STUDIES ON PNEUMOCOCCUS VARIATION

II. SMOOTH VIRULENT VARIANTS PRODUCED BY DAUGHTER-COLONY DISSOCIATION OF SMOOTH PNEUMOCOCCUS STRAINS

MONROE D. EATON

Department of Bacteriology, Yale University, School of Medicine, New Haven, Connecticut

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INTRODUCTION

A previous paper (Eaton, 1934) described variants of the pneumococcus characterized by a tendency to undergo rapid spontaneous lysis under certain cultural conditions at 37°C. These variants were produced by daughter-colony dissociation of smooth pneumococcus strains on blood agar at 25°C. A further investigation of this method of dissociation revealed that a second and independent type of variation is also produced by the formation of daughter colonies. This consisted in the appearance of a stable smooth variant in the daughter colonies.

The purpose of this paper is to demonstrate that dissociants obtained from the daughter colonies which first appear at 25°C on the colonies of S pneumococci are smooth strains distinguishable in colony form and other characteristics from the parent smooth strains. The production of these variant S forms has been observed with great regularity in the daughter colony dissociation of most of the pneumococcus cultures of types I, II, III and group IV so far studied. Daughter-colony formation occurs with especial ease in recently isolated strains.

Hadley (1927) has pointed out the frequency of daughter-colony formation in many species of bacteria. He believes that the dissociants formed in daughter colonies are usually not true rough variants. Sometimes these dissociants form large smooth mucoid colonies on subculture, as with organisms of the typhoid
group, and these mucoid colonies may regenerate the parent culture. Other daughter-colony dissociants revert on subculture to the parent colony form, or give rise to rough strains. Daughter-colony variants usually show an increased vigor of growth.

Studies on daughter-colony formation by the pneumococcus on various solid media at 37°C. have usually failed to demonstrate the existence of any distinct dissociants in the daughter colonies except the R forms as reported by Griffith (1928). Often subculture of the papillae has given only smooth mouse-virulent forms apparently similar to the parent type as observed by Paul (1927), Grumbach (1931), and Klumpen (1932). The opaque appearance of the daughter colonies of the pneumococcus was noted by Grumbach. Atkin (1926) reported that the daughter papillae apparently formed no autolysin and were insoluble in bile but recovered their bile solubility and susceptibility to autolysis after being transplanted to fresh medium.

The only essential difference in the method of daughter-colony dissociation described in this paper from that of other workers was that the dissociation was effected and subsequent subcultures were kept at 25°C. instead of at 37°C. This modification has led to the isolation of the phantom colony variants described in the previous paper and the smooth variants previously mentioned and described below.

For the purposes of comparison, the colony form and other characteristics of freshly isolated smooth strains of pneumococcus will be considered normal and these strains will be designated by the letter "N." The variant smooth strains derived from the daughter papillae formed on colonies of the N forms will be designated by the letter "V." In the previous paper these N and V strains were referred to as translucent and opaque colony forms respectively.

METHODS

The media used in the course of these studies were the same as those described in the previous paper, except that 5 per cent rabbit blood instead of horse blood was added to the infusion agar medium used in the latter part of the work. No significant effect
of this change in the source of blood used for the medium was observed.

"Chocolate" agar was made by heating the rabbit-blood infusion agar to 70°C. for thirty minutes with frequent shaking to break up the coagulum.

The N and V strains of pneumococcus were kept in stock culture by cultivation on blood agar at room temperature with transplants every three to four days. Cultivation on blood agar at temperatures around 25°C. appears to result in greater stability of the pneumococcus cultures without affecting their properties in any other way. Once every week or ten days, colonies from the blood agar cultures were transferred to blood broth and grown for twenty-four hours at 37°C. Small amounts of these blood-broth cultures were then injected into mice and after the death of the animals the organisms were recovered from the heart blood by cultivation on blood agar at room temperature.

Capsules were stained in smears made by mixing the exudate from the peritoneum of mice dead of pneumococcus infection with a small drop of saline on a slide. The gentian violet and copper sulphate staining method of Hiss, and Wright's stain as ordinarily employed, were the two methods most generally used for demonstrating capsules.

Agglutinin absorptions were done with centrifuged sediments from cultures of pneumococcus in infusion broth containing 0.2 per cent glucose and 5 per cent normal horse serum. Agglutination studies were carried out with organisms centrifuged down from broth cultures and washed, and also with suspensions of organisms from the peritoneum of mice.

**PRODUCTION OF S VARIANT STRAINS BY DAUGHTER-COLONY DISSOCIATION**

The method of causing daughter-colony formation has been described in the previous paper (Eaton, 1934). Well separated colonies are allowed to age on blood agar at 25°C for three to eight days. At some time during this period, usually shortly after autolysis and flattening of the colonies has begun, nuclei consisting of cells of the variant organism appear and these grow above
the surface of the original colony to form the daughter colonies. Subculture of such colonies with daughter papillae to blood agar at 25° gives a mixture of two well defined colony types, the small flat colonies of the original (N) form and the larger more convex colonies of the smooth variant (V). The variant colony forms may be subcultured and passed through mice indefinitely and continue to breed true. They are bile soluble and ferment inulin.

If cultures are allowed to age further at 25°, the first daughter colonies consisting of the smooth variants disappear leaving pits, and later a second growth of daughter colonies appears. These may be shown by subculture to consist of a variety of dissociants most of which are of low virulence or rough.

**TABLE 1**

*The source and type of pneumococcus strains which have been shown to produce smooth variant (V) forms by daughter-colony dissociation*

<table>
<thead>
<tr>
<th>Source of Cultures</th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock cultures</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pneumonia sputum</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Blood cultures</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Other human sources*</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Sources of these strains as follows: type III, mastoid infection; and group IV, pneumococcus meningitis.

When daughter-colony dissociation is allowed to occur at 37° the dissociative processes appear to be more rapid than at 25°. At the higher temperature only rough dissociants have been isolated, in the course of the present studies, from the daughter colonies.

The source and type of the cultures studied in the course of this work are shown in table 1. The seventeen strains tabulated produced smooth V forms by daughter colony dissociation. With three additional stock cultures, one each of types I, II, and III, the formation of smooth V forms could not be demonstrated. It is suspected that these stock cultures may have undergone some dissociative change during long continued artificial cultivation.
None of the freshly isolated strains so far studied has failed to produce smooth variant forms.

Special mention must be made here of the type III culture isolated from a case of mastoid infection. No smooth variants could be obtained from this culture by the ordinary method of daughter colony dissociation. However, when this strain was aged on "chocolate" agar instead of blood agar daughter colonies appeared. By repeated subcultures of the resulting variants and daughter colony formation on blood agar at 25° two distinct V colony forms in addition to the original N colonies were finally isolated. These will be described in the next section.

COLONY CHARACTERISTICS OF THE N AND V FORMS

Colonies of the N and V forms are best differentiated by cultivating the organisms on blood agar at 25° to 28°, but the differences noted in the two forms at room temperature are also quite obvious in colonies grown at 37° under appropriate conditions. Colonies that are well separated exhibit the most striking differences.

The diagrammatic sketches of the colonies in cross section shown in figure 1 represent the comparative changes which take place in the N and V colonies during growth on blood agar at 25°. At the end of twenty-four hours the colonies are small and convex with a smooth shining surface. After two days colonies of the N form have begun to flatten slightly while those of the V form are larger and more convex. At three days a distinct autolysis and flattening has occurred in the colonies of the N form while those of the V form remain convex and continue to grow larger. At four days these changes are still more noticeable. At this time the colonies of most N strains have formed a well defined rim; but occasionally autolysis of the N colonies occurs at the periphery so that pointed colonies are produced. Colonies of the V forms have never been observed to form rims by central autolysis. At four days the surface of the N colonies appears dull and slightly granular while that of the V colonies is mirror-smooth and shining. By transmitted light the N colonies appear translucent
and the V colonies more opaque. By a horizontal light the N colonies appear dull grey and transparent and the V colonies have a greyish white appearance.

Daughter colonies consisting of V dissociants usually appear on colonies of the N form between four and seven days. The V colonies seldom form daughter colonies and these appear only after about ten days. Although autolysis of the N colonies begins on the third day, no autolysis of the V colonies is apparent until after six or seven days.

![Diagram showing the appearance of N and V colonies at various stages of growth on rabbit blood agar](image)

**Fig. 1. Sketches Representing Appearance in Cross-section of Typical Colonies of N and V Strains at Various Stages of Growth on Rabbit Blood Agar at 25°C.**

Differences similar to those just described are apparent in some N and V cultures grown at 37° for seventy-two hours, as is pictured in plate 1, figures 9 and 10. With some cultures, however, the differences may be brought out only by growing the colonies in the presence of about one per cent of carbon dioxide. As has been stated in the previous paper the appearance of the V forms in the daughter colony dissociation coincides with the appearance of certain lytic properties in both the N and V strains. These lytic properties are most evident when the organisms are grown at 37° on solid media, and may entirely prevent the appearance of visible colonies or may greatly modify the form of the colonies. How-
ever, the lytic process may be inhibited by small amounts of CO₂ so that the colonies obtained at 37° are similar to those produced at 25°C.

Comparison of the colonies of several type I S stock cultures under identical cultural conditions has revealed that some stock cultures give flat translucent colonies at 25° and 37° which correspond to the N forms, and other stock cultures give convex white colonies corresponding to the V forms.

From one culture of type III as mentioned in the previous section two variant S forms of especial interest were obtained. The first variant, designated V₁ formed flat colonies totally unlike the characteristic large smooth mucoid colonies of the normal strain from which it was derived. These V₁ colonies formed daughter colonies with great ease and the strain corresponded in its other properties to the N forms of other types. From the daughter colonies of the V₁ strain there was isolated a second variant (V₂) which formed moderately large convex, opaque, non-mucoid, colonies and corresponded to the V forms of other types in its general properties. These V₁ and V₂ dissociants of type III bred true, were fully virulent for mice, and agglutinated rapidly and specifically with type III antiserum. They are pictured in plate 1, figures 11 and 12.

ACTION OF THE N AND V FORMS ON BLOOD

Besides the differences in colony shape and surface just described colonies of the N and V forms also exhibit differences in the amount of green coloration produced on chocolate agar and blood agar. On blood agar at 25° to 37° the N colonies produce much wider and deeper zones of greening than do the V colonies. On chocolate agar these differences are somewhat more marked. After four days at 25° the N colonies bleach out the brown color of the medium, but the V colonies cause only a change of the brown color to green. When incubated in whole blood for twenty-four hours at 37°C, the N strains blacken the blood much more rapidly than do most V strains. Both forms grow at about the same rate in whole blood when large inocula are used.

These differences in action on blood by the N and V strains are
presumably due to differences in the metabolic mechanism responsible for hydrogen peroxide formation.

**MORPHOLOGY AND CAPSULE FORMATION**

By Gram's stain the N and V forms appear identical in morphology, except that pleomorphism and swollen forms are more frequent among organisms of the N strains.

Capsules may be demonstrated by the Hiss capsule stain on both the N and V forms when stained in smears directly from the peritoneum of mice, or after growth in serum or whole blood. In general the capsules of the V forms stain more lightly, by the method of Hiss, and appear thinner than those of the N forms. The capsules of the N forms sometimes appear to contain dark-staining granules. These differences are illustrated in plate 1, figures 1 and 2.

By using Wright's stain it has also been possible to demonstrate differences in capsule-staining reactions not only among some of the N and V forms but also between the pneumococcus types I, II, and III. Seastone (1934) has recently used Wright's stain to demonstrate capsules in young cultures of streptococci. By this staining method the following differences in capsule formation in smears from the mouse peritoneum have been noted in the pneumococcus.

Type I-N capsules appear as red-staining ragged fringes around the organism. Type I-V, capsules do not stain by the method of Wright (plate 1, figures 3 and 4). These differences have been observed in the N and V forms derived from four strains of type I.

Type II-N and V capsules do not stain by Wright's method although they are readily demonstrated by the Hiss Capsule stain (plate 1, figures 5 and 6).

Type III-N, -V₁, and -V₂ capsules all stain by the method of Wright as broad pink fringes about the organisms. The capsules of the N and V₁ forms appear similar in staining reaction, but the V₂ capsules appear slightly narrower and more striated than those of the N and V₁ forms. Differences between the V₁ and V₂ capsules are illustrated in plate 1, figures 7 and 8.
Dissociative behavior of the N and V forms

Significant differences have been observed between the N and V forms as regards dissociative behavior. The N forms readily produce daughter colonies on blood agar at 25° and also at 37°, thus giving rise to many dissociant forms including the smooth V, intermediate forms, and R forms. The V forms produce daughter colonies slowly or not at all at 25° or 37° and dissociants differing appreciably from the parent V strains can seldom be isolated from the daughter colonies. Certain strains which appear to be intermediate between N and V forms in their properties may give rise to more typical V strains by daughter-colony dissociation after a period of ten to twelve days at 25°.

Unless cultivated with frequent transplants, mouse passage, and colony selection the N forms cannot be kept in pure culture unmixed with V or R dissociants. When N forms are transplanted repeatedly at 25° or 37° without mouse passage they dissociate abruptly after several subcultures to the R variant. If allowed to remain without transplanting for any length of time at temperatures between 25° and 37° on agar or in broth the N forms may dissociate to smooth V forms.

The V strains are much more stable than the N forms. They do not readily dissociate to the R variant even after prolonged cultivation without mouse passage. When grown under cultural conditions which readily produce R dissociants from the N strains, the V strains show no change in colony form and only a gradual diminution in virulence for mice. After cultivation for several months under such unfavorable conditions V strains may eventually become rough by a transition through intermediate forms but abrupt dissociation to the R variant does not occur.

These statements are based on observations of about twenty N and V strains of pneumococcus which were carried as stock cultures under various conditions and for various lengths of time over a period of three years.

Type specificity and virulence for mice

The variant smooth strains which have just been described agglutinated specifically with the same type of pneumococcus.
antiserum as did the N strains from which they were derived. No cross-agglutinations with sera of other types have been observed with fully virulent smooth V strains. Both the N and V strains of types I and II killed mice in forty-eight hours at dilutions of $10^{-7}$ to $10^{-8}$. The N, V₁, and V₂ strains of type III killed mice in forty-eight hours at dilutions of $10^{-8}$ to $10^{-7}$.

As was indicated in the previous section “degraded” V forms may be produced by prolonged cultivation of the smooth V strains with infrequent transplants. Under these conditions the colony form remains smooth and convex and the organisms grow diffusely in 10 per cent normal horse serum broth but the virulence for mice gradually decreases, and eventually there is a partial loss of type specificity as indicated by agglutination tests. These degraded V forms apparently correspond in a general way with the forms intermediate between S and R, described by Klumpen (1932), Blake and Trask (1933) and Paul (1934). The virulence and type specificity of many of these “degraded” strains may be restored by repeated mouse passage.

**AGGLUTININ ABSORPTION AND MOUSE PROTECTION TESTS WITH ANTI-SERA FOR THE N AND V STRAINS**

Anti-sera were prepared in rabbits by intravenous injection of heat-killed cultures of the N and V forms of types I and II, and also by injection of formalinized cultures of N and V forms of type I in two animals.

In table 2 are presented the results of agglutination tests with absorbed and unabsorbed anti-sera against the N and V forms of type I and II. The N anti-sera were cross-absorbed with V organisms of the same type, and the V anti-sera were absorbed with N organisms. The absorptions were repeated until no agglutination of the absorbing organisms occurred. From the results tabulated it may be seen that V organisms absorb the agglutinins for the N and vice versa. The antigenic compositions of the V and N strains, by these tests, therefore appear to be identical.

Tests for cross protection by type I-V and -N anti-sera in mice were done by injecting simultaneously 0.1 cc. of the N or V anti-sera and various amounts of twenty-four-hour blood broth
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cultures of the N or V strains. The results may be summarized as follows.

1. Mice protected with 0.1 cc. of N antiserum: all lived with 0.02 cc. or less of either N or V cultures; and all died with 0.05 cc. or more of either N or V cultures.1

2. Mice protected with 0.1 cc. of V antiserum: all lived with 0.15 cc. or less of either N or V cultures; and all died with 0.5 cc. or more of either N or V cultures.1

Although the I-V antiserum had a protective value about ten times as great as the I-N antiserum, it may be seen from these results that either serum produced the same degree of protection against the I-N as it did against the I-V strain.

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{ANTISERUM} & \text{ABSORBED WITH} & \text{STRAIN AGGLUTINATED} & \text{AGGLUTININ TITRES OF SERA} \\
\hline
\text{I-N} & \text{I-V} & \text{I-N} & 1:120 & <1:7 \\
\text{I-V} & \text{I-N} & \text{I-V} & 1:50 & <1:7 \\
\text{II-N} & \text{II-V} & \text{II-N} & 1:200 & <1:10 \\
\text{II-N} & \text{II-V} & \text{II-V} & 1:60 & <1:10 \\
\text{II-N} & \text{II-V} & \text{II-N} & 1:50 & <1:5 \\
\text{II-V} & \text{II-N} & \text{II-V} & 1:20 & <1:5 \\
\hline
\end{array}
\]

ACTIVE IMMUNIZATION OF MICE AGAINST N AND V STRAINS OF TYPE I

Mice were immunized with blood-broth cultures of the N and V strains of type I killed with merthiolate. Each mouse received into the peritoneum one dose of 0.3 cc. and two doses of 0.5 cc. over a period of twelve days. Sixteen mice were immunized with each strain. Of the group of mice receiving the N vaccine five became emaciated and died (with sterile hearts' blood) during the course of immunization. None of the mice receiving the V vaccine died.

1 Each 0.1 cc. of cultures contained approximately 5 million minimal lethal doses of I-N or I-V.
The mice were tested sixteen days after the last immunizing injection. Test doses of 0.1 and 0.01 cc. of twenty-four-hour blood-broth cultures of the N and V strains were injected intraperitoneally into mice immunized with each strain. The results of these tests are presented in table 3. Only one of the mice receiving 0.1 cc. of the test cultures survived while over half of those receiving 0.01 cc. survived. Therefore, the fatal dose of either N or V cultures for mice immunized with either N or V strains is between 0.1 and 0.01 cc. There seems to be little difference in the immunity conferred by either strain.

**TABLE 3**

Active immunization of mice with type I-N and I-V strains

<table>
<thead>
<tr>
<th>IMMUNIZING STRAIN</th>
<th>TEST STRAIN</th>
<th>AMOUNT OF TEST STRAIN INJECTED*</th>
<th>NUMBER OF MICE TESTED</th>
<th>NUMBER OF DEATHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-N</td>
<td>I-N</td>
<td>0.1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>I-N</td>
<td>I-N</td>
<td>0.01</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>I-N</td>
<td>I-V</td>
<td>0.1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>I-N</td>
<td>I-V</td>
<td>0.01</td>
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<tr>
<td>I-V</td>
<td>I-N</td>
<td>0.1</td>
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<tr>
<td>I-V</td>
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<tr>
<td>I-V</td>
<td>I-V</td>
<td>0.1</td>
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<tr>
<td>I-V</td>
<td>I-V</td>
<td>0.01</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

* 0.1 cc. of a twenty-four-hour blood broth culture contains approximately 5,000,000 minimal lethal doses.

DISCUSSION

The daughter-colony dissociation of the pneumococcus on blood agar at 25°C. resembles the dissociation of other bacterial species on solid mediums. It is interesting to note that the N cultures which are very susceptible to autolysis on solid mediums form daughter colonies and dissociate much more readily than do the V dissociants which are relatively resistant to autolysis and show much less tendency to dissociation. Although no bacteriophage for the pneumococcus has as yet been demonstrated, this dissociative behavior is analogous to that of other species of bacteria in the presence or absence of bacteriophage. In the pneumococcus, stability toward dissociation and increased
vigor of growth on solid media are also accompanied by a decreased activity of the metabolic mechanism which oxidizes hemoglobin to methemoglobin and its decomposition products. It is possible that further studies along these lines may help to elucidate the mechanism of bacterial dissociation.

As regards virulence for mice and antigenic composition as studied in the course of this work the daughter-colony V dissociants are indistinguishable from their smooth parent strains. For this reason they cannot be classed as forms intermediate between smooth and rough. It is possible that differences in antigenic composition between the N and V strains may be detected by some means other than those reported in this paper. Perhaps such antigenic differences are indicated by the staining reactions of the capsules. On the basis of present evidence it seems probable that the pneumococcus variations reported in this and the previous paper are changes independent of the smooth to rough dissociation.

Differences in the colony form of various smooth strains of pneumococcus of the same type have been noted by many investigators. The present paper represents, to our knowledge, the first description of the production of stable smooth variants under controlled experimental conditions. In view of the observed marked instability of freshly isolated N strains of pneumococcus on blood agar at 25° or 37°C. it seems reasonable to postulate that many freshly isolated cultures of pneumococcus dissociate to forms resembling the smooth V strains during prolonged cultivation on artificial mediums even if virulence is maintained by mouse passage. We have definitely observed at least one such change from the N type of colony to the V form in a stock culture maintained by daily transplants in blood broth with mouse passage once a week. Many of the stock cultures examined during the course of this work formed colonies intermediate between the N and the V type in appearance, and practically all of the stock cultures studied produced daughter-colony dissociants less easily than freshly isolated strains, thus resembling the V forms. A few stock strains showed decreased methemoglobin formation on blood agar, formed daughter colonies with
great difficulty or not at all, and resembled typical smooth V dissociants in colony form.

SUMMARY

Some stock strains and all of the freshly isolated strains of smooth pneumococcus studied during the course of this work gave rise by daughter-colony dissociation on blood agar at 25°C. to smooth variants which differ in colony form from the parent strains.

The smooth variant (V) forms and the smooth parent (N) strains from which they were derived have the same virulence for mice and do not differ in antigenic composition as determined by agglutination, agglutinin absorption, and mouse protection studies.

The smooth V strains are very stable. They form daughter colonies and dissociate to R forms much less easily than do the N or freshly isolated strains.

The N and V strains appear also to differ in capsular staining reactions, and in the ability to form methemoglobin in blood.

The significance of these observations with regard to bacterial dissociation, and the maintenance of smooth pneumococci in stock cultures is discussed.

REFERENCES

PLATE 1

In figures 1 to 8 the pairs of stained preparations shown in each horizontal row were stained on the same microscope slide so that in comparing the capsule stains of the N and V forms the effects of variations in the method of staining were minimized. All stained preparations were made from the mouse peritoneum.

Fig. 1. Type I-N strain from mouse peritoneum stained by Hiss capsule stain. Capsules appear dark and granular.

Fig. 2. Type I-V strain stained by Hiss method. Capsules colored a light pink.

Fig. 3. Type I-N strain by Wright's stain. Capsules appear as ragged red stained fringes.

Fig. 4. Type I-V strain by Wright's stain. Capsules are not stained.

Fig. 5. Type II-N strain stained by Hiss capsule stain. Capsules darkly colored.

Fig. 6. Type II-V strain by Hiss stain. Capsules are stained very lightly on some of the organisms, not at all on others.

Fig. 7. Type III-V₁ strain stained by method of Wright. Capsules appear as wide pink staining halo.

Fig. 8. Type III-V₂ strain by Wright's method. Capsules are narrower than those of V₁ and appear striated.

Fig. 9. Colony of type I-N strain after growth of forty-eight hours on blood agar at 37°C.

Fig. 10. Colony of type I-V strain on other half of same agar plate as colony in figure 9.

Fig. 11. Colonies of type III-V₁ strain after growth of four days at 25°C. on blood agar.

Fig. 12. Colony of type III-V₂ strain on other half of same agar plate as colony in figure 11.
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