

# Culture of Serum-induced Spheroplasts from *Vibrio cholerae*

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Spheroplasts of *Vibrio cholerae* types Inaba, Hikojima, and Ogawa were produced in serum. An excess of lysozyme was added to expedite transformation, and  $\text{CaCl}_2$  was used as the stabilizing agent. At the optimal time for each strain, when less than 1% classical rod forms were observed microscopically, samples were plated on both conventional and modified L-form media. No growth occurred on conventional media; L-form type colonies appeared on L-form media after 2 to 6 days of incubation. L-form colonies could be subcultured on conventional media and required from four to six passages for complete reversion to classical parent forms. Reaction mixtures of Hikojima and Inaba types were passed through membrane filters ( $0.45 \mu$ ); L-form colonies were grown from both strains after spheroplast transformation. Appropriate controls were negative. It is suggested that the replicative particle may be filtrable.

The role of the antibody-complement system in the killing of gram-negative bacteria is well recognized. Many workers have reported on the consequences of bactericidal action and the role of lysozyme in the production of the spheroplast. In a recent report, Davis et al. (1) provided a working model for the sequential stages from rod to spheroplast. In the absence of lysozyme, viable rods are converted to an apparently nonviable form ( $R^*$ ) by the antibody-complement system. Lysozyme acts on these nonviable rod forms ( $R^*$ ) with the production of spheroplasts. Through what appears to be loss of intercellular contents, the spheroplast undergoes transformation and becomes a ghost-like form. Our findings show that the kinetics and the sequential stages for *Vibrio cholerae* conform to the above scheme both qualitatively and quantitatively.

Although there have been many reports on the culture of spheroplasts produced by agents such as glycine, penicillin, etc., only Dienes et al. (2) have reported the isolation in culture of L forms from serum-induced spheroplasts. The present report chiefly concerns the replicative ability of the spheroplast stage and shows that, when appropriately modified media are utilized, spheroplasts can be cultured.

## MATERIALS AND METHODS

**Normal serum.** Serum from one healthy donor was stored at  $-64 \text{ C}$  until used; it was the source of both complement and "natural" antibody.

**Bacteria.** *V. cholerae* types Hikojima NCTC 7270, Inaba NCTC 4693, and Ogawa NCTC 5596 were obtained from the Central Public Health Laboratories, London.

**Conventional media.** *V. cholerae* cultures were maintained on Blood Agar Base (BBL) with 1.0% sodium chloride and 0.1% dextrose added. Broth cultures were made in Brain Heart Infusion (BBL). Both media were adjusted to pH 8.0 with 1 M tris(hydroxymethyl)aminomethane buffer (Sigma Chemical Co., St. Louis, Mo.). Cultures were maintained at room temperature after an initial overnight incubation at  $37 \text{ C}$ .

**L-form media.** L forms were grown on a modification of the medium described by Gutman and associates (4) and contained the following in grams per liter: sucrose, 100.0; Phytone (BBL), 20.0; NaCl, 10.0;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.0; cholesterol, 0.04 dissolved in 95% ethyl alcohol; Ionagar No. 2 (Consolidated Laboratories, Chicago Heights, Ill.), 8.0; and yeast extract (Difco Laboratories), 10.0. Heat-inactivated horse serum (BBL) was added to 20% final concentration after the medium was autoclaved. The liquified medium was then poured into 12 by 50 mm petri dishes with tight covers (Falcon Plastic, Div. of B-D Laboratories, Inc., Los Angeles, Calif.).

**Lysozyme.** Crystalline egg-white lysozyme (Nutritional Biochemicals Corp., Cleveland, Ohio) was added to the reaction mixture in a final concentration of 0.001 mg/ml to insure prompt conversion of killed rods to spheroplasts.

**Absorbed serum.** Serum was batch-absorbed with either bentonite or carboxymethyl cellulose (Cellex-CM; Bio-Rad Laboratories Richmond, Calif.). Absorbed serum did not cause significant lysis of *Micro-*

*coccus lysodeikticus* during 30 min of incubation and was found to retain complement activity.

**Antisera.** Rabbits were immunized with acetone-killed, dried whole bacteria preparations of either *V. cholerae* type Ogawa or type Inaba suspended in Freund's complete adjuvant. The rabbit antisera were made monospecific by cross absorption with the heterologous bacterial preparations.

**Calcium chloride.**  $\text{CaCl}_2$  was added in final concentration sufficient (0.008 to 0.015 M) to stabilize spheroplasts.

**Experimental procedures.** For each study, bacteria from overnight agar cultures were subcultured 3 to 4 hr in broth in a 37 C shaker water bath. During subculture in broth, it was necessary to make repeated subcultures to prevent autolysis of the bacteria. The bacteria were then collected by centrifugation and suspended in 0.15 M NaCl to an optical density of 0.15 at 650  $m\mu$  ( $2.5 \times 10^9$  organisms per ml). Human serum was added to a final concentration of 10 to 15%. (The serum concentration selected was one which yielded at least 99.9% killing of rods in 10 to 15 min as determined by colony count on conventional media.) Egg-white lysozyme was added in excess to insure prompt conversion of rods to spheroplasts. The reaction mixtures were then incubated at 37 C. At intervals, samples were removed, suspended in 1% Formalin, and examined under a phase-contrast microscope. When less than 1% of classical rod forms re-

mained, additional samples were taken from the reaction mixture for spheroplast cultures. Serial 10-fold dilutions of these samples were made in cold saline, and 0.1-ml samples of each dilution were inoculated on the conventional media and on the modified L-form media.

In some experiments, absorbed serum, which had the lysozyme activity removed, was utilized to evaluate the viability of R\* forms. Reaction mixtures were prepared as above and plated on both conventional and modified L-form media.

In other experiments, identical reaction mixtures with normal serum were prepared and passed through a 0.45- $\mu$  membrane filter (Millipore Corp., Bedford, Mass.) in a Swinney-type syringe adapter. By use of a 1.0-ml plastic disposable syringe, excessive pressure could be avoided and filtration could be regularly accomplished without membrane rupture. Samples of the filtrates were inoculated on conventional and modified L-form agar.

## RESULTS

Microscopic observations of the reaction mixture substantiated the findings of Freeman et al. (3). The transformation of the normal *Vibrio* form to spheroplast to ghost is shown in Fig. 1 and 2.

When positive, colonies of the classical rod forms of all three *V. cholerae* strains studied appeared on both conventional and L-form media within 24 hr. L-form cultures were considered positive only when colonies appeared on the

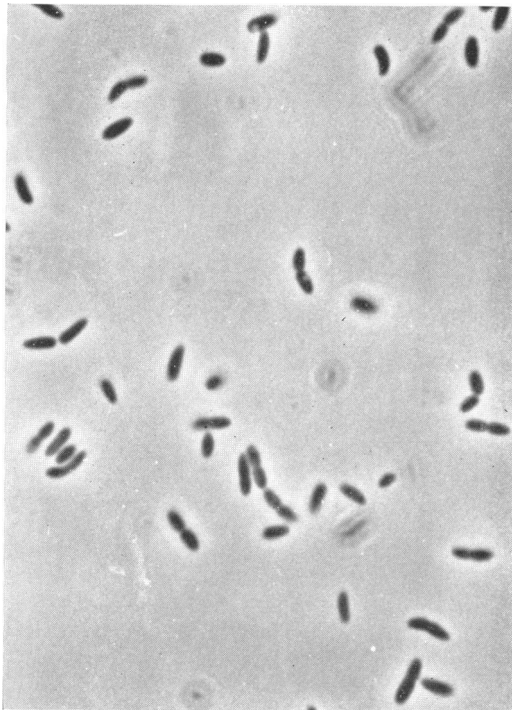


FIG. 1. Wet mount in Formalin of classical form of *Vibrio cholerae*, type Hikojima.  $\times 2,000$ .

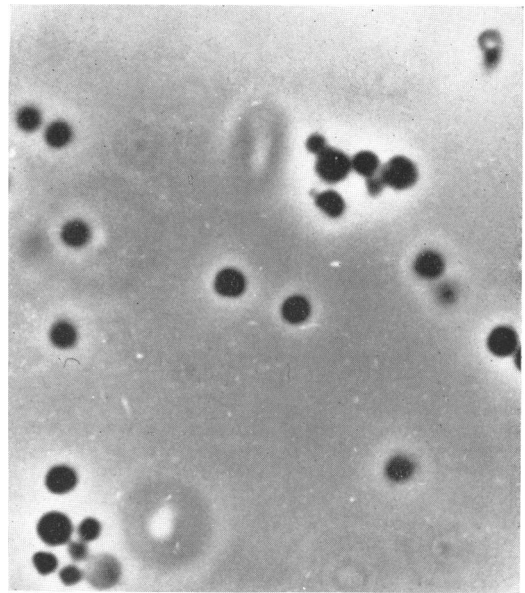


FIG. 2. Wet mount in Formalin of serum spheroplasts of *Vibrio cholerae*, type Hikojima, showing a ghost-like form at lower left.  $\times 2,000$ .

modified L-form media after 48 hr and when no growth occurred on control plates of conventional media. No cultures for L-forms were ever positive in less than 48 hr; easily identifiable colonies appeared from 2 to 6 days.

Growth of L-form colonies was observed on L-form media in studies with all three *V. cholerae* strains. When compared with the appearance of colonies of classical *V. cholerae* rods on L-form media, the L-form colonies were smaller, more translucent, and mucoid. When examined in wet mounts and Gram strains, the L-form colonies consisted of spherical organisms similar to the spheroplasts seen in the initial reaction mixtures with serum. The L-form colonies which required 5 days to grow were more pleomorphic than those which appeared after the minimal incubation of 48 hr. Growth of spheroplast cultures was observed in three of six studies with Inaba type, in three of three with Hikojima, and in three of four with Ogawa. In every instance, cultures on conventional media were negative, even with 100-fold larger inocula.

Subcultures of the L-form colonies were grown on both conventional and L-form media. After initial isolation, L forms generally could be grown in subculture on conventional media. The

L-form colonies grown in this manner were smaller and grew slowly. The three *V. cholerae* strains reverted to typical forms after four to six passages on conventional media and reverted more slowly on the L-form media. Figures 3-5 show several stages during the reversion process.

Microscopic observations on the absorbed serum reaction mixtures showed that the R\* stage was produced without conversion to the spheroplast stage. Colony counts on conventional media indicated better than 99.9% killing. Under these conditions, we were unable to culture the R\* on either the conventional or the L-form media, even with inocula containing  $10^4$  organisms.

Both microscopic and cultural observations in multiple experiments demonstrated that the classical rod forms of Hikojima and Inaba strains did not pass through an intact  $0.45\text{-}\mu$  membrane filter. Cultures of filtrates of suspensions of classical rod forms were routinely sterile on both conventional and L-form media. (The Ogawa strain was not studied.) After conversion of the rod form to spheroplasts, neither rod forms nor spheroplasts from reaction mixtures could be identified by phase microscopy in the filtrates. The filtrates of reaction mixtures were inoculated

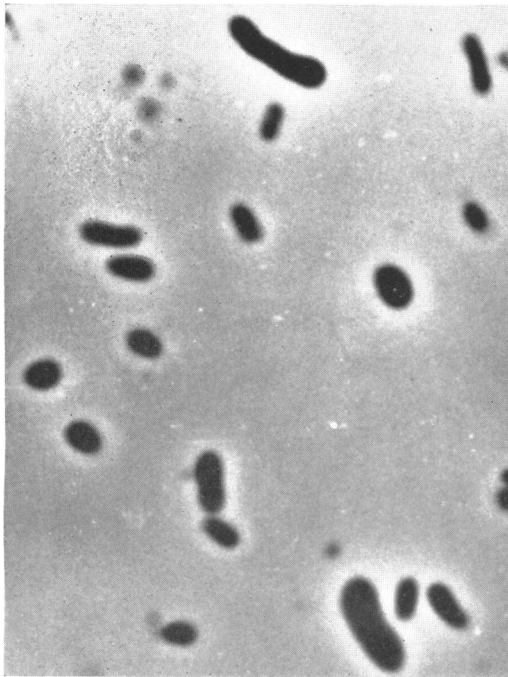


FIG. 3. Gram stain of an L-form colony of *Vibrio cholerae*, type Hikojima, in process of reversion after one subculture.  $\times 2,000$ .

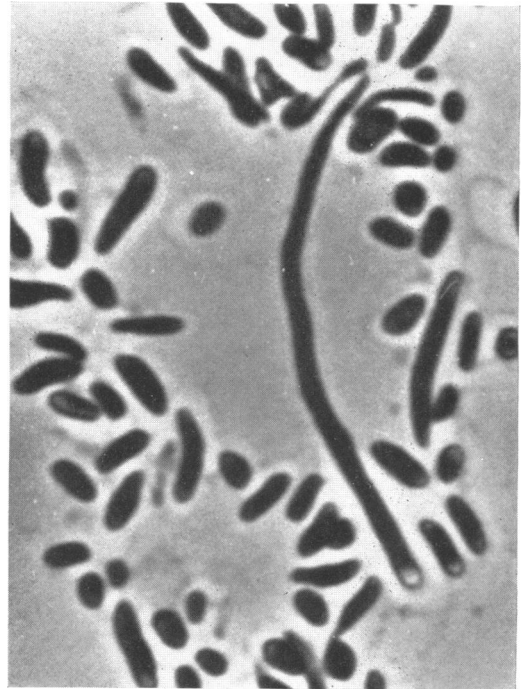


FIG. 4. Gram stain of an L-form colony of *Vibrio cholerae*, type Hikojima, showing further reversion after two subcultures.  $\times 2,000$ .

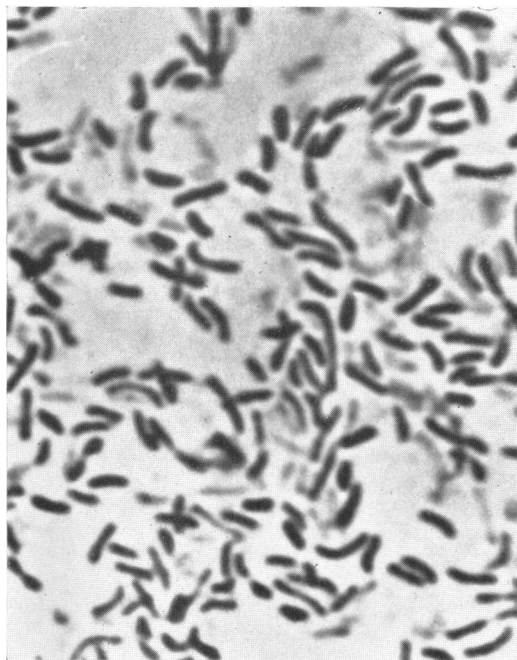


FIG. 5. Gram stain of an L-form colony of *Vibrio cholerae*, type Hikojima, after four subcultures demonstrating almost complete reversion to classical forms.  $\times 2,000$ .

on conventional and L-form media. L-form colonies appeared on the L-form media; there was no growth on the conventional media. L-form colonies of Inaba were tiny and, in spite of several attempts, did not grow on subculture. L-form colonies of Hikojima were similar to those previously described and grew on subculture without difficulty. Wet mounts of L-form colonies from Hikojima filtrates showed dramatically pleomorphic organisms (Fig. 6).

The identity of the L-form and reverted colonies was confirmed by slide agglutination tests. Saline suspensions of both the L-form colonies and the reverted rod form colonies gave prompt agglutination when tested with the specific antisera. No agglutination occurred with the heterologous antisera or with normal rabbit serum.

#### DISCUSSION

These studies show that spheroplasts of *V. cholerae* produced in serum are viable and can be cultured on modified L-form media, and suggest that the replicative particles of the spheroplast can pass through a  $0.45\text{-}\mu$  membrane filter. L forms of all three strains were demonstrated and cultured at a time and dilution when no classical rod forms appeared. These studies suggest, fur-

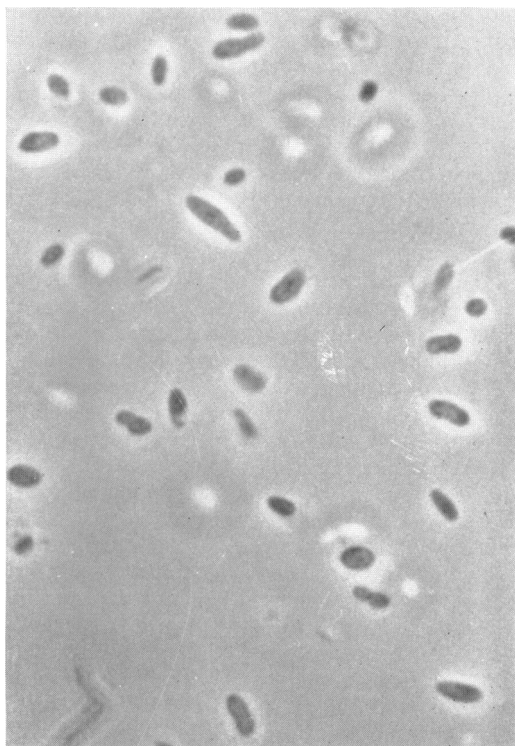


FIG. 6. Wet mount in Formalin of an L-form colony grown from a membrane filtrate of *Vibrio cholerae*, type Hikojima, demonstrating pleomorphic forms.  $\times 2,000$ .

thermore, the nonviability of the R\* stage since the R\* could not be grown on either conventional or the present modified L-form media.

The mechanisms involved in *Vibrio* spheroplast production appear not unlike that of the gram-negative bacilli, and the processes may be regarded as general phenomena. Since Dienes et al. (2) have reported on the growth in culture of serum L forms of typhoid bacilli, it is possible that serum spheroplasts of all gram-negative bacteria are viable in culture when appropriate media are utilized.

Some technical points seemed to be essential for the successful production and culture of serum spheroplasts. Yields of spheroplasts in the serum reaction mixture were better when the appropriate concentration of calcium (0.008 to 0.015 M) was added to the saline diluent. We have found that excess calcium inhibits the process. Better growth of L forms was obtained on media which were freshly prepared and still moist. Since the L forms were grown on moist plates, isolated colonies were not generally obtained. Therefore, it was not possible to make meaningful L-form

colony counts and an accurate estimate of the proportion of spheroplasts which were viable. Qualitatively, recovery rates of L-form colonies from spheroplasts were low. However, although these studies show that the constituents of the modified L-form media were adequate for isolation, additional studies to identify the basic growth requirements and the composition of optimal media will be required to provide quantitative data.

The culture of serum spheroplasts of *V. cholerae* under conditions when regular forms cannot be grown raises a serious question as to the use of conventional cultural techniques for the study of the epidemiology and pathogenesis of cholera. Negative cultures on standard media can no longer be interpreted as absence of replicative, and presumably infective, organisms.

These studies also shed light on the nature of the bactericidal system. It would appear that the antibody-complement system, although not visibly altering bacterial morphology under routine phase microscopy when forming the nonviable rod (R\*) in the absence of lysozyme, affects the organism in such a way that it is no longer replicative. Serum absorbed to remove lysozyme will kill gram-negative bacteria but will not cause transformation to spheroplasts. In preliminary studies, we were unable to obtain growth on such killed rods even on media which supported growth of L forms. The action of lysozyme and production of spheroplasts would appear in some way to restore viability.

The nature of the viable particle is not clear; it may not be the intact spheroplast itself, since replicative particles, which could not be identified as either classical or spheroplast form under phase microscopy, were passed through a 0.45- $\mu$  membrane filter.

#### ACKNOWLEDGMENTS

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#### LITERATURE CITED

1. DAVIS, S. D., D. GEMSA, AND R. J. WEDGWOOD. 1966. Kinetics of the transformation of gram-negative rods to spheroplasts and ghosts by serum. *J. Immunol.* **96**:570-577.
2. DIENES, L., H. J. WEINBERGER, AND S. MADOFF. 1950. The transformation of typhoid bacilli into L forms under various conditions. *J. Bacteriol.* **59**:755-764.
3. FREEMAN, B. A., G. M. MUSTEIKIS, AND W. BURROWS. 1963. Protoplast formation as the mechanism for immune lysis of *Vibrio cholerae*. *Proc. Soc. Exptl. Biol. Med.* **113**:675-680.
4. GUTMAN, L. T., M. TURCK, R. G. PETERSDORF, AND R. J. WEDGWOOD. 1965. Significance of bacterial variants in urine of patients with chronic bacteriuria. *J. Clin. Invest.* **44**:1945-52.