strains H37Rv and H37Ra), *Mycobacterium bovis* (strain Ravenel) and BCG (strain Takeo); 7–12 day old cultures grown on Sauton's medium or 9–12 day old cultures in 0.05 per cent Tween 80–bovine plasma albumin medium prepared according to the original method of Dubos (1947) or the modified method of Schaefer *et al.* (1949).

Corynebacterium diphtheriae (strain Park-Williams No. 8, Toronto Harvard); 24–48 hour old cultures grown in the medium used for toxin production in the Research Institute for Microbial Diseases, Osaka University (a modified Taylor's medium) which was mechanically shaken at 120 strokes per minutes at an amplitude of 6 cm or 18–24 hour old cultures on the slants of Loeffler's coagulated serum medium.

Staphylococcus aureus (strains 209P and Newman-1); 24–48 hour old cultures on nutrient agar medium.

Streptococcus pyogenes, Lancefield group A (strain O89); 24–48 hour old cultures grown in Todd-Hewitt's medium.

Bacillus subtilis (strain NRRL-B558) and *Bacillus megaterium* (strain KM); 18–24 hour old cultures grown in nutrients broth which was mechanically shaken.

Bacillus cereus (strain No.2); 72 hour old cultures in nutrient broth mechanically shaken (under these cultural conditions, most bacteria sporulated).

Micrococcus lysodeikticus (strain No.2665) and *Escherichia coli* (strain UKT-B); 18–24 hour old cultures on nutrient agar medium.

Nocardia asteroides (strain Blanchard-Duke); 48–96 hour old cultures grown on Sabouraud's liquid medium with mechanically shaking.

Candida albicans (strain No.1033) and *Saccharomyces cerevisiae* (strain IFO337); 18–24 hour old cultures on Sabouraud's agar medium.

All bacteria used were cultivated at 37°C and each was harvested by filtration or centrifugation, washed twice with physiological saline and finally suspended in a small amount of saline as thick suspension (in case of *Mycobacterium* and *Corynebacterium,* 0.03 per cent Tween 80 solution was used as both washing and suspending fluid instead of saline).

2. *Preparation of cell walls:* BCG and *C. diphtheriae* cell walls—The procedures adopted were essentially the same as those described by Kotani *et al.* (1959) and Kitaura *et al.* (1959) with a slight modification.

The cultures of BCG grown on Sauton's medium and those of *C. diphtheriae* grown in the medium for toxin production as described in the foregoing section were used as starting materials. The former was harvested by filtration through a sintered glass filter (Iwata and Co., 17G3) and the latter by centrifugation with the Sharples-type centrifuge (Tomoe Shoko Co., Open-Type 5A). Both cells thus harvested were washed with a large quantity of distilled water or 0.067 M phosphate buffer (pH 7.8) and kept frozen in a dry-ice box until

* All, but *Myc. bovis* (strain Ravenel), of the strains of bacteria used in the present study were supplied by the Research Institute for Microbial Diseases, Osaka University. *Myc. bovis* (strain Ravenel) was received from the National Sanatorium, Toneyama Hospital.

used.

The cells, approximately 20 g wet weight, of BCG or *C. diphtheriae* were suspended in 100 ml of the phosphate buffer (as described above) and a 10 ml aliquot of the suspensions was disintegrated for 30 minutes in the Raytheon 50 watt, 9-Kc, Magnetostriction Oscillator (Model S-102A) run at a plate voltage of 90 and an output voltage of 75. To remove unbroken cells, clumps of cell walls and other debris, the disintegrated cell suspensions were centrifuged for 20 minutes at 330 *g* in case of BCG and at 900 *g* in case of *C. diphtheriae*. The supernatant was separated and centrifuged twice more under the same conditions as described above. The supernatant of the third centrifugation, then, was centrifuged at 9,500 *g* for 20 minutes and a cell wall fraction was collected as a sediment. The sediment, after being washed with about 100 ml of the buffer, was suspended in 200 ml of the buffer containing 10 mg/ml of trypsin (E. Merck Co.) and the suspension was incubated at 40°C for 1 hour with shaking in a water bath. The trypsin-treated suspension was centrifuged at 9,500 *g* for 20 minutes and the precipitated cell walls were treated once or twice more with trypsin until little or no reduction in turbidity was caused by the trypsin treatment. The purified cell walls prepared in this manner were centrifuged at 9,500 *g* for 20 minutes, washed twice with the buffer and three times with distilled water of about 200 ml each. The washed cell walls were taken up in a small amount of distilled water as a thick suspension and kept in a refrigerator or in a dry-ice box.

Staph. aureus and *Strep. pyogenes* cell walls—Cultures of *Staph. aureus* grown on nutrient agar plates for 48 hours were harvested by scraping them off the agar with a spatula and those of *Strep. pyogenes* grown in Todd-Hewitt's medium for 48 hours were collected by centrifugation. They were washed with a large quantity of the buffer or distilled water and used for preparation of cell wall materials.

Since cocci in general are more resistant than bacilli to the distintegrating effect of sonic oscillator, the disintegration of the cells was performed as follows. About 50 g (wet weight) of the cells harvested and washed as described above were suspended in 50 ml of the phosphate buffer with about 50 g of glass beads (100–150 meshes) as abrasives. A 5 ml aliquot was disintegrated by placing it in the sonic oscillator run at a plate voltage of 130 and output voltage of 120 for 20 minutes. The disintegrated cell suspension was left standing for 10 minutes and the supernatant fluid was separated by decantation from the bulk of the glass beads which had settled down. It was centrifuged twice at 400 *g* for 20 minutes to remove intact cells and debris.

The further procedures adopted for separation and purification of cell walls were essentially the same as those used in the preparation of BCG and *C. diphtheriae* cell walls.

The purity of the cell wall preparations isolated as described in the foregoing paragraphs was checked by electron microscopy. The cell wall suspensions to be tested were diluted to an appropriate concentration, mounted on collodion films, shadowed with chromium at an angle of 15 degrees to the plane of the film and examined with the Japan Electron Optics Laboratory electron microscope, Model JEM-5G. Electron micrographs of the cell walls used in the present study are shown in Figs. 1–4.

3. *Preparation of opaque agar plate media containing cell walls or living cells of various bacteria:* According to the method of Salton (1955a), cell wall or living cell agar plates were prepared as follows; about 8 ml of the medium consisting of 2.5 per cent agar, 0.1 per cent K_2HPO_4 and 0.05 per cent $MgSO_4 \cdot 7H_2O$, which was sterilized by autoclaving at 15 pounds for 15–20 minutes, was poured into the Petri dishes (8 cm diameter) and allowed to set as a bottom layer; about 1.6 ml of the thick suspension of cell walls (0.1–0.2 g wet weight cell walls/ml of distilled water) or living cells (0.8–1.0 g wet weight cells/ml of physiological saline or 0.03 per cent Tween 80 solution) was added to about 6.4 ml of melted 3 per cent agar, which was maintained at 50°C after sterilization by autoclaving. This mixture was poured on as a top layer. The uniformly opaque agar plates thus obtained, the surface of which was dried by incubating at 37°C for several hours, were used for the isolation of cell wall lytic bacteria from soil or for testing the lytic activities of isolated

bacteria. Use was made of the non-treated, autoclaved and ultra-violet light irradiated*1 cell wall preparations and essentially the same results were obtained with all of them.

4. *Preparation of soil samples for the isolation of cell wall lytic bacteria:* About 2 g samples of soils, collected from various places, were suspended in 15 ml of distilled water and the supension, after vigorously shaking, was left standing for about 30 minutes at room temperature. The supernatant fluid was separated by decantation or low speed centrifugation and used as a source of bacteria capable of lysing cell walls of lysozyme-resistant bacteria.

RESULTS

1. *Isolation of bacteria capable of producing a lysed zone on BCG cell wall agar plates:*

Experiment 1: In August of 1957, twelve samples of soil were collected from various places in Nara and Osaka and used as sources for the isolation of cell wall lytic bacteria. BCG cell wall agar plates were used as an isolation medium and inoculated with the supernatant fluids separated from the water suspension of the soil samples. The inoculated plates (four samples/one plate) were incubated at 24-30°C for five weeks and examined at intervals for the appearance of the colonies surrounded by a lysed zone. To prevent the plates from drying, the gap between the receptacle and lid of the Petri dish was sealed with cellulose tape.

After incubation for twenty six days, eight colonies surrounded by clear lysed zones appeared on one quandrant of one plate. The growth of the colony, which showed the most extensive lysed zone, was picked off and streaked out on another plate. A number of isolated colonies surrounded by distinct zones of lysis appeared after several day incubation (Fig. 5). These colonies, although appearing pure, when streaked out on the plates of nutrient-rich medium*2 containing no cell walls, showed some contamination by bacteria and fungi, the growth of which had so far been inhibited on the cell wall agar by the absence of nutrients other than cell walls. These grew rapidly and luxuriantly and made it very difficult to isolate colonies of cell wall lytic bacteria. Such being the case, the plan for the use of a medium without added cell walls was abandoned and successive transfers of the single colony on the agar plate containing autoclaved BCG cell walls were made until a pure culture of the bacteria in question could be isolated. An examination for the presence of contaminants by microscopy and cultivation on several different media was made. The cell wall lytic bacterium thus isolated was named tentatively L_1 bacterium.

Experiment 2: In June of 1958, eight samples of soil were collected from various places. Through a similar process to that in experiment 1, two bacteria which lyzed BCG cell walls (named L_5 and L_{11} bacteria) were isolated as pure cultures.

The three bacteria thus obtained were maintained on a 0.1 per cent pepton

*1 Sterilization of the cell wall preparations by irradiation with ultra-violet light was performed as follows; cell wall suspensions, about 3 ml, contained in the sterilized Petri dishes (9 cm diameter) were irradiated with the Matsuda germicidal lamp, GL 15 (Tokyo Shibaura Electric Co.) at a distance of about 10 cm for 20 minutes, with constant rotation of the dish. Sterilization was not complete, but satisfactory for the present purpose.

*2 Shinobu's glycerol potato agar (Shinobu, 1953), Sabouraud's agar, nutrient agar and others.

agar slant containing 0.1 per cent Bacto-peptone, 0.025 per cent K_2HPO_4, 0.025 per cent $MgSO_4 \cdot 7H_2O$ and 2.0 per cent agar (with or without BCG cell walls).

2. *Classification of the bacteria isolated on BCG cell wall agar plate from soil:*

The bacterium (L_1) isolated in the first experiment and one (L_5) of those obtained in the second experiment were tentatively identified with a *Micromonospora* sp. and a *Streptomyces* sp. respectively, by Dr. R. Shinobu, Hirano Blanch School, Osaka University of Liberal Arts and Education. The other bacterium (L_{11}) isolated in the second experiment was classified as a *Flavobacterium* sp. according to the description of Bergey's Manual of Determinative Bacteriology (1957, 7 th edition).

3. *The lytic activities of the bacteria isolated from soil on cell wall preparations or living cells of various bacteria:*

To test the lytic activities of L_1, L_5 and L_{11} bacreria on various micro-organisms, the agar plates were prepared with the walls or living cells of a number of bacteria and yeast as presented in the Figures and Table and inoculated by streaking some of the 3 to 7 day old growth of the three isolates on 0.1 per cent Bacto-peptone agar slants. The plates were incubated at 27°C and examined daily for the appearance, extent and clearness of the lysed zones around the growth of test organisms.

The results of the test of the lytic activities of the three isolates on the agar plates prepared with cell walls or living cells of BCG, *C. diphtheriae, Staph. aureus and Strep. pyogenes* are illustrated in Figs. 6-13 (7-10 day incubation at 27°C) It can be seen from these figures that all test organisms exhibited distinct lytic activity on both isolated cell walls and living cells of the four lysozyme-resistant, pathogenic bacterial species examined but on the living cells of BCG. On the plate containing living BCG cells, contrary to expectation, no distinct lysed zone could be produced by any of the lytic bacteria. This is in marked contrast with the distinct lysis observed on the BCG cell wall agar medium. Similar negative results were

Table 1. The lytic activities of the bacteria isolated from soil on the agar plates containing living cells of various bacteria

Test organisms	*M. lysodeikticus* (2665)	*B. megaterium* (KM)	*B. subtilis* (NRRL-B 558)	*B. cereus spores* (No. 2)	*Esch. coli* (UKT-B)	*N. asteroides* (Blanchard-Duke)	*Cand. albicans* (1038)	*Sacch. cerevisiae* (IFO 337)
L_1	+ (T)	+ (C)	+ (C)	—	—	±	—	±
L_5	(+)	++ (C)	—	—	—	±	+ (T)	±
L_{11}	+++ (C)	++ (C)	—	—	—	±	+ (T)	+ (T)

Plates were incubated for 7–10 days at 27°C.

+++, lyzed zone 6mm. or more; ++, lyzed zone 2–6 mm.; +, lyzed zone 0.2–2 mm; (+), lyzed zone confined to the portion of bacterial growth; ±, doubtful lysis; —, no lysis.

C: clear lyzed zone; T: turbid lyzed zone.

obtained with living cells of *Myc. tuberculosis* (H37Rv and H37Ra) and *Myc. bovis* (Ravenel).

Table 1 summarizes the results of the experiments, in which the lytic acitivities of the three bacteria were tested on the agar plates prepared with living cells of the organisms other than the four species described above. It can be seen that L_1 bacterium exhibits weak, but definite, lytic acitivity on *B. megaterium*, *B. subtilis* and *M. lysodeikticus* and the L_5 and L_{11} bacteria produces distinct zones of lysis on *B. megaterium*, *M. lysodeicticus* and *Candida albicans*. None of the lytic bacteria showed evidence of lytic activity when tested on the agar medium prepared with living cells of *Esch. coli* and spores of *B. cereus*. The results of the test on living cells of *Sacch. cerevisiae* and *N. asteroides* were obscure.

DISCUSSION

In recent years a considerable number of reports have been published on cell wall lytic principles of microbial origin, especially those produced by *Actinomycetes* and spore-bearing bacilli (see the reviews of Salton, 1955 b, 1956; Cummins, 1956; Work, 1957; Welsch, 1957, 1958; Strange, 1959 and the original papers of Richmond, 1959 a, b, c).

However, the greater part of these reports is concerned with the studies on the active principles capable of lysing the cell walls of lysozyme-sensitive bacteria and as for studies in which cell wall lytic principles active on lysozyme-resistant bacteria have been dealt with, there are found in the literature only those of Salton (1955) who observed the lytic activities of *Nocardia gardneri*, *Micromonospora chalceae* and others on *Staph. aureus* cell wall agar plates, McCarty (1952 a and b) on the streptolytic enzyme produced by *Streptomyces albus* and Ghuysen (1957) and Salton and Ghuysen (1957) on the staphylolytic principle (actinolysopeptidase) of *Streptomyces albus*. There have been no reports on cell wall lytic agents active on bacteria of *Mycobacterium* or *Corynebacterium*. Although Welsch (1947), in his short communication on actinomycetin, reported that the culture filtrate of *Streptomyces albus*, strain G, was capable of lysing living cells of *C. diphtheriae*, it cannot be decided from this and subsequent papers whether the lysis observed of *C. diphtheriae* cells was really due to lysis of the cell walls or to other mechanisms.

In the present report, results have been presented on studies in which three strains of bacteria capable of producing clear lysed zones on the opaque agar plates containing cell wall preparations of BCG, *C. diphtheriae*, *Staph. aureus* and *Strep. pyogenes* (Lancefield group A) were isolated from soil. What relationships the active principle(s) produced by these cell wall lytic bacteria have with one another or with the streptolytic or staphylolytic enzymes produced by *Streptomyces albus* cannot be decided from the present work and further investigations are needed to answer the question.

As for BCG, all of the bacteria exhibited a distinct lytic activity on the isolated cell wall preparations, but little or no activity on the living cells. The reason why such a contradictory result was obtained remains unexplained (the possibility that Tween 80 used as the dispersing agent for cells of BCG inhibited the activity of the lytic principle was exculded from the result of control experiments on BCG

cell wall agar plates containing a comparable concentration of Tween 80). It should be pointed out in this connection that even when the extensive lysed zones were observed on living cell agar plates, the possibility exists that the lysis observed might be due to a mechanism other than the lysis of the cell wall, for example to acceleration of autolytic processes.

Studies are in progress to isolate the active principle(s) produced by the cell wall lytic bacteria isolated in the present study in a cell free state and to concentrate and purify it and the results will be published in subsequent papers.

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Fig. 1 BCG,

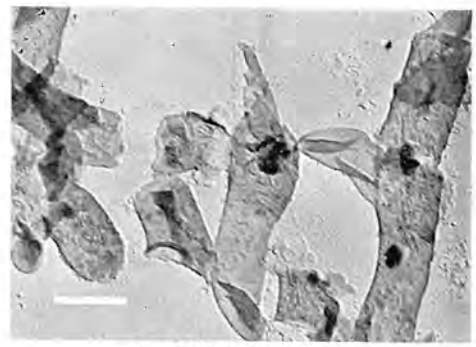


Fig. 2 C.*diphtheriae*,

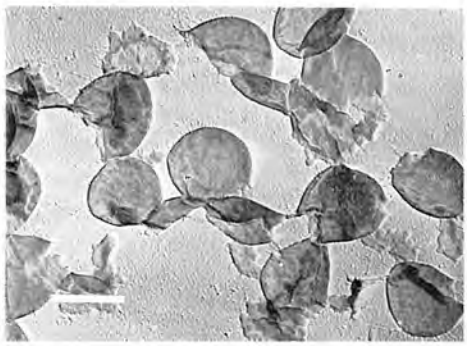


Fig. 3 *Staph.aureus*

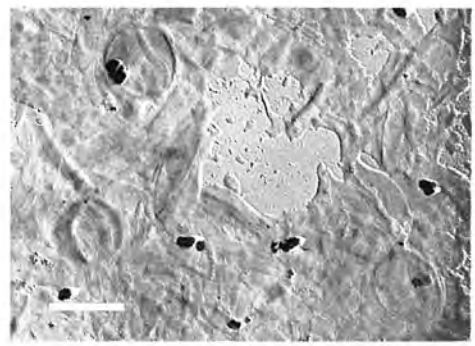


Fig. 4 *Strep.pyogenes*

Figs. 1-4 Electron micrographs of the cell wall preparations used for the assay of the lytic activities of the bacteria isolated from soil. The cell wall samples were mounted on collodion films, shadowed with chromium at an angle of 15 degrees to the plane of the film and examined with the Japan Electron Optics Laboratory electron microscope, Model JEM-5G. Scale : 1μ

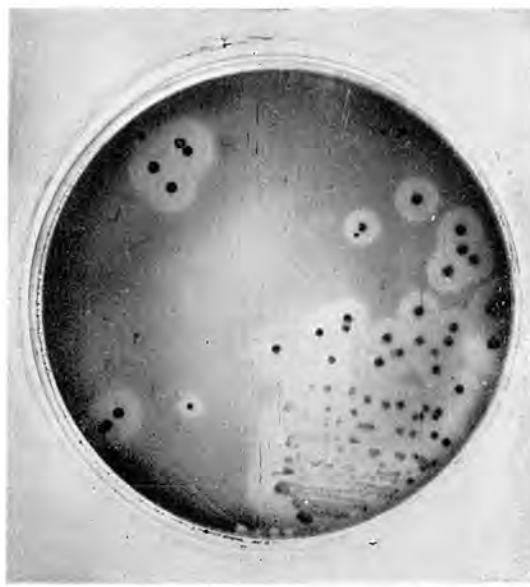
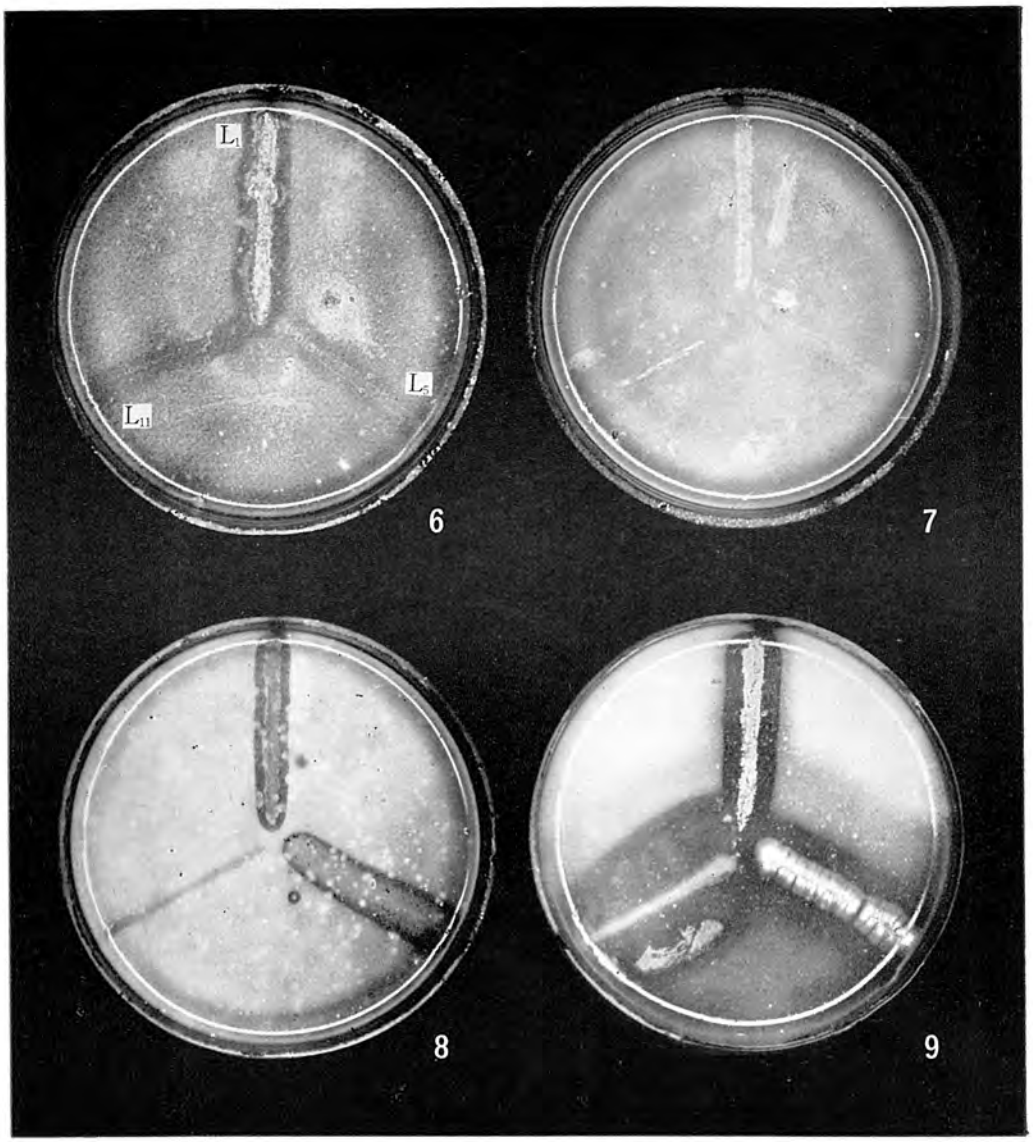


Fig. 5 Isolated colonies of L_1 bacterium surrounded by a distinct zone of lysis.



Figs. 6-13 The lytic activities of the three bacteria isolated from soil, examined on the opaque agar plates containing cell walls or living cells of various bacteria. Photographs were taken after the incubation of the inoculated plates for 7-10 days.

Fig. 6 BCG cell walls, Fig. 7 BCG living cells

Fig. 8 C.*diphtheriae* cell walls Fig. 9 C.*diphtheriae* living cells

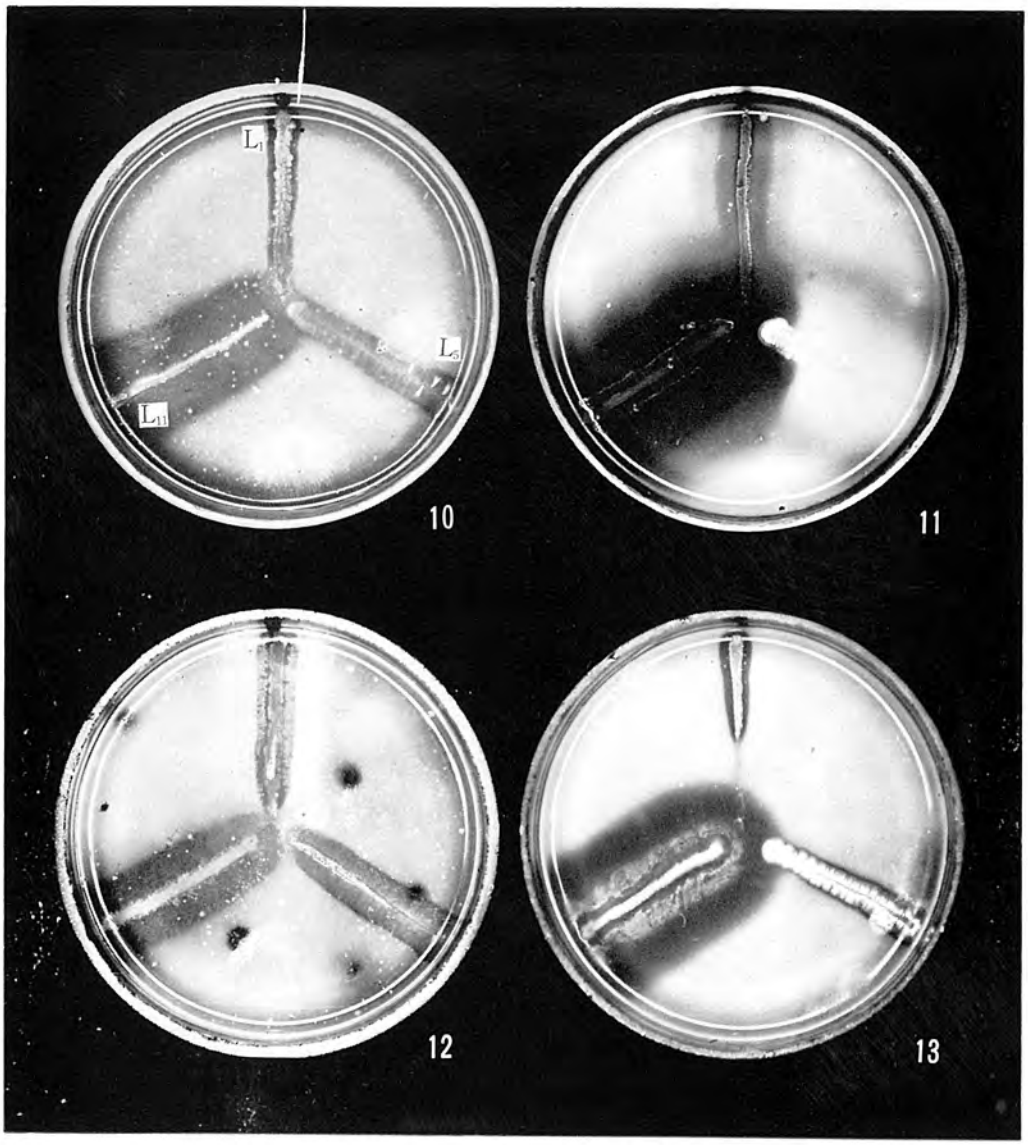


Fig. 10 *Staph.aureus* cell walls
Fig. 11 *Staph.aureus* living cells
Fig. 12 *Strep. pyogenes*cell walls
Fig. 13 *Stred. pyogenes* living cells