INDUCED BIOCHEMICAL MUTANTS OF AZOTOBACTER AGILIS

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Through the intense study of biochemical genetics in recent years an insight has been gained into the mechanism of gene action, which in conjunction with previously known facts has made it possible to investigate the existence of gene-like determinants in organisms that do not lend themselves to ordinary genetical analysis. The application of these developments has opened up the previously almost nonexistent field of bacterial genetics, and on the basis of recent evidence (Gray and Tatum, 1944; Lederberg, 1947) it is already fairly well established that at least some bacteria possess organizers that correspond to the genes in higher forms.

Bacteria, like higher organisms, are characterized by a great hereditary stability, even with regard to characteristics that are only potentially existing, such as adaptive enzymes. This makes it necessary to assume the existence of a precise mechanism whereby the parental characteristics are exactly duplicated in the offspring. A certain degree of mutability is still exhibited, the direction of which is as a rule independent of external factors, and new characters thus established are passed on with the same precision as the original ones. The frequency of appearance of new forms is increased by destructive agents that increase the mutation rate in higher organisms. And, as far as has been established, the function of the bacterial hereditary determinants seems to be the same as that of the genes in higher forms. This is indicated by the fact that artificially induced variants with specific growth factor requirements are obtainable by the same methods as in sexually reproducing organisms (Gray and Tatum, 1944). Presumably these variants are unable to carry out a particular chemical process in a synthetic reaction chain like similar mutants of Neurospora, although this has not been specifically demonstrated.

The work presented in this paper was started early in 1944, when the possibilities of new developments in the field of bacterial genetics were just beginning to be realized. But even at that time a close resemblance between the basic hereditary mechanisms of bacteria and higher forms seemed probable that it was decided to make an attempt to isolate not only the already known types of biochemical mutants, but also variants with specific genetic blocks in some major metabolic pathways, which then could be studied with the aid of such mutants. The main interest was in the oxidative dissimilation of organic compounds. Although variants of this type had not been obtained artificially, they were known to occur in nature, so that no good reason was evident why they could not be produced. But since it was not certain that such mutations could be isolated from irradiated material, the process of nitrogen fixation was selected as a second choice, as it is known to be dispensable in the presence of nitrogenous compounds.
Azotobacter offers suitable material for the study of the mechanisms of both oxidative dissimilation and nitrogen fixation and was therefore chosen for this investigation.

MATERIALS AND METHODS

The organism employed in this study was Azotobacter agilis, strains 4.4 and 4.5, obtained from Professor C. B. van Niel. After several single colony isolations uniformity in colony size was attained, and subsequently all experiments were started from single colonies. A. agilis is more satisfactory than other Azotobacter species for these experiments because the cells are more easily separated and more easily identified by microscopic examination.

The following basal medium (medium 1), made up in distilled water, was used when nothing else is specified: 1 per cent (vol.) ethyl alcohol, 0.1 per cent K2HPO4, 0.02 per cent MgSO4, 0.01 per cent CaSO4, 0.003 per cent FeSO4, and 0.0002 per cent NaMoO4. The pH was adjusted to 7.2 with hydrochloric acid. In solid media 1.5 per cent agar was included. The incubation temperature was 28 to 30°C. Liquid media were usually agitated on a horizontal shaker to ensure uniform and rapid growth.

For the isolation of irradiated bacteria a partially selective medium of the following composition was employed (medium 2): 1.0 per cent (vol.) ethyl alcohol, 0.01 per cent glucose, 0.02 per cent yeast extract (Difco), 0.04 per cent K2HPO4, 0.02 per cent MgSO4, 0.01 per cent CaSO4, 0.003 per cent FeSO4, 0.0002 per cent NaMoO4, 1.0 per cent (vol.) of a 0.04 per cent aqueous bromthymol blue indicator solution; the pH was adjusted to 7.2. The important features of this medium are: (a) alcohol is the only abundant energy source, so that colonies unable to utilize it for growth will appear small; (b) the low buffering capacity and the indicator permit the formation of a small amount of acid or alkali to be detected; (c) the total amount of nitrogen, added in the form of yeast extract, is so standardized as to permit only limited growth in the absence of nitrogen fixation; (d) the presence of the yeast extract should permit development of mutants requiring growth factors. The use of this selective medium made the detection of variants with disturbances in the nitrogen fixation or alcohol oxidation mechanisms more probable, but did not discriminate against any types of mutants. Thus the probability of finding growth factor variants or mutants unable to oxidize glucose was the same as if colonies were tested at random.

Mutations were induced by X-ray treatment of 1 ml of a 40-hr culture in the basal medium. The irradiation was carried out with an oil-cooled X-ray tube operated at 200 kilovolts and 15 milliamperes. The distance from the target was 47 cm when the dosage was 1,250 roentgens per minute. The total exposure was 150,000 roentgens, which killed approximately 99.99 per cent of the cells. The remaining viable cells were plated out on the surface of medium 2 and incubated for 3 to 4 days. Small and otherwise aberrant colonies from these plates were tested for growth on medium 1 with alcohol alone or with glucose alone. As a rule these tests were carried out on solid media to avoid loss of unstable variants.
If abnormal behavior was again observed, further tests were carried out in accordance with the suspected characteristics.

Growth was measured with the Evelyn photoelectric colorimeter and expressed as \( 2 - \log G \).

**RESULTS**

Several physiological mutants of *A. agilis* were obtained by isolation from X-ray-treated cultures. The data are not extensive enough to warrant a statement as to the mutation frequency, particularly since the methods do not ensure detection of all mutants, but the indications are that the frequency is in the same order of magnitude as in other bacteria. The data from the irradiation experiments are summarized in table 1.

As shown in the table almost 8,000 single cell isolations were made from irradiated material, but only 383 colonies were specifically tested for altered physiological characteristics. The majority of these were small or otherwise aberrant colonies.

Of great interest is the high degree of mutability with regard to colony form and size and possibly other characteristics that is exhibited on subsequent transfer of colonies from X-ray-treated material. A further study of this latent variation would certainly be pertinent, but little attention was given to it in this work, since its significance was not realized at the outset. In all probability this phenomenon is a manifestation of the same mechanism as the latent variation in *Escherichia coli*, which has been reported by Demerec (1946). The possibility seems very attractive that the same factors might also be responsible for the instability of some of the biochemical strains.

Morphological variants, involving colony form and color, are quite frequent. The parent strain forms a greenish-yellow water-soluble pigment, but approximately 20 per cent of the viable irradiated population in each experiment were no longer able to form the pigment. Similarly, a high proportion of rough colony variants, up to 40 per cent, was found in the treated material, whereas the original strain consisted entirely of smooth colony variants.

**TABLE 1**

<table>
<thead>
<tr>
<th>EXPERIMENT</th>
<th>X-RAY DOSAGE</th>
<th>NO. AT START</th>
<th>PERCENTAGE KILLING</th>
<th>COLONIES ISOLATED</th>
<th>COLONIES SPECIFICALLY TESTED</th>
<th>PHYSIOLOGICAL STRAINS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>1.1 ( \times 10^4 )</td>
<td>2 ( \times 10^6 )</td>
<td>99.92</td>
<td>1,500</td>
<td>93</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>1.5 ( \times 10^6 )</td>
<td>99.40</td>
<td>4,950</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1.5 ( \times 10^4 )</td>
<td>1.5 ( \times 10^6 )</td>
<td>99.997</td>
<td>26</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>5 ( \times 10^6 )</td>
<td>99.95</td>
<td>1,338</td>
<td>192</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1.5 ( \times 10^4 )</td>
<td>5 ( \times 10^6 )</td>
<td>99.996</td>
<td>70</td>
<td>70</td>
<td>1</td>
</tr>
</tbody>
</table>

* A Coolidge type X-ray tube, operated at 100 kv and 5 ma, was used for this experiment.
Physiological variants other than colorless ones are less frequently encountered, but they are of the same kind as those already described in other organisms in the sense that they lack the ability to form certain compounds required in the normal metabolism.

A discussion of the biochemical characteristics of the mutants is present below.

Pigment variants. As already mentioned, among the most common variants are those lacking the ability to form the diffusible greenish pigment that is characteristic of A. agilis. These mutants appear like the normal strain when grown in shaken liquid cultures without combined nitrogen, but growth is very slight on solid media of the same composition and is slow in stationary test tubes. Solid media supplemented with yeast extract support good growth, and at least some of these variants give a response to added ammonia, which inhibits the normal strain to some extent. The slight growth obtained on the solid basal medium appears glossy, and microscopic examination reveals large, refractile globules that fill the cells. Motility is absent. These mutants are rather stable and have never been observed to revert. The fact that they differ from the other kinds of physiological mutants, occurring more frequently and being more stable, suggests that they may be formed by a different mutation path.

Variants requiring growth factors. The first clear-cut nutritional variant isolated, strain A3, was so unstable as to make further characterization difficult. For this reason the particular growth factor required was never identified, but it was definitely established that something present in yeast extract was needed, growth on alcohol being proportional to the amount of yeast extract supplied, as is shown graphically in figure 1.
The second physiological mutant, strain A5, was also unstable, possibly no less so than A3; but since more experience had been gained at that stage, this variant was characterized as quickly as possible without any transfers except to the first plate, where growth was very uniform. The colonies from this plate were then used for all the subsequent tests. Growth was proportional to the amount of yeast extract added as shown by figure 2, and casein hydrolyzate seemed to contain roughly three times as much of the required substance as the yeast extract. Finally the amino acid leucine was identified as a growth factor for this strain, as is indicated in figure 3.
When the first transfer of strain A5 to 0.5 per cent yeast extract was made, growth was very homogeneous, so that all the colonies appeared alike, slightly rough, and average in size. From then on difficulties were encountered because of instability, and a uniform culture with the original characteristics could not be re-established. Growth occurred even on the basal medium after a long initial lag period, during which the cells were filamentous, similar to those shown by Den Dooren de Jong (1938). When adapted, these elongated forms transformed into the ordinary spherical cell type.

Variants with disturbed energy metabolism. The last physiological variant isolated, strain A13, was detected as a colony unable to utilize glucose for growth. When grown on alcohol, it was indistinguishable from the parent strain. A high degree of stability was indicated by the finding that in a liquid medium containing both alcohol and glucose the total growth corresponded only to the alcohol, showing that not a single reversal occurred during the growth of millions of cells. However, reversion was effected several times by long-continued shaking in liquid media, and the resulting organism was identical with the original strain, as far as substrate utilization and morphological characters were concerned. These facts, namely, that reversal can occur and that all the characteristics of the mutant revert at the same time, indicate that only a single alteration is responsible for the disturbances in the energy metabolism of this strain.

The biochemical characteristics of strain A13 will be described elsewhere. It has lost the ability to oxidize pyruvate to acetate, which seems to be the main channel of pyruvate breakdown in the parent strain. A13 is unable to grow on any substrate that is arrested at the pyruvate stage, since acetate or its derivatives are essential for development. Growth is supported only by alcohol, acetate, and malonate. The following compounds used by the parent strain cannot serve as growth substrates for the mutant: glucose, fructose, gluconate,
tartarate, trans-aconitate, cis-aconitate, \(\alpha\)-ketoglutarate, succinate, fumarate, malate, lactate, and pyruvate.

Figure 4 is a photograph of the growth of the original and mutant strains with alcohol or glucose as an energy source.

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SUMMARY

It has been demonstrated that X-ray-induced mutants are obtainable in *Azotobacter agilis*. Morphological variants, involving colony form and color, occur frequently, and physiological mutants have also been produced.

A special search was made for variants with disturbed energy metabolism, and a rather stable mutant was discovered which, because of a complete genetic block, is unable to convert pyruvate to acetate. Since acetate or its derivatives are essential for growth, this mutant is not able to develop on substrates such as glucose and succinate, which are arrested at the pyruvate stage.

REFERENCES


Lederberg, J. 1947 Gene recombination and linked segregations in *Escherichia coli*. Genetics, 32, 505-525.