Mechanism of 'Vegetative Hybridization' in Streptomyces

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SUMMARY: A population of *Streptomyces griseus*, strain W107, exposed for several growth cycles to a sterile culture filtrate of *S. griseus*, strain M142, acquired several genetic characteristics similar to those of strain M142. The changes observed were:

1. streptomycin sensitivity to resistance;
2. bacteriophage V sensitivity to resistance;
3. absence to presence of soluble pigment;
4. presence to absence of pigment in the vegetative mycelium.

The filtrate contained streptomycin and a temperate bacteriophage. The low concentration of streptomycin did not grossly inhibit the growth of strain W107 but streptomycin-resistant mutants were selected. Resistance to the temperate phage frequently conferred resistance to bacteriophage V. The observed morphological changes were coupled with bacteriophage- and streptomycin-susceptibility. Vegetative hybridization was the result of selection of mutants rather than gene transfer.

The streptomycetes are filamentous bacteria which resemble the true moulds in many respects. Hyphal fusion with resulting heterokaryosis has been demonstrated for several true moulds (Beadle & Coonradt, 1944) and for different species of *Streptomyces* (Bradley, 1957a). Specialized sexual structures have not been found in the streptomycetes. Sexuality in *Streptomyces* (Sermonti & Spada-Sermonti, 1956) probably resembles either parasexuality in *Aspergillus* or sexuality in *Escherichia coli* (Lederberg, 1952).

As is the case among true bacteria, many streptomycetes are lysogenic (Bradley, 1957b; Welsch, 1956). Temperate streptomyces-phages might serve as vectors for the transfer of genetic material. Alteration of the genetic composition of *Streptomyces* populations by extracts which might contain bacteriophage has been reported (Horvath, Marton & Oroszlan, 1954). Several growth cycles in the presence of the extract were required before phenotypic shifts were detected. The characteristics affected were colonial morphology, pigmentation and antibiotic production. The mechanism responsible for this 'vegetative hybridization' was not amply studied. Similar changes in populations of *Streptomyces griseus* treated with a culture filtrate have now been observed. These phenotypic shifts might result from (1) phage-mediated transduction; (2) deoxyribonucleic acid-mediated transformation; (3) selection for particular phenotypes. This paper presents evidence that the culture filtrate used contained selective agents. Some of these have now been identified.

METHODS

Mutant substrains of *Streptomyces griseus* were used throughout this investigation. The genetic markers employed were growth-factor requirements, antibiotic sensitivity, bacteriophage sensitivity and morphology. The
characteristics of the mutant and ancestral substrains are shown in Table 1. The mutant substrains were obtained after ultraviolet irradiation or by direct selection using methods previously described (Bradley & Lederberg, 1956).

Table 1. Principal stocks of Streptomyces griseus used and their ancestors

<table>
<thead>
<tr>
<th>Strain</th>
<th>Derived from</th>
<th>Genotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td>W 86</td>
<td>Nature</td>
<td>V⁺ V₂ V₃ V⁴ B' S⁺ Ad⁺ Ar⁺ P⁺⁻ P⁺⁻</td>
</tr>
<tr>
<td>W 107</td>
<td>W 86</td>
<td>V₁ V₂ V₃ V⁴ B' S⁺ Ad⁺ (Ar⁻) P⁺⁻ P⁺⁻</td>
</tr>
<tr>
<td>W 104</td>
<td>Nature</td>
<td>V₁ V₂ V₃ V⁴ B⁺ S⁺ Ad⁺ Ar⁺ P⁺⁺ P⁺⁻</td>
</tr>
<tr>
<td>M 142</td>
<td>W 104</td>
<td>V₁ V₂ V₃ V⁴ B⁺ S⁺ (Ad⁻) Ar⁺ P⁺⁺ P⁺⁻</td>
</tr>
</tbody>
</table>

* V⁺ or V⁻ denotes sensitivity or resistance to bacteriophage. V₁, V₂ and V₃ represent bacteriophages WSP-3, WSP-2 and MSP-2 respectively. B⁺ or B⁻ denotes sensitivity or resistance to 2500 units bacitracin/l. S⁺ or S⁻ denotes sensitivity or resistance to 25 mg. dihydrostreptomycin sulfate/l. Ad⁺ or Ad⁻ and Ar⁺ or Ar⁻ denote independence or dependence for exogenous adenine or arginine 20 mg/l. P⁺ or P⁻ and P⁺⁺ or P⁻⁻ denote presence or absence of soluble pigment and pigment in the vegetative mycelium respectively.

( ), genetic markers obtained after ultraviolet irradiation.
———, genetic markers obtained by direct selection.

The basal medium was composed of: glucose, 20 g; KNO₃, 2 g; K₂HPO₄, 2 g; MgSO₄·7H₂O, 0·5 g; CaCO₃, 0·25 g; water, 1 l. For complete medium, 0·1% (w/v) Difco yeast extract was added. The medium was solidified with 1·5% (w/v) agar. Glucose and salts solutions were autoclaved separately at double strength.

The culture filtrate was prepared as follows. Complete medium was inoculated with *Streptomyces griseus*, strain M 142, to yield 10⁶–10⁷ spores/ml and incubated at 30° on a rotary shaker. The culture underwent spontaneous lysis after 4 days and the culture was then filtered through coarse filter-paper to remove floating debris and remaining mycelium. The culture filtrate was further clarified by centrifugation and rendered bacteriologically sterile by filtration.

RESULTS

*Streptomyces griseus*, strain W 107, was grown in complete medium to which 5% (v/v) strain M 142 filtrate had been added. After several serial subcultures exposed to the filtrate, non-parental types appeared, with respect to the streptomycin and bacteriophage V₁ characters. The strain M 142 culture filtrate did not affect the bacteriophage V₂ and arginine characters. These data, taken from a typical experiment, have only slight quantitative significance because ‘colony forming unit’ is not a distinct entity. Colony forming unit refers to a hyphal fragment of any size which produces a single discrete colony. The number of subcultures required to establish non-parental types varied considerably as between different experiments. Rarely non-parental types arose as early as the second subculture and in one experiment, non-parental types did not become established until the twelfth subculture.

Treated strain W 107, selected for one non-parental character, frequently differed for other characters as well (Table 3). Growth-factor requirements, bacitracin sensitivity and bacteriophage V₃ sensitivity were not altered in the
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selected streptomycin- or bacteriophage V<sub>1</sub>-resistant substrains. Other factors were affected to different degrees, exceeding the spontaneous mutation rate, which was less than 10<sup>-3</sup>.

Table 2. *Selection for non-parental phenotypes of Streptomyces griseus strain W107*

<table>
<thead>
<tr>
<th>No. subcultures in strain M142 filtrate</th>
<th>Characteristics selected</th>
<th>V&lt;sub&gt;1&lt;/sub&gt;</th>
<th>V&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Ar&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colonies/10&lt;sup&gt;7&lt;/sup&gt; colony-forming units</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>20</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>31</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>18</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>59</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>132</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>897</td>
<td>1021</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>8.8 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.7 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3. *Characteristics of non-parental isolates of Streptomyces griseus strain W107*

Strain W107 was grown for 9 subcultures in the presence of 5% (v/v) strain M142 or W104 culture-filtrates.

<table>
<thead>
<tr>
<th>Filtrate used</th>
<th>Character selected</th>
<th>V&lt;sub&gt;1&lt;/sub&gt;</th>
<th>V&lt;sub&gt;3&lt;/sub&gt;</th>
<th>B</th>
<th>S</th>
<th>P&lt;sub&gt;s&lt;/sub&gt;</th>
<th>P&lt;sub&gt;v&lt;/sub&gt;</th>
<th>Ad</th>
<th>Ar</th>
</tr>
</thead>
<tbody>
<tr>
<td>M142</td>
<td>Control, no selection</td>
<td>13</td>
<td>4</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>35</td>
<td>2</td>
<td>9</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>M142 V&lt;sub&gt;1&lt;/sub&gt;</td>
<td>27</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>100</td>
<td>9</td>
<td>14</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>M142 V&lt;sub&gt;3&lt;/sub&gt;</td>
<td>100</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>13</td>
<td>0.6</td>
<td>15</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>W104</td>
<td>Control, no selection</td>
<td>24</td>
<td>7</td>
<td>0.5</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>2</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

A filtrate of strain M142, heated at 65<sup>o</sup>C for 30 min., was added to strain W107 cultures. No strain W107 organism, resistant to bacteriophage V<sub>1</sub>, was found during any of the serial subcultures. The development of streptomycin resistance was not affected. This indicated that at least two distinct agents were involved.

Strain M142 is lysogenic (Bradley, 1957<sup>c</sup>). Antisera against the temperate phage L8 was added to the strain W107 cultures, in addition to the M142 culture-filtrate. No bacteriophage V<sub>1</sub>-resistant organisms were isolated during the serial subcultures. As with heat-treated filtrates, antisera did not affect the development of streptomycin resistance. The temperate phage L8 was responsible for the increased proportion of V<sub>1</sub> cells.

The ancestor of strain M142, i.e. W104, is streptomycin sensitive, phage V<sub>1</sub> sensitive and lysogenic for the temperate phage L8. Filtrates of strain W104 added to growing cultures of W107 produced the change from phage V<sub>1</sub> sensitivity to resistance as effectively as filtrates of strain M142. However,
the W104 filtrate did not affect the streptomycin character (Table 3). As before, the number of subcultures required to attain corresponding yields of the non-parental types varied considerably as between different experiments.

Table 4. Factors affecting the activity of Streptomyces griseus strain M142 filtrates

<table>
<thead>
<tr>
<th>Treatment</th>
<th>V1 colonies/10^6</th>
<th>V2 colonies/10^6</th>
<th>S colonies/10^6</th>
<th>Ar+ colonies/10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>65° for 30 min.</td>
<td>88</td>
<td>0</td>
<td>7.1 x 10^6</td>
<td>0</td>
</tr>
<tr>
<td>Anti-L3 serum</td>
<td></td>
<td></td>
<td>4.9 x 10^6</td>
<td>0</td>
</tr>
<tr>
<td>Activated charcoal</td>
<td>9.6 x 10^6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pH 2 eluate from charcoal</td>
<td>61</td>
<td>0</td>
<td>1.0 x 10^7</td>
<td>0</td>
</tr>
<tr>
<td>Control, no treatment</td>
<td>1.4 x 10^6</td>
<td>0</td>
<td>3.2 x 10^6</td>
<td>0</td>
</tr>
</tbody>
</table>

Bacteriophages L3 and V1 are demonstrably different. However, resistance to L3 might concurrently confer resistance to V1. This was shown to occur. Of 68 mutants of strain W107 selected for resistance to L3, five were also resistant to V1. Moreover, two were resistant to V2 and one lacked pigment in the vegetative mycelium. Resistance to the temperate phage L3 might result by selection of a mutant or by lysogenization. If W107 were lysogenized, free phage should be demonstrable in culture autolysates. Bacteriologically sterile filtrates of phage V1-resistant derivatives of strain W107 were plated with sensitive indicator strains. No evidence of lysogenization was found. The temperate phage L3 was irregularly found in lysates of strain M142. Therefore the temperate phage L3 was the selective agent responsible for certain changes in morphology and bacteriophage susceptibility.

Activated charcoal removed the factor in the strain M142 filtrate (pH 7.0) responsible for the change from streptomycin sensitivity to resistance. An eluate made by extracting the activated charcoal with acidified water (pH 2) was able to alter streptomycin susceptibility. The eluate inhibited completely the growth of strain W107 unless the eluate was adequately diluted. This indicated that strain M142 might produce an antibiotic. The filtrate inhibited streptomycin-sensitive but not streptomycin-resistant strains of Escherichia coli. Comparing the inhibitory effect of the culture filtrate and dihydrostreptomycin sulphate standards, it was found that strain M142 produced antibiotic equivalent to 30-40 mg. streptomycin/l.

The low concentration of streptomycin added to the experimental flasks was not sufficient to alter grossly the growth of strain W107. However, W107 grown for 10 subcultures in 2 mg. dihydrostreptomycin sulphate/l. became resistant to 25 mg./l. A few of these colonies on complete agar produced soluble pigment (7 of 1291) and several lacked pigment in the vegetative mycelium (122 of 1291).

DISCUSSION

Culture filtrates of Streptomyces griseus, strain M142, brought about certain genetic changes in S. griseus, strain W107. Several growth cycles in the filtrate were required before these changes became detectable. Selective agents pro-
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duced by strain M142 were responsible. Two of the agents, temperate bacteriophage L3 and streptomycin, were isolated from the M142 filtrates.

To demonstrate conclusively that the genesis of resistance to bacteriophage V1 was the result of selection of mutants resistant to temperate bacteriophage L3, it was necessary to rule out DNA-mediated transformation, phage-mediated transduction and cross-resistance conferred by lysogenization. Antibody against temperate phage L3 prevented the development of V1 resistance. Temperate phage L3 was essential, therefore DNA-mediated transformation was not the mechanism involved. Moreover, L3 grown on a V1-sensitive donor promoted the development of V1 resistance as effectively as phage L3 grown on strain M142. This was inconsistent with both DNA-mediated transformation and phage-mediated transduction. Temperate phage L3 could not be isolated from autolysates of the V1-resistant derivatives of strain W107. This indicated that lysogenization had not occurred.

Quantitative comparisons among the streptomycetes were difficult because 'cell number' could not be accurately enumerated. Moreover, genetically-altered nuclei might be masked by normal nuclei in the same cytoplasm. The alterations resulting from dihydrostreptomycin sulphate and phage L3 did not precisely duplicate the effect of the culture filtrate. It is possible that other selective agents were involved. Metabolic products such as alanine and kinetin select for particular phenotypes of Brucella (Mika, Braun, Ciaccio & Goodlow, 1954; Braun, Firshein & Whallon, 1957). Horvath et al. (1954) interpreted similar data as transduction or transformation. Selection of mutants was ruled out because the observed changes grossly occurred during a single subculture, after several subcultures in presence of the cell extract. Gross populational changes cannot be used as an accurate test of selection. The choice of genetic markers is important for interpretation of experimental data. In Streptomyces, antibiotic resistance may be coupled with antibiotic synthesis. Cross-reaction among streptomycetes-phages and the relatively high mutation rate from phage sensitivity to resistance makes this genetic marker difficult to score. Morphological expressions seemed to be related to antibiotic and bacteriophage sensitivity. Growth-factor requirements have not been found, in this study, to be coupled with morphology or susceptibility. Growth-factor requirements are invaluable in genetic studies of the Streptomyces.

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REFERENCES


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