GENETIC TRANSFORMATION OF RHIZOBIUM: A REVIEW
OF THE WORK OF R. BALASSA

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Prefatory Note

Dr. Rozsi Balassa, who died in Budapest in 1960, devoted the last years of her life to the genetic study of Rhizobium. This important work remained incomplete, and only part of it has been published, often in languages and periodicals that are not readily accessible to most research workers. For this reason, I have gathered together her results in the form of a review, which I hope will be useful to specialists in symbiosis as well as to those working in the field of bacterial genetics.

This article will be limited to a description and an analysis of the results obtained in the laboratory of R. Balassa. A few fundamental notions concerning transformation and the physiology of symbiosis will be presented at the outset. For a bibliography of transformation, the reader is directed to the recent reviews of Ravin (10) and Schaeffer (12), and, for a bibliography concerning Rhizobium, to the reviews of Allen and Allen (1, 2). A detailed description of the methods used in this kind of research may be found in these references.

My principal concern was with the objectivity of this presentation. The rapid development of this field sometimes obliged me to reconsider the value of the methods that were used as well as the interpretations proposed; it also made it necessary to introduce some new hypotheses. Responsibility for the latter should fall only upon the author of this review.

The experimental work described here was accomplished by R. Balassa, in collaboration with M. Gabor, and with the technical assistance of
Mrs. I. Goth, Mrs. S. Burszan, Mrs. S. Letenay, and Mrs. E. Persz. The work was carried out in the Institute of Genetics of the Hungarian Academy of Sciences (Director: B. György) during the years 1950–1960. A list of the original publications is given at the end of the article.

INTRODUCTION

The genetic transformation of bacteria, in the broadest sense of this term, is the transfer of certain hereditary properties from one bacterium to another by means of cellular constituents extracted from the former. The study of transformation is of great interest to basic genetics in addition to being a useful tool for the genetic analyses of specific bacterial characters. First of all, it permits us to obtain direct information on the nature and properties of the genetic material, deoxyribonucleic acid (DNA), and of the mechanism of genetic recombination; second, it affords an opportunity to study specific mutations in certain bacteria, to observe the expression of a property newly introduced into a bacterium, and to study the relationships between bacterial species.

Before describing the results obtained in Rhizobium, it would seem useful to discuss the advantages and disadvantages of this organism. At the present time, a relatively large number of bacterial species are known to be transformable. However, the present research was begun at a time when the existence of transformation had been proven only in pneumococcus, in Haemophilus, and in the meningococcus. The discovery of transformation in Rhizobium, an organism which is not closely related to the other groups, contributed to establishing the generality of the phenomenon.

For those studying such biological properties of Rhizobium as symbiosis and nitrogen fixation, the use of transformation in this organism is certainly very advantageous. It permits one to study the genetic determination of these properties and the relationship among the different characters of a strain; in addition, it opens a new path for the study of the biochemical mechanism of symbiosis.

The geneticist who is looking for a useful material for studying the mechanism of transformation can also find advantages in Rhizobium. In the first place, the properties of the members of this genus have been very well studied. Moreover, the ease of using a synthetic medium and of selecting mutants, as well as the existence of a large number of species (which are unambiguously defined by their specificity with respect to different groups of leguminous plants), some of which harbor lytic and temperate phages, offer many opportunities for investigation.

On the other hand, the presence of several nuclei per bacterium, and the rather complex morphological variations that Rhizobium species frequently undergo, constitute the principal disadvantages of this material.

In the first two parts of this review, I will present, in turn, the methods that have been used and the results of transformation in Rhizobium and of the study of certain nonsymbiotic properties, such as resistance to streptomycin and auxotrophy. A great deal of knowledge has been accumulated over the years from experiments performed with the most thoroughly studied of the transformable bacteria, namely, the pneumococcus, the Haemophilus bacterium, and, more recently, Bacillus subtilis. Although the observations obtained with Rhizobium do not differ, in general, from those obtained with other species, they will be summarized briefly before analyzing in greater detail certain of the results in Rhizobium. The third part will be devoted entirely to the experiments on symbiosis and to an analysis of the genetic determination of the symbiotic properties of Rhizobium. This presentation will not follow the chronological order of the research, and certain of the earlier experiments, which were accomplished under less well-defined conditions, will be described last.

RESEARCH ON TRANSFORMATION

The study of bacterial transformation began of necessity with a search for transformable strains, for conditions that favor the development of competence, and for methods for the extraction and purification of the transforming substance. I will describe briefly the strains, the media, and the methods that have been utilized in the transformation of Rhizobium. This description should be useful because it will reflect the principal difficulties generally encountered when beginning work on transformation, and because some of the results to be analyzed were obtained by the early, more rudimentary methods.
have been obtained of the Institut strains can be obtained from the

These isolations were made in Hungary during a 2-year period preceding the beginning of the experiments on transformation. Table 1 indicates the origin and certain characteristics of these strains as well as a list of some mutants that have been obtained from them. (Some of these strains can be obtained from the culture collection of the Institut Pasteur.)

**Strains**

Strains of four species of *Rhizobium* (*meliloti, lupini, japonicum,* and *leguminosarum*) were isolated from soil cultures of various leguminous plants. These isolations were made in Hungary during a 2-year period preceding the beginning of the experiments on transformation. Table 1 indicates the origin and certain characteristics of these strains as well as a list of some mutants that have been obtained from them. (Some of these strains can be obtained from the culture collection of the Institut Pasteur.)

**Media**

*Synthetic medium.* Synthetic medium contained (per liter): K$_2$PO$_4$, 3.6 g; KH$_2$PO$_4$, 0.4 g; MgSO$_4$·7H$_2$O, 0.05 g; NaCl, 0.5 g; (NH$_4$)$_2$SO$_4$, 1 g; and ferric ammonium citrate, 4.0 mg. The pH was adjusted to 7.0, and the medium was autoclaved. Glucose was added for a final concentration of 10 g per liter, and, if necessary, biotin was also added (0.3 g per liter).

*Complex medium.* To synthetic medium (without ammonium sulfate) were added (per liter) casein hydrolysate, 1 g; and yeast extract, 1 g.

**Method of Transfer**

It was while growing bacteria of a certain type in the sterile cultures of bacteria of another type that the first in vitro observations of genetic transformation were made. Specifically, Krasilnikov (8) succeeded in introducing symbiotic characters into a strain of *Rhizobium*, while growing it during several passages in the sterile filtrate of a culture of another strain. This initial observation led to the first work on the transformation of *Rhizobium*.

As it was impossible to know *a priori* which species would be transformable, the first experiments, which employed the method of serial passage, utilized a mixture of receptor species. (The strain from which the transforming substance has been extracted is referred to as the donor strain, and the one subjected to the action of this substance is referred to as the receptor strain.)

The mixture of receptor strains gave positive results (see Transformation of Symbiotic Properties of *Rhizobium*), and examination of the transformed bacteria that were so obtained permitted one to choose the most readily transformable species. However, this method had numerous defects, among which were its slowness, its poor reproducibility, and the large number of factors which remained unknown.

**Use of Ultrasound and Penicillin**

In searching for better techniques, the use of ultrasound gave the first encouraging results. Transforming substance was obtained by disintegrating the donor bacteria by means of ultrasonic waves, and the material thus obtained was added to a culture of the receptor strain to be transformed.

Another method, described by Hotchkiss (7) for the transformation of *pneumococcus*, was also used. Penicillin was added to a culture of the donor strain (carrying a streptomycin or a host-specificity marker) which is penicillin-sensitive, and after lysis the culture was inoculated with the receptor strain which had been previously rendered penicillin-resistant. One can also add penicillin to a mixed culture which contains both strains at the same time. The results obtained by the use of penicillin were similar to those obtained by ultrasonic extraction of the transforming substance.

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**TABLE 1. Origin and properties of the strains of *Rhizobium* employed**

| Strain | Species      | Original host                          | Growth    | Biotin independence | Natural resistance to streptomycin
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td><em>R. meliloti</em></td>
<td>Alfalfa</td>
<td>Slow</td>
<td>-</td>
<td>0.1 g/ml</td>
</tr>
<tr>
<td>L</td>
<td><em>R. lupini</em></td>
<td>Lupine</td>
<td>Rapid</td>
<td>+</td>
<td>2 g/ml</td>
</tr>
<tr>
<td>H</td>
<td><em>R. lupini</em></td>
<td><em>Lupinus hartwegii</em></td>
<td>Very rapid</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>J</td>
<td><em>R. japonicum</em></td>
<td><em>Sainfoin (Onobrychis vicifolia)</em></td>
<td>Rapid</td>
<td>+</td>
<td>0.1 g/ml</td>
</tr>
</tbody>
</table>

* Strains listed in the table are wild strains. Mutants of strain H are cys$^-$ and val$^-$; A, B, and C are rough mutants, differing by their colonial morphology, isolated from strain H.
Transformation with DNA

Avery, MacLeod, and McCarty (3) and Hotchkiss (6) demonstrated that the transforming agent, at least in pneumococcus, is DNA. Consequently, after the experiments just described permitted a choice of the most desirable strains and genetic markers, it was natural to verify the role of DNA in the transformation of *Rhizobium* and thereby to develop a method of transformation using purified DNA. For these experiments DNA was prepared and purified by the usual methods, starting with bacteria lysed by deoxycholate (DOC). The bacteria were centrifuged at the end of exponential growth, resuspended in 0.1 M NaCl and 0.01 M sodium citrate, and lysed by the addition of 0.2 volume of 5% DOC. The lysis was usually allowed to occur at 55°C for 10 min. The material thus obtained was deproteinized several times by the Sevag method and further purified by repeated precipitation with ethanol. The high resistance of *Rhizobium* to DOC necessitated a rather high temperature during the course of lysis. To determine whether a significant loss of biological activity took place under these conditions, the method was occasionally replaced by lysis in the presence of penicillin. In this latter method, bacteria in their exponential phase of growth were centrifuged and resuspended, at a density which still permitted growth (maximum of $10^8$ bacteria/ml), in an optimal medium to which a high concentration of penicillin (100 μg/ml) was added. After 3 hr, citrate was added for a final concentration of 0.01 M, the suspension was centrifuged, and finally the same procedure was followed as after lysis by DOC. The yield was from 20 to 50%.

Except for a quantitative difference in the biological activity of preparations obtained by the ultrasonic and penicillin methods (see below), the results were essentially the same with the two kinds of preparation.

The transformation experiment was carried out as follows. A 20-hr-old culture (40 hr in the case of strain M) was diluted into 2 ml of fresh minimal medium for a final concentration of $10^8$ bacteria/ml. DNA, added at the end of the latent phase of growth (3 to 6 hr), acted over a period of two to three generations. The transformation reaction was stopped, if necessary, by the addition of deoxyribonuclease. If selection of transformants was accomplished by means of an antibiotic, the cultures were incubated for the necessary period of time to allow phenotypic expression, before applying the antibiotic.

Each experiment was accompanied by a certain number of controls to verify that the number of spontaneous mutations bearing the marker under study was insignificant, that the transforming extracts did not contain any viable bacteria, that the transformation was sensitive to deoxyribonuclease, and that the action of the DNA was specific (only DNA from a donor strain bearing the genetic marker being active).

**Results of Transformation of Nonsymbiotic Properties**

**Mechanism of Transformation**

The mechanism of transformation has been analyzed in great detail in pneumococcus and *Haemophilus*, and in these organisms the following stages have been distinguished: (i) development of competent bacteria, (ii) adsorption of DNA on the bacterial surface, (iii) penetration of DNA, (iv) genetic integration of the transforming marker, and (v) expression of the new phenotype.

In this section, I will bring together some observations made on *Rhizobium* which relate to the first stages of transformation.

**Competence.** Competence (or transformability), defined as the permeability of cells to DNA, depends on a number of factors (the medium, the physiological state of the cells, etc.) which are not yet fully understood. One of the experiments permitting us to follow the appearance of competence is the following. At various times, bacteria grown under specified conditions were placed in contact with an excess of DNA; after a period of time sufficient for adsorption and penetration of the DNA, deoxyribonuclease was added to stop the reaction; the new phenotype was allowed to be expressed; and, finally, the number of transformed bacteria was determined. The results in pneumococcus show that competence appears at culture densities that depend upon the size of the inoculum. In *Haemophilus* it reaches a maximum at the end of the exponential phase of growth. *Rhizobium*, on the other hand, is competent, under the conditions that have been studied, especially at the beginning of the exponential phase of growth, the most propitious moment seeming to occur during the first two divisions after the latent phase (at bacterial densities that still permit several divisions in the medium). Under the
conditions used, therefore, the behavior of Rhizobium in regard to competence is different from that of other transformable species of bacteria.

Adsortion and penetration of DNA. When the number of competent bacteria is constant, the frequency of transformation is proportional to the concentration of DNA up to a limiting, saturating concentration. This concentration depends, in Rhizobium, on the method of preparing the transforming DNA. DNA prepared after lysis by DOC is partially inactivated, probably because of the high temperature necessary for lysis. The saturating concentration of such DNA is of the order of 0.2 to 0.5 \( \mu g \) per ml, which is higher than that of DNA obtained from penicillin lysates (0.1 \( \mu g \) per ml).

Nontransforming DNA (lacking the genetic marker or of foreign origin) can also be adsorbed by the bacterium, and it can prevent transformation or lower its frequency. Thus, the transformation of strain J is completely inhibited by the prior addition, in excess, of DNA from the sensitive strain (homospecific DNA); calf thymus DNA, under the same conditions, inhibits only up to 90%.

Upon penetrating the bacterium, transforming DNA becomes inaccessible to deoxyribonuclease, a fact which permits measurement of the time necessary for penetration. In the pneumococcus, this time is extremely short; several minutes are sufficient for the DNA to be entirely protected against the enzyme. In Rhizobium, a longer period seems to be needed for penetration; the optimal time of contact between bacteria and DNA is 30 min.

Transformation of Auxotrophic Mutants

Although auxotrophic mutants of Escherichia coli and Salmonella have been in use for a long time in genetic research, the transformation of such mutants has been accomplished only recently. The classic transformable species of bacteria, pneumococcus and Haemophilus, require complex nutrients and, hence, are not suitable for such studies.

It was in Rhizobium that the first case was described of transformation by wild-type DNA of a mutant having a metabolic deficiency. The same year and independently, Spizizen (13) demonstrated the possibility of such transformation in B. subtilis.

Description of the cysteine-dependent strain.

From strain H, treated by nitrogen mustard, several auxotrophic mutants were isolated by the method of Davis (4), among which was a cysteine-dependent (cys-) mutant. The colonies of this cys- mutant differ from those of the original strain by their specific appearance, being glossy and entirely homogeneous (the wild type having mucoid colonies with centers that become creamy-yellow).

Deprived of cysteine, cys- bacteria undergo a few divisions. This residual growth leads to the formation of microcolonies in which reverse mutations arise more or less early. Thus, over a period of 5 to 6 days, one observes on minimal medium a daily increase in the number of visible colonies. These colonies are of three types: (i) reverse mutant (cys+) colonies, which reach the size and have the appearance of wild-type colonies; (ii) colonies of partial reversions, which are morphologically cys-, grow very slowly on minimal medium, remain small, and give rise, when replated, to a variable frequency of cys+ reversions; and (iii) cys- microcolonies. Thus, there is a complex system of reversions in which intermediary steps are involved. Considering only the true reversions (the large colonies of wild-type appearance), the rate of spontaneous reversion (cys- \( \rightarrow \) cys+), as measured by the fluctuation method, is around 5 \( \times 10^{-9} \) per bacterium per division.

Transformation of the cysteine-dependent strain.

In transforming the cys- strain by DNA from the wild-type strain, one finds, after plating the transformed culture on minimal medium, a large number of colonies of the cys+ type, being normal in size and appearance, as well as colonies of the "partial reversion" type. Only the large "wild-type" colonies are considered in the estimation of the frequency of transformation, which can reach 0.5%. The small colonies are probably due in part to partial transformations, but the background of spontaneous mutations of this type prevents a systematic study of these transformations.

Essentially the same results are obtained using DNA from a valine-dependent, cysteine-independent strain (cys+; val-).

Intra- and Interspecific Transformation of Resistance to Streptomycin

Streptomycin resistance is the genetic marker of choice in the study of transformation in different species of Rhizobium. Starting with wild-type strains of Rhizobium, which are sensitive to strep-
tomycin (see Table 1), resistant mutants have been obtained by selection in a medium containing a high concentration of streptomycin as well as by successive passages in increasing concentrations of the antibiotic. The lysate or the DNA from these mutants serves to transform the original sensitive strain and strains belonging to other species of *Rhizobium*.

In the hope of obtaining more transformable strains, three "rough" morphological mutants (A, B, and C) were isolated from strain H. The transformation of these strains by DNA from different streptomycin-resistant mutants was studied. Since bacteria transformed for antibiotic resistance can be selected only after expression of the new phenotype has occurred, the time necessary for this expression, therefore, had to be determined.

In pneumococcus, resistance to streptomycin begins to appear about 5 to 10 min after penetration of the transforming DNA. During recombination in *E. coli*, on the other hand, several generations and the segregation of the nuclei must take place for conjugation to be followed by the appearance of resistant cells. The process in *Rhizobium* resembles the latter case, since several divisions precede the full expression of resistance.

Table 2 brings together the results of intraspecific and interspecific transformations. The three species that have been studied, *R. japonicum* (J), *R. meliloti* (M), and *R. lupini* (L and H), were transformed by extracts of different streptomycin-resistant mutants. The frequency of transformation varied according to the combination of receptor and donor strains. In general, transforming principle from mutants derived from the receptor strain transformed the latter at frequencies higher than that derived from mutants of other species. But the nature of the mutation had an influence as well; one observes, for example, a 100-fold difference between two independent mutants of the same species J (J100 and J1,000). The wild-type strain L, which is more resistant than strains J and H, is also capable of conferring resistance upon them. Finally, certain mutants do not transmit their resistance to any of the three species studied.

Among the rough strains, the most transformable one, C, was used as receptor. Transformation of this strain by DNA from smooth strains, H and, in particular, M occurred at very low frequencies. DNA of mutants A and B also gave frequencies lower than that obtained with DNA from C. These transformations confirm the preceding results. Finally, DNA from the resistant strain CSmtr, obtained as a transformant in strain C by the DNA from strain A Smr', was compared with DNA from strain A Smr'; the former gave the highest frequency of transformation. Since the same marker introduced from A into C was involved, the difference is attributed to the distant relationship of the strains in question.

Examination of the results shows that three factors determine the frequency of transformation: (i) the transformability of the receptor strain, (ii) the nature of the mutation being transformed, and (iii) the degree of relationship between the donor and receptor strains.

### Table 2a. Interspecific transformation of streptomycin resistance

<table>
<thead>
<tr>
<th>Donor strain</th>
<th>Receptor strain*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>Resistance</td>
</tr>
<tr>
<td></td>
<td>µg/ml</td>
</tr>
<tr>
<td>J†</td>
<td>0.1</td>
</tr>
<tr>
<td>M†</td>
<td>0.1</td>
</tr>
<tr>
<td>L†</td>
<td>2</td>
</tr>
<tr>
<td>J</td>
<td>10</td>
</tr>
<tr>
<td>J</td>
<td>100</td>
</tr>
<tr>
<td>J</td>
<td>1,000</td>
</tr>
<tr>
<td>M</td>
<td>100</td>
</tr>
<tr>
<td>M</td>
<td>500</td>
</tr>
<tr>
<td>L</td>
<td>10,000</td>
</tr>
</tbody>
</table>

* The figures indicate the ratio of transformation-spontaneous mutation.
† Wild strain.

### Table 2b. Transformation of streptomycin resistance in receptor strain C

<table>
<thead>
<tr>
<th>Donor strain</th>
<th>Resistance</th>
<th>No. of transformed bacteria/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>µg/ml</td>
<td></td>
</tr>
<tr>
<td>M Smr'</td>
<td>10,000</td>
<td>20</td>
</tr>
<tr>
<td>H Cys&lt;sup&gt;-&lt;/sup&gt;Smr'</td>
<td>1,000</td>
<td>300</td>
</tr>
<tr>
<td>A Smr'</td>
<td>1,000</td>
<td>100</td>
</tr>
<tr>
<td>B Smr'</td>
<td>1,000</td>
<td>900</td>
</tr>
<tr>
<td>C Smr&lt;sup&gt;-&lt;/sup&gt;</td>
<td>500</td>
<td>6,900</td>
</tr>
<tr>
<td>C Smr&lt;sup&gt;r&lt;/sup&gt;</td>
<td>1,000</td>
<td>28,000</td>
</tr>
</tbody>
</table>
The level of resistance of the transformed bacteria depends upon the nature of the receptor strain. Thus, if M is the receptor strain transformed by the lysate of a resistant mutant of strain H, resistant bacteria appear in the presence of the mixture of five amino acids, but not with lysine. The resistant bacteria thus obtained have the characteristics of strain H: they grow on the amino acid mixture and not on lysine. It seems, therefore, that the specific requirement is determined by the streptomycin marker itself. The nature of this peculiar requirement is still unknown.

**Transformation of Streptomycin Dependence**

The mutant A Sm\(^2\), which was isolated from strain A and possesses a high level of resistance, description of the experimental observations (Table 4).

The two strains can grow on minimal medium; they can also utilize several amino acids as a sole source of nitrogen. In the case of strain M (as opposed to strain H) lysine is one of these utilizable amino acids. In strain M, after mutation (resistant bacteria were selected by passage in increasing concentrations of antibiotic) or after transformation by DNA from strain M Sm\(^2\), resistance does not develop in minimal medium (containing ammonium sulfate as a sole nitrogen source). Casein hydrolysate, or lysine, is necessary for phenotypic expression, which does not occur in the presence of the other amino acids utilized by this strain. The situation is similar in the case of strain H, except that casein hydrolysate is not replaceable by lysine (on which this strain does not grow) but by a mixture of five amino acids (which are insufficient individually).

In the case of interspecific transformation, the development of resistance requires in the transformed bacteria the same factor as that required by the donor strain and not that required by the receptor strain. Thus, if M is the receptor strain, transformed by the lysate of a resistant mutant of strain H, resistant bacteria appear in the presence of the mixture of five amino acids, but not with lysine. The resistant bacteria thus obtained have the characteristics of strain H: they grow on the amino acid mixture and not on lysine. It seems, therefore, that the specific requirement is determined by the streptomycin marker itself. The nature of this peculiar requirement is still unknown.

**Metabolic Requirements for the Expression of Resistance**

A curious phenomenon concerning the expression of streptomycin resistance has been revealed by the study of transformation between species M and H. The nature of this phenomenon remains obscure, so we will present only a brief

| Table 3. Level of resistance of the transformed bacteria* |
|-----------------------------------|-----------------|-----------------|-----------------|-----------------|
| Donor strain                      | No. of streptomycin-resistant transformants |
| Name | Resistance | 10 μg/ml | 100 μg/ml | 1,000 μg/ml |
| C Sm\(^2\)... | 1,000 | 4,600 | 3,000 | 1,800 |
| B Sm\(^2\)... | 1,000 | 1,800 | 970 | 80 |

* Strain C was the receptor strain.

These results are in accord with those obtained in studies of other bacteria such as, for example, *Haemophilus* (11).

The level of resistance of the transformed bacteria depends upon the mutation transferred as well as upon the receptor strain. For example, in the course of transforming strain C by DNA from C Sm\(^2\) (a strain rendered resistant by transformation), the entire resistance of the latter strain was transmitted in every case. On the other hand, the transfer of the marker B Sm\(^2\) was of the disseminative type; one can distinguish two levels of resistance among the transformed bacteria, and only 10% of the transformants resisted the higher concentration (Table 3). As is sometimes the case, the marker B Sm\(^2\) is composed of two closely linked mutations having an additive effect.

**Table 4. Metabolic requirements for expression of resistance**

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Growth</th>
<th>Mutation to streptomycin resistance</th>
<th>Intraspecific transformation</th>
<th>Interspecific transformation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lysine</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mixture of amino acids†</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* H/M: H = donor, M = receptor; M/H: M = donor, H = receptor.
† A mixture containing serine, threonine, phenylalanine, leucine, and cystine.
is a streptomycin-dependent mutant. It does not grow in the absence of streptomycin, grows very slowly in a medium containing 100 μg/ml of streptomycin, and does not attain a maximal rate of growth until a concentration of 1,000 μg/ml is reached.

The genetic analysis of streptomycin-dependent mutants in E. coli and in Salmonella typhimurium have revealed the existence of a complex locus. Dependence is controlled by two regions of the streptomycin-resistance locus, one of which confers resistance upon the bacterium, the other dependence (5). If the situation were similar in Rhizobium, one would expect that transformation of the sensitive strain by DNA of the dependent mutant would produce several types of transformants, resistant and dependent. (To simplify the presentation, we will refer to the streptomycin-independent bacteria as resistant.)

Since the homologous strain A was not transformable, it was strain C which was treated by the DNA of A Sm^d. Transformation was brought about in the absence of streptomycin. To reveal the different types of transformed colonies, the bacteria were plated, 48 hr after the addition of DNA, in media containing a low, and in media containing a high, concentration of streptomycin (in general, 100 and 1,000 μg/ml, respectively). Testing of these colonies, by replicas in media containing or lacking streptomycin, permitted identification of the following types (Fig. 1): (a) small colonies which contain only sensitive, untransformed bacteria (these colonies are not taken into further consideration); (b) normal-sized colonies containing bacteria that are streptomycin-independent and have a high level of resistance; (c) normal-sized colonies containing bacteria that are streptomycin-independent and have a low level of resistance; (d) colonies that are generally small, consisting of streptomycin-dependent bacteria having a low level of resistance and optimal growth at a concentration of 300 μg/ml of streptomycin; and (e) colonies containing bacteria which, in isolation, give rise to colonies, in agar media lacking streptomycin, only at the end of 48 hr (these colonies are said to be of “unstable dependence”).

The colonies of type e merit a more detailed study. Their growth is slow in low concentrations of streptomycin, and they do not resist 1,000 μg/ml. Isolated, transferred in liquid medium, and plated in medium lacking streptomycin, no colony appears by 24 to 48 hr, indicating their dependence. After 48 hr, colonies containing bacteria of type c (independent) appear on the plates lacking streptomycin. If, after the appearance of these colonies, one detaches the layer of agar, places it on a layer that does contain streptomycin (in an amount sufficiently high to produce a final concentration of 100 μg/ml of streptomycin throughout both layers), and re-incubates it for 48 hr, new colonies arise, in numbers corresponding to 5 to 15% of the originally plated population. These new colonies behave exactly like the original colonies of type e, proving that a fraction of the bacteria remained streptomycin-dependent.

DNA of strain C Sm^d, derived from a colony of “unstable dependence,” can also transform the sensitive strain C. The majority of colonies obtained are of type c, but one finds again several colonies of “unstable dependence,” that is, of type e. This finding proves that dependence can be transmitted by DNA from an “unstably dependent” strain.

DNA of the streptomycin-dependent strain

\[
\text{C + DNA of A Sm}^d \rightarrow \text{transformation} \\
\downarrow \quad \quad \downarrow \quad \quad \downarrow \\
\text{unstable dependence} \quad \text{stable dependence} \quad \text{transfer} \\
\downarrow \quad \quad \downarrow \\
\text{dependence} \quad \text{dependence} \\
\downarrow \quad \quad \downarrow \\
\text{unstable} \quad \text{unstable} \\
\text{dependence} \quad \text{dependence}
\]

**FIG. 1.** Transformation by DNA from the streptomycin-dependent strain.
can, therefore, transfer either resistance to a low concentration of streptomycin, or the entire resistance of the donor strain, or, more rarely, dependence itself. This suggests that the mutation responsible for dependence is found in the same locus as the complex streptomycin-resistance mutation. The colonies of type e exhibit the possibility of continuous segregation by loss of the "dependence" factor. This is the first description of such a segregation after transformation.

The experiments on transformation of streptomycin dependence are still in progress, and it is difficult to interpret them at the present time. The phenomenon of "unstable dependence" recalls observations of abortive transduction. It would be tempting to think that the transforming molecule, which bears the sites of resistance and dependence, cannot be entirely integrated into the recipient genome, for some unknown reason. Bacteria of type e would possess the integrated resistance mutation, accompanied by the dependence mutation in a form which is easily lost.

Transformation of Symbiotic Properties of Rhizobium

As was mentioned in the Introduction, the transformation of Rhizobium opens the way to genetic analysis of the role played by bacteria in the symbiotic relationships they establish with leguminous plants. The existence of symbiosis between the legumes and bacteria of the Rhizobium species has been known for a long time by microbiologists and physiologists. The nodules formed on the roots of the plants fully justify the term of symbiosis, since they manifest properties that neither of the participants possesses, among which is the capacity to fix atmospheric nitrogen. The mechanism of this fixation and the respective roles played by the plant and the bacteria are still unknown. The only certain information that we possess at the present time is that the genetic factors permitting symbiosis are distributed between the plant host and the micro-organism (see below).

The genetic determinants of the plant that affect symbiosis can be analyzed by the classic methods of genetics (9). However, the direct study of these determinants in bacteria was impossible before the discovery of transformation in Rhizobium. Only an indirect approach was achieved by examining the properties of the different natural strains and the mutants obtained from them. These studies showed that symbiosis involves three bacterial characters: infectivity, host specificity (both recognized by the ability to form nodules only on certain plants), and effectiveness (measured by the amount of nitrogen fixation in the nodules). Naturally, none of these characters can be studied outside the plant host; the loss either of specificity for every known host or of infectivity would prevent the study of other characters. Consequently, effectiveness can be easily separated from infectivity and specificity, but only genetic analysis of interspecific crosses can demonstrate whether there is independence of the latter two properties.

None of the symbiotic characters of the strains of Rhizobium seems to be linked physiologically to properties observable in vitro, such as colonial morphology, serotype, phagotype, or biochemical properties. Correlations observed up to the present time by comparing strains are either fortuitous or are due to the effect of selective advantages, without there being a precise physiological connection between the properties in question.

In the following experiments, the selective agent is the plant host. Selection bears upon the symbiotic characters, and eventually on the other factors which favor bacterial penetration and multiplication. The study of transformed bacteria permits a genetic analysis of these factors. In particular, the following questions can be posed. (i) Is infectivity independent of specificity? (ii) Does a change in specificity brought about by transformation affect other properties of the bacterium? (iii) Are bacteria that are transformed, or selected in vitro for other properties, changed in their specificity?

Methods

Before describing the results, we will examine the principal methods used for the study of the plant-bacterium system.

Because it is easily cultured, alfalfa was the only plant utilized as host. One can germinate sterile alfalfa seeds in petri dishes, and after 2 days one may choose well-developed seedlings of a specific length (5 mm). These seedlings were planted in tubes containing 8 ml of a Thornton nutritive agar medium containing (per liter): KCl, 0.74 g; KH₂PO₄, 0.30 g; K₂HPO₄, 0.30 g; MgSO₄·7H₂O, 0.50 g; CaSO₄, 0.50 g; FeCl₃, 0.04 g; and agar, 10 g. Infection by the bacterial cul-
ture was done at the time of formation of the first leaves.

Under these conditions, strains of *R. meliloti* formed normal nodules in 1 to 2 weeks (nodules that are effective in 80 to 100% of the cases). The nodulated plants survived and grew for several weeks without the addition of a mineral source of nitrogen. On the other hand, plants inoculated with bacteria of other species never formed nodules; they yellowed and died in nitrogen starvation at the end of a few weeks. Inoculation by killed bacteria or by their extracts never brought about nodulation. The described test, therefore, permits one to detect, without ambiguity, the presence of infectious bacteria possessing the specificity of *R. meliloti*.

Nitrogen fixation was measured by the total nitrogen in the plants or, simply, by the total weight. Qualitative checks could replace these measurements. After infection by an ineffective strain, nodules were formed, but the plants did not develop normally and often showed signs of nitrogen starvation (yellowing, etc.). Observations made earlier on a large number of natural populations revealed a correlation between effectiveness and early formation of nodules. As a general rule, nodules that form only a long time after infection never fix nitrogen. In effective nodules, moreover, *Rhizobium* takes on a characteristic bacteroidal form. Microscopy of sections can, therefore, also furnish an index of the behavior of the strains.

To examine the properties of the bacteria, they must be re-isolated from the nodules formed. For that purpose, nodules previously washed with alcohol were crushed with alumina. Since bacteria of other species, or non-infectious bacteria, are able to penetrate the nodules by secondary infection, the individual clones isolated from the nodules must be tested for their symbiotic properties.

**Transformation Affecting Host Specificity**

In the first experiments, Krasilnikov's method of serial transfer (8) was utilized. The donor strain was cultivated for 8 days in a complete medium. This medium, filtered on a Seitz filter and diluted five times in fresh medium, was then inoculated with the receptor bacteria. After 24 hr of incubation with shaking, the cultures thus obtained served for the inoculation of a new filtrate from the donor strain, and so on continu-

<table>
<thead>
<tr>
<th>Receptor strain</th>
<th>No. of passages</th>
<th>No. of infected plants</th>
<th>No. of nodulated plants</th>
<th>Per cent nodulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Smr.</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 5 contains a few results from these experiments, which, among other things, allowed strains H and J to be chosen as receptor strains.

In the following experiments, strains J, L, and H were transformed by DNA of strain M. The transformed cultures were used to inoculate the plants in which the appearance of nodules was then followed. In these experiments, transformation is demonstrated only by the appearance of nodules. The plant exercises an absolute selection, but the system does not permit one to determine the number of bacteria initially transformed. If a few clones of transformed bacteria are placed in contact with the plant, secondary characters may give one of them an important selective advantage, which calls for prudence in analyzing the connections between the different characters. The results (Table 6) show that it is possible to introduce the host specificity of strain M, by means of its DNA, into strains J, H, and L. When strain J is transformed, around 20% of the inoculated plants are nodulated. These nodules, which are of normal appearance, are formed at the same

<table>
<thead>
<tr>
<th>Receptor strain</th>
<th>No. of infected plants</th>
<th>No. of nodulated plants</th>
<th>Per cent nodulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>J</td>
<td>40</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>16</td>
<td>28.5</td>
</tr>
<tr>
<td>L</td>
<td>20</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td>H Cys*</td>
<td>60</td>
<td>10</td>
<td>16.6</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>14</td>
<td>11.7</td>
</tr>
</tbody>
</table>

* Donor strain was M or M Sm*.
time as on plants inoculated by M. They are generally effective, containing bacteroidal forms and fixing nitrogen. Among the bacteria isolated from these nodules, one finds the following types: (i) type J (in terms of colonial morphology, antigenic and fermentative properties), which does not form nodules on alfalfa and is derived, therefore, from a secondary infection of the nodules; (ii) type M, which forms nodules on alfalfa in repeat experiments; (iii) type $J_M$, which contains colonies of type J but is capable of forming nodules on alfalfa in repeat experiments (only one clone of this type has been observed).

The latter two types have acquired a new hereditary specificity by transformation. Type iii proves that there is no absolute linkage between such characters of strain M as antigenic structure and morphology, and specificity for alfalfa. The fact that the majority of the bacteria transformed in regard to specificity are of type M is explained either by genetic linkage of several characters of the donor strains or by selective advantage of this type in the course of infection and multiplication in the nodules.

A strain of *R. lupini* (H) can also be transformed for specificity. The nodules obtained in this case are generally small, late in appearance, and ineffective, and they do not contain any bacteroids. Bacteria isolated from these nodules produce, in repeated experiments, nodules of the same type. On the other hand, if one transforms them a second time by DNA of strain M, the transformed cultures form several nodules, which contain bacteroidal forms, arise early, and are effective on alfalfa. From these nodules one may isolate bacteria which behave in symbiosis like strain M. This experiment shows, therefore, that early formation of nodules and effectiveness are also under the genetic control of the bacterium. Antigenic analysis of the transformed clones has revealed their diversity; serological types of donor and receptor strains, as well as intermediate types, are found.

If the transforming DNA comes from a streptomycin-resistant mutant of strain M, the transformed bacteria isolated from the nodules remain sensitive to streptomycin. On the other hand, bacteria selected in the same transformation experiment for their resistance to streptomycin do not form nodules on alfalfa. These characters are independent, therefore, and are not transmitted simultaneously.

Finally, it should be added that the cysteine-dependent mutant of strain H can also be transformed for host specificity by the DNA of strain M. The transformed clones isolated from nodules are either $\text{cys}^-$ or $\text{cys}^+$, the latter probably being spontaneous revertors.

All the experiments show, therefore, that specificity for alfalfa is a genetic character which is transferable from one bacterial strain to another by DNA. Unfortunately, the existence of clones with double specificity (i.e., for both alfalfa and the lupine) has not been looked for in these experiments, because of the technical difficulties involved. It remains to be determined, therefore, whether the different specificities are mutually exclusive, genetically linked, or independent.

**Increase in Effectiveness Brought About by Transformation**

The effectiveness of *Rhizobium* strains in symbiosis is a distinct character. The results of interspecific transformations show that it is not linked, at least in certain cases, to a given host specificity. Its study is difficult because of the lack of a convenient means of selection. In the course of these experiments, a preliminary study was undertaken with a specifically practical aim. This was to find out whether one could introduce into strain M, by interspecific transformation, factors of heterospecific origin which would increase the rate of appearance of nodules and their capacity to fix nitrogen.

An experiment done with strain M, using a mixture of extracts from several foreign species, showed a decrease in the time of appearance of nodules and an increase in the quantity of nitrogen fixed. The change produced by transformation was small but significant. If transformation was followed, before the plants were inoculated, by selection of biotin-independent strains of the donor type, the effectiveness of the transformed bacteria was not decreased.

<table>
<thead>
<tr>
<th>Donor strain</th>
<th>Net wt of nodulated plants % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>J + L</td>
<td>127</td>
</tr>
<tr>
<td>H</td>
<td>137</td>
</tr>
<tr>
<td>H</td>
<td>36.5</td>
</tr>
</tbody>
</table>

* Receptor strain was M. After transformation, clones consisting of biotin-independent transformants were used to infect plants.
transformants, the results were more striking. Among three clones isolated from the nodules, two proved to be more effective than the initial strain; the third produced a slow and ineffective nodulation (Table 7).

Naturally, it is not possible to demonstrate loss of specificity or of effectiveness as a result of transformation, since there is no way of selecting for such transformed bacteria.

Transformation In Vivo

In experiments done with the mouse, it has been shown that bacterial transformation can occur in vivo. Since this discovery, the role of this phenomenon in evolution and in epidemiology has often been considered. It is, therefore, interesting to note the transformation of *Rhizobium* in vivo, in the interior of nodules. Application of the penicillin technique (described above) permits one to do the following experiment. One infects plants with the sensitive strain M. If penicillin is added at the beginning of formation of nodules, it lyases the sensitive bacteria. The development of the nodules is stopped and does not begin again until after formation of a new population from the few spontaneous, penicillin-resistant mutants which survive. If at the same time that the penicillin is added, one introduces penicillin-resistant bacteria belonging to another species, they are able to penetrate the nodule and are there transformed by the lysate of M bacteria. Such transformations have been observed in the nodules of alfalfa after addition of penicillin and a mixture of penicillin-resistant strains of *R. japonicum* and other species. Bacteria isolated after this penicillin treatment possess not only resistance to this antibiotic but also other properties of the secondarily infecting bacteria, which may be considered as the receptors. Thus, for example, forms are found which have the colonial morphology of *R. japonicum* and, like the latter, do not reduce saccharose. These forms possess an antigenic structure which is intermediate between J and M.

Conclusions

The results described above show unambiguously the occurrence of genetic transformation in *Rhizobium* mediated by DNA. The mechanism of transformation, to the extent it has been elucidated, is apparently quite similar to that generally described in other bacterial species. A first step in the genetic analysis of *Rhizobium* has been made by the transformation of antibiotic-resistant and cysteine-dependent mutants. These results are incomplete, but they indicate that *Rhizobium* constitutes a promising material for the study of bacterial genetics. A detailed study of the development of competence should certainly bring about an improvement in the yield and the synchronization of the system. A more precise understanding of the mechanism of transformation, particularly of genetic integration, should help to determine the features of transformation that are general and those that are peculiar to each species. Genetic and biochemical analyses of the mutants will undoubtedly supplement in a useful way the results obtained with other species. Lastly, further results on the nature of unstable streptomycin dependence should yield information about genetic integration.

Despite the importance of the problems just mentioned, the interest in genetic transformation in *Rhizobium* would still seem to reside primarily in the possibility it offers for the study of plant-microbial symbiosis and especially that dealing with nitrogen fixation. Indeed, only a genetic study will permit us to distinguish the several factors intervening in these phenomena and to determine whether an observed correlation between the different characters of a strain is fortuitous or reflects an underlying physiological relationship.

The results obtained thus far, however, do permit us to discuss several problems. The first concerns the nature of host specificity. The species of *Rhizobium* are defined by the groups of leguminous plants on which they can form nodules. (We will not embark here on a discussion of the general notion of species in bacteria, a notion which has been amply discussed since the discovery of numerous examples of genetic exchanges between species and between genera.) Variations of host specificity have been described in this group, but there has been no definite observation of intermediate types or of types possessing two specificities at once. In genetic terms, two extreme hypotheses could account for these facts. (i) The *Rhizobium* species are very distant one from another, differing by a large number of factors. (ii) Specificity is determined by only one gene, the alleles of which, being mutually exclusive, correspond to the groups of host plants on which nodules can be produced. The possi-
bility of changing specificity by transformation favors the second hypothesis. Indeed, although a quantitative statement is difficult to make, specificity for alfalfa is certainly transferred into other species like a single gene or like a group of closely linked genes. Research on types having double specificity and a more thorough quantitative study of transformation (if possible with the help of linked genetic markers) will probably allow a definitive choice between these hypotheses. If, as we believe, specificity depends on a single gene (or a group of linked genes), one or a small number of proteins may be involved in its expression, perhaps in forming a specific structure in the bacterial cell wall. The existence of secondary factors affecting specificity is not excluded. Only the simultaneous analysis of the genetic factors of plant and bacterium will allow this problem to be resolved.

The second question raised by results of interspecific transformation is that dealing with the relation between host specificity and infectivity. Three possibilities present themselves. (i) Infectivity is independent of specificity; mutants which lose one of these properties retain the other (although this cannot be revealed by infection of the plant). (ii) Only specificity is necessary to initiate infection, noninfectious mutants losing all specificity. (iii) Special infectivity corresponds to each group of hosts, the simultaneous presence of this infectivity and the corresponding specificity being necessary for the formation of nodules. Results of interspecific transformation, as well as other indirect arguments, exclude the third possibility and are in favor of the first. Two experiments should be able to provide a direct proof: (i) the transfer of infectivity into a noninfectious mutant of *R. meliloti* by DNA from an infectious strain belonging to another species; and (ii) the introduction of specificity for alfalfa into a foreign species by the DNA of a noninfectious mutant of *R. meliloti*. If the first hypothesis is correct, transformation will give positive results in the two cases.

Effectiveness is the third question to be discussed. As we have seen, a complex relationship between plant and bacteria determines the capacity of the nodules to fix nitrogen. Undoubtedly, fixation requires the formation of a complex enzymatic system, a visible sign of which is the production of leghemoglobin in the nodules.

Neither the plant nor the bacterium possesses the total genetic information necessary for this system, but the distribution of this information between the two participants is unknown. Mutations may occur in either the host or the bacterium, rendering it incapable of fixing nitrogen in symbiosis with the normal partner. A question to which we can give an answer, albeit partial, is that dealing with the relation between specificity and effectiveness: do the bacterial factors differ according to the host? We have seen that, if a strain of *R. japonicum* acquires the specificity of *R. meliloti*, it forms effective nodules. Therefore, the bacterial factors that affect nitrogen fixation are the same in the case of these two hosts. On the other hand, the same experiment done with strains of *R. lupini* (the original effectiveness of which is unfortunately not known) yields only ineffective transformants, into which effectiveness can be introduced by means of a second transformation. This finding suggests the existence of a factor which intervenes in fixation only when the host is alfalfa. But the majority of the factors seem to be the same in the different species of *Rhizobium* and to function whatever the host happens to be. Crossing different ineffective mutants may provide detailed information on the nature and the number of these factors.

To conclude, it seems that, in making possible genetic analysis, transformation can solve by itself certain problems of symbiosis and nitrogen fixation. In addition, it constitutes an important tool supplementing other methods, especially biochemical ones, for attacking other aspects of these phenomena.

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**Bibliography of R. Balassa on Transformation in Rhizobium**


LITERATURE CITED


