SPONTANEOUS LYSIS AND PHAGE-CARRIER STATE IN BRUCELLA CULTURES

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Abstract

RENoux, GERARD (Université de Montpellier, Montpellier, France), and ANDREE SUIRE. Spontaneous lysis and phage-carrier state in Brucella cultures. J. Bacteriol. 85:642-647, 1963.—When broth or saline suspensions of 60 randomly chosen Brucella strains were directly poured onto plates of Albimi Agar, distinct plaques, indicative of phage activity, developed. Unselected Brucella cultures containing cell types that gave rise to several morphologically distinct colonial types all proved to be naturally phage-infected. Selection and study of some of these colonial types led to the following conclusions: (i) S or SI colonies do not carry the phage and are sensitive to it; (ii) pure R colonies are phage-resistant and do not carry the phage; (iii) butyrous or sticky white P ("Porteuses") colonies develop from "carrier cells" resistant to phage; and (iv) the progeny of cells of the P colony type segregate into cells that give rise to P, S, or SI colonies. However, when plates were streaked with a cotton swab soaked in the Brucella suspension, no visible plaque developed. The phenotypic changes occurring after phage infection are believed to play a role also under natural conditions; they are able to explain most of the natural behavior of Brucella. Their occurrence, however, does not exclude other genetic mechanisms that may produce similar phenotypic effects.

In the course of a previous study, it was observed that some Brucella strains are lysed by their own filtrates (e.g., B. melitensis strains H. 38 and H. 107, or B. suis strain 8.6, in Table III of Lazuga and Renoux, 1960), suggesting that a lytic agent was already present in the culture. Sometimes the area surrounding phage lysis on agar media showed a distinct white border of growth (see Fig. 2 of Lazuga and Renoux, 1960), suggesting the possibility of "changes" in the behavior of certain phage-exposed cells.

Some observations made to investigate this phenomenon further are reported here.

Materials and Methods

Strains. Table 1 summarizes the origin and characteristics of the 60 Brucella strains employed. Of them, 36 had been kept lyophilized for from 3 months to 3 years prior to use; 24 had been kept at 4 C and had been subcultured at monthly intervals. All strains were used prior to selection of any particular colonial type.

Dye-inhibition tests with basic fuchsin and thionin, agglutination by monospecific sera, CO2 dependence, and H2S production were employed to identify the strains (Expert Committee on Brucellosis, 1953). B. intermedia is the very widespread variety of Brucella which have the biochemical features of B. melitensis and the serological pattern of B. abortus (Renoux, 1952; Renoux, Amarasinghe, and Sacquet, 1955).

Cultural techniques. The lyophilized cultures were reconstituted with sterile distilled water and seeded on agar slants which were then incubated for 24 to 48 hr at 37 C. From such growth on slants, homogeneous suspensions in broth or saline containing approximately 10⁶ cells per ml were used to prepare agar plates: (i) 1 ml of a given suspension was poured onto agar plates, and after a few minutes of standing, the excess of supernatant fluid was pipetted off and the plates were incubated at 37 C ("distribution technique"); (ii) agar plates were streaked with a cotton swab soaked in the suspension mentioned above ("swab technique").

Albimi Agar was used as solid medium and Albimi broth as liquid medium or diluent fluid (Albimi Laboratories, Brooklyn, N.Y.).

Selection of colonial types. Selection was made according to the technique of Henry (1933) and
TABLE 1. Origins and characteristics of 60 Brucella strains

<table>
<thead>
<tr>
<th>Source</th>
<th>Location</th>
<th>Identification characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Iran, Mexico, Argentina, Italy, Argentina, France, Italy</td>
<td>B. melitensis: 2, B. abortus: 3, B. suis: 10</td>
</tr>
<tr>
<td>Bovine</td>
<td>France, Puerto-Rico, Chad</td>
<td>B. melitensis: 3, B. suis: 2</td>
</tr>
<tr>
<td>Caprine</td>
<td>Tunisia, France, Switzerland, Poland</td>
<td>B. melitensis: 3, B. suis: 2</td>
</tr>
<tr>
<td>Ovine</td>
<td>France, Iran, South Africa</td>
<td>B. melitensis: 4, B. suis: 6</td>
</tr>
<tr>
<td>Porcine</td>
<td>Bulgaria, France, U.S.A.</td>
<td>B. melitensis: 1, B. suis: 19</td>
</tr>
<tr>
<td>Hare</td>
<td>France</td>
<td>B. melitensis: 4</td>
</tr>
<tr>
<td>Unknown</td>
<td>Iran, Poland</td>
<td>B. melitensis: 19, B. suis: 4</td>
</tr>
</tbody>
</table>

Huddleston (1952) under a low-power stereoscopic microscope with oblique lighting.

Preparation of filtrates. Broth cultures were centrifuged at 6000 rev/min in an International centrifuge, and the supernatant liquid was filtered through Millipore HA filters. The filtrates were checked for sterility and stored at 4°C.

Results

Agar media inoculated with the bacterial suspension were frequently inspected, beginning at the 15th hr of incubation; the following observations are typical for plates initiated with a “distribution of a suspension.”

(i) The presence of a few round disseminated plaques within a homogeneous lawn of culture was observed (Fig. 1). (ii) A greater number of plaques, some of them oval-shaped with a tendency to join together was also observed. Figures 1 and 2 show two aspects of the same type of phage activity, found after cultivation of the same 17 strains (5 B. melitensis, 2 B. abortus, 4 B. suis, and 6 B. intermedia). The observed differences apparently depend only on the random distribution of phage particles.

(iii) Presence of large plaques with clear-cut borders (Fig. 3) was obtained with 17 strains (7 B. melitensis, 4 B. abortus, 4 B. suis and 3 B. intermedia).

(iv) The growth occurred in waves with borders of denser appearing growth and some isolated colonies, most of them being white ones, and tiny pin-point plaques were found in areas of quite homogeneous growth (Fig. 4) on the cultures from 26 strains (16 B. melitensis, 5 B. abortus, and 5 B. intermedia).

In most instances, plaques were still recognizable at the fourth day of incubation. In a few cases, secondary growth developed and plaques were overgrown by the fourth day of cultivation.

An enrichment technique proved that Brucella strains are sensitive to their own phage. A few drops of the filtrate of a broth culture were added to the saline or broth suspension of the same strain 1 hr before pouring it onto a plate. The number of plaques that developed was greater than when the Brucella suspension was used alone. Figure 5 shows the result obtained after such an enrichment and is to be compared with Fig. 1, which is a culture from the same suspension, at the same time, but without addition of homologous filtrate.

When the secondary growth which developed eventually in the plagues was subcultured, the resulting growth gave rise to a homogeneous lawn with disseminated plaques, just as in the case of the initial cultivation.

Several colonial types were selected from the above cultures, and a study of their descendants gave information that can be summarized as follows.

(i) Smooth and smooth-intermediate colonies are sensitive to the action of the lytic agent obtained in the filtrate of a broth culture of the same strain. When phage-infected, the bacteria of such colonies will either be lysed or develop into sticky white colonies which are apparently resistant to lysis.
FIG. 1. *Brucella intermedia* strain 0.28. Round plaques in a homogeneous lawn of culture obtained after direct distribution of a broth suspension.

FIG. 2. *Brucella intermedia* strain 0.28. After direct distribution of a broth suspension, the number of plaques is greater than in Fig. 1; most of them are oval-shaped and show a tendency to join together. Figures 1 and 2 are two different aspects of the same phenomenon.


FIG. 4. *Brucella melitensis* strain D. 194. After direct distribution of a broth suspension: a part of the growth occurs in waves; note white isolated colonies in large area of lysis and pin-point plaques in the homogeneous area of growth.

(ii) White, homogeneously sticky colonies are very difficult to distinguish morphologically from true rough types; they may be those which are described as butyrous in consistency by Huddleston (1952). They are not mucoid forms. The *Brucella* cells of such colonies always carry active phage against smooth and smooth-intermediate type cells of the same strain. They are not lysed.
by this phage. The P ("Porteuses") colonies thus are resistant and are phage carriers.

(iii) After propagation of bacteria of a P colony, they give rise either to new P bacteria or to smooth (smooth-intermediate) phage-sensitive Brucella cells.

In summary, sticky (or butyrous) white colonies, when streaked again, always yield white and blue (various values of blue, blue-gray, or green-blue according to the Brucella variety involved) colonies. Phage was not recovered from S or SI type colonies.

(iv) The rough colonies do not carry the phage and are resistant to the action of this lytic agent.

On the plates inoculated by the swab technique, no plaques visible to the naked eye developed. Figures 5 and 6 illustrate typical differences: after "distribution of the suspension," many plaques are visible on the plate (Fig. 5); after "swab" streaking of the same suspension, the plate contains only a homogeneous lawn of culture (Fig. 6).

**Discussion**

Of 60 arbitrarily chosen stock cultures of *Brucella*, all were found to contain phage particles which are easily revealed under ordinary conditions of cultivation on solid media. It is difficult to explain why the use of a cotton swab for streaking on the plates suppressed the appearance of plaques, but this may be due to an adsorption of phage particles to the cotton.

The very simple technique of spreading the bacterial suspension onto plates, employing *Brucella* cultures without prior selection of any given colonial type, demonstrates that phage infection of *Brucella* stock cultures is quite a rule. The practice of selecting S or SI colonies before studying a strain is undoubtedly the reason this phenomenon was relatively unknown until now.

Jones, McDuff, and Wilson (1962) have made comparable observations, showing that smooth-intermediate colonies of *B. abortus* may develop a sticky white growth in the area of a phage drop. Both white and blue-gray colonies developed when such growth was streaked on agar plates, and the white colonies proved to be phage carriers.

The selection and study of different colonial types reported here and by Jones et al. (1962) indicate that the phenotypic changes to resistant and carrier P colonial types able to segregate subsequently into P and S or SI colonies may be one explanation for the survival of *Brucella* cells under natural conditions where phage infection of the *Brucella* cells may occur in the natural host. Consequently, most and presumably all the cul-
tures when first isolated may still be phage-infected.

When, in natural environment of *Brucella*, phage infection occurs, P cells appear (an effect that may be caused by the action of bacteriophage enzymes on the cell wall). P cells are able to segregate into other P cells, or into S or SI cells. New P cells may arise from other P cells, or as the result of the action of the liberated phage particles on S (SI) cells. S or SI cells can either give rise to the same type of cell, to R or M cells by mutation (?), or by infection by bacteriophage some S cells are lysed and others are transformed into P cells.

Drozhevkina (1956a, b), Ostrovskaya and Knyazeva (1957), Dubrovskaya, Ostrovskaya, and Glubokina (1958), Dubrovskaya (1960), Dubrovskaya and Ostrovskaya (1961), and Ostrovskaya (1961) also have demonstrated that, under the influence of phage, *Brucella* cells are able to change in regard to morphology, chemical composition, or antigenic constitution. Such phenotypic modifications created by phage activity within *Brucella* cultures resemble observations in other bacterial species, e.g., in *Salmonella* (Uetake, Luria, and Burrous, 1958) and mycobacterium (White, Foster, and Lyon, 1962).

Brucellaphage activity in the natural hosts may explain, at least in part, the primary isolation of “dissociated” (Alton, 1960) or “atypical” (Sacquet and Renoux, 1957; Renoux, 1961, 1962) cultures from naturally or artificially infected animals. The existence of a lytic agent in *Brucella* cultures probably also contributes to the well-known instability of *Brucella* cultures, which now may be considered as being partly due to the selective advantage of rough types in the presence of phage and to the here-described phenotypic alteration of phage-infected cells. Nevertheless, these phage-mediated phenomena do not exclude the existence of other genetic mechanisms able to produce comparable phenotypic effects in *Brucella* and other bacterial species.

**Literature Cited**
