RELATION BETWEEN CHANGES IN THE STABILITY OF PASTEURELLA TULARENSIS SUSPENSIONS AND IN ITS BACTERIAL POPULATION

II. MUTUAL INFLUENCE BETWEEN SALT-AGGLUTINABLE AND SALT-NONAGGLUTINABLE TYPES

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Population changes in Pasteurella tularensis were noticed thirty years ago by Francis (1922) who found that serial transfers of a virulent strain on serum-glucose agar resulted in loss of virulence. This phenomenon has been confirmed since then by other investigators (Foshay, 1932; Snyder et al., 1946).

In a recent publication, Eigelsbach, Braun, and Herring (1951) describe the existence of several types of colonies in P. tularensis; however, they were unable to find a definite correlation between colony appearance and biological properties. In liquid media, a rough variant appeared which became dominant in time. The authors assumed that in old cultures toxic factors accumulate and create conditions unfavorable to the smooth parent type. As in this microorganism the properties of the surface of the individual cell, as revealed by the stability of the bacterial suspension, show a better parallelism with biological properties than colonial morphology (Eigelsbach et al., 1951; Avi-Dor and Yaniv, 1953); the population dynamics were studied as function of changes occurring in agglutinability.

MATERIALS AND METHODS

In most of the experiments, the virulent (non-agglutinable) Dor strain and the avirulent (salt-agglutinable) 176 strain were used. Both were of smooth appearance. The bacteria were cultivated on glucose-cysteine-blood-agar. The population changes were followed by agglutination tests with Na/160 NaCl as described before (Avi-Dor and Yaniv, 1953). In order to secure a more even distribution of the inoculum, the usual method of streaking the plate with a loop was replaced by seeding it by means of strips of filter paper. The paper was cut to the desired shape, and the pieces were autoclaved, soaked in the bacterial suspension, drained, and brought in contact with the surface of the plate for a period of five minutes.

RESULTS

Population changes in the virulent strains and in mixed cultures during serial transfers. Three virulent strains were transferred every 24 hours on glucose-cysteine-blood-agar slants (pH 6.8 to 7.0). After various numbers of transfers, the percentage of salt-agglutinable microorganisms was determined in the presence of Na/160 NaCl by the salt agglutination test. The results are given in table 1.

It shows that in cultures, in which no salt-agglutinable variants could be detected initially, these variants appeared after approximately 35 transfers. Their proportion increased progressively until they became dominant.

In another series of experiments 24 hour cultures of the virulent and avirulent types were washed from slopes with saline adjusted to the turbidity of 40 per cent light transmission and were combined in varying proportions (1:10 to 1:1). These mixtures were seeded on slopes, a transfer was carried out daily, and the composition of the population was determined after every transfer. The shift in the composition of the populations is shown in figure 1.

Addition of the avirulent to the virulent type affects the population dynamics of the cultures decisively. A culture, containing initially 1 per cent of avirulent type, changed its composition after two transfers to 67 per cent of the avirulent and 33 per cent of the virulent type. Addition of the avirulent organism in a proportion as low as 1:10 caused rapid establishment of the avirulent variant.

The smallest percentage of the agglutinable type which can be detected by turbidity measurement after salt agglutination is about 1 to 2 per cent. Below this limit, the agglutinable type could be revealed only after a certain number of transfers. Some correlation seems to exist be-
tween the number of transfers necessary to reach the lower sensitivity limit of the salt agglutination test and the proportion of the agglutinable type present in the original culture.

*Inhibition of virulent by avirulent types.* The possibility was tested that one of the strains exhibits an inhibitory effect on the growth of the other or on its own growth. The strain to be tested was seeded on a glucose-cysteine-blood-agar plate by means of a strip of filter paper (length 85 mm, width 3 mm), which divided the plate into two semicircles, and at the same time vertically on one side of the dividing line while from the other side of the line the other type was inoculated. After every 24 hours of incubation, additional vertical lines of the two strains were streaked out, and the growth of the previous streaks was observed. The results are summarized in Table 2.

**TABLE 2**

*The mutual effect on growth of strains of Pasteurella tularensis belonging to virulent (strain Dor) and avirulent (strain 176) types, when grown in close vicinity on solid medium.*

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>ZONE OF INHIBITION (IN MM) INDUCED BY THE STRAINS</th>
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<tbody>
<tr>
<td></td>
<td>Time of incubation</td>
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<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Dor</td>
<td>0</td>
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<td>176</td>
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*Figure 1.* Population changes during serial transfers in mixtures of avirulent and virulent types of *Pasteurella tularensis.* (Each curve represents changes obtained in an inoculum of different composition.) Ratio of avirulent to virulent organisms in the inoculum—1. 1:10⁷; 2. 1:10⁶; 3. 1:10⁵; 4. 1:10⁴; 5. 1:10³; 6. 1:10²; 7. 1:1; 8. 1:1.

*Figure 2.* Inhibition of growth of the virulent type of *Pasteurella tularensis* by the avirulent type. Both strains were seeded simultaneously. The blocks represent the growth of the avirulent type; the line between them, that of the virulent type.

The avirulent type inhibited the growth of the virulent organisms very distinctly, particularly if seeding of the virulent type was delayed until growth of the avirulent strain had been established. When the virulent type of the organism was streaked on the line passing between two blocks of the avirulent type, growth of the former was inhibited completely in the zone between...
the two blocks, even though both strains were inoculated simultaneously. When blocks and line consisted of the same type, no inhibition was observed, but when the line was seeded with the avirulent type and the blocks with the virulent type, the growth of the blocks was inhibited in the vicinity of the line.

The inhibitory effect of the avirulent type was shown also when the blood in the medium was replaced by 1 per cent neutralized corn steep liquor.

In some of the experiments, a slight growth was observed in the inhibited zones inoculated with virulent organisms. Propagation of these slight growths for 48 hours at a distance of 2 mm from avirulent organisms (which had been grown for 48 hours) led to the rapid establishment of populations containing appreciable proportions of avirulent forms. From 16 virulent lines two salt-sensitive lines were derived by one such exposure, and seven were derived by a repeated exposure. These results indicate that cultivation of a virulent type in close proximity to avirulent type causes the induction of salt-sensitivity after only one or two transfers, instead of the 35 to 40 transfers which were necessary for the appearance of the avirulent type in the first experiments described.

**DISCUSSION**

The establishment of preponderance by spontaneously arising mutants may be due to a variety of causes. It may be dynamic in the sense that one strain will grow faster than another (Monod, 1946), or it may be due to a directive role of the environment. This latter factor may assert itself by a variety of mechanisms. The composition of the medium may be more favorable to the development of the mutant, as Zamenhoff (1946) found for citrate-unstable strains of *Escherichia coli* in citrated media. A direct effect of the composition of the medium on variation was found also by Dickenson (1945) in *Brucella bronchi septica* and by Van Lanen, Baldwin, and Riker (1940) in *Phytononas tumefaciens*. In other cases, the conditions may become favorable for the mutant only at a certain age of the culture, due to the exhaustion of a metabolite essential for the parent strain, but not for the mutant (histidineless variants of *E. coli*; Rayon and Schneider, 1949), or due to the excretion, by either the parent strain or the variant, of factors specifically inhibitory for the former. Thus, Goodlow, Mika, and Braun (1950) found that their parent strain of *Brucella* excretes, and is inhibited by, d-alanine. In *P. tularensis*, Eigelsbach, Braun, and Herring (1951) observed that population changes in the direction from S to R in aged liquid media were due to the accumulation of a product which favored the establishment of the R type. The source of these toxic metabolites has not been established.

In the present paper, the mutual influence between salt-agglutinable (nonvirulent) and non-agglutinable (virulent) strains of *P. tularensis* has been investigated. Nonvirulent strains originating from mutation in the original virulent population established themselves gradually during serial transfers. The observed strong inhibition of the growth of the parent virulent strain by the nonvirulent mutant explains fully the progressive development of dominance of the mutant. The same fact accounts for the rapid rate at which the avirulent type establishes itself in mixtures of the two types. The alternative assumption that the phenomenon was due to the faster growth of the mutant is not borne out by the experiments. The fact that the parent strain did not show any self-inhibitory activity excludes the possibility that the mechanism proposed for *Brucella* by Goodlow, Mika, and Braun (1950) plays a part in the population changes observed in *P. tularensis*.

To account for the observed inhibition of the virulent type by the avirulent type, two assumptions could be made: (a) excretion of a toxic substance by the avirulent type or (b) depletion of the medium of an essential growth factor in the region of the avirulent strain growth. In view of the absence of self-inhibition, the first alternative seems to be in better accordance with the present state of our experimental knowledge. The surprising rapidity with which the virulent strain changes to the nonvirulent type, when grown repeatedly in the vicinity of the latter, may be due to an influence, on the mutation of the virulent strain, of a substance excreted by the avirulent type, in addition to its inhibitory effect on the former; however, there is no necessity for introducing this complicating hypothesis.

**SUMMARY**

Population changes in *Pasteurella tularensis* were investigated, using the salt agglutination
test for the differentiation of avirulent and virulent types. When serial transfers of virulent type strains were made on glucose-cysteine-blood-agar slants, avirulent variants appeared after approximately 35 transfers, their proportion in the population increasing progressively. By experiments in which the initial inoculum contained mixtures of the two types in proportions varying from $1:10^7$ to $1:1$, it was found that the addition of the avirulent type to the virulent type affects decisively the population dynamics; the former type establishes domination rapidly. The assumption that the avirulent type excretes substances inhibitory to the growth of the parent virulent strain accounts for all observations recorded.

REFERENCES


