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Complete and Reduced Life Cycles in *Rhizobium*

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SUMMARY: Strains of *Rhizobium* from a wide variety of host plants were found to possess either a complete or a reduced life cycle. In the former, which was characteristic of strains from certain wild legumes and garden flowers, the bacteroids within the nodule were branched and septate; the free-living stages included small Bacterium-like forms which were responsible for infection of the host plant, and large, occasionally Gram-positive forms resembling *Bacillus*, which produced both specialized, coccoid swimmers and resistant endospores. The reduced cycle was commonly found in *Rhizobium* from cultivated field crops. The bacteroids were single cells of irregular outline; the free-living stages were reduced to the Bacterium-like stage alone, sometimes so actively motile as to perform the function of swimmers. This condition is regarded as degenerate and due to a parasitic habit. The mode of formation of the endospores, which differs in detail from that of *Bacillus*, is described. The morphology of various stages in the life cycle and a slight tendency to Gram-positivity render it apparent that *Rhizobium* is a specialized genus of Bacillaceae, probably related to the plant parasitic *B. polymyxa* group, with which it also has biochemical affinities.

Although *Rhizobium* has been much studied, attention has been almost exclusively diverted towards the strains or species which exist in symbiosis with important field crops. In a recent review Allen & Allen (1951) pointed out that many strains from less familiar legumes do not accord so closely with the classical descriptions, and Bisset & Hale (1951) reported that true swimmers are more readily produced by *Rhizobium* from lupins, sweet-peas, etc., than from cultivated peas and clovers, in which the function of swimmers is performed by small, motile bacilli, produced by normal cell division. In the present work further differences between the life cycle of these different types of *Rhizobium* are indicated.

The problem of the existence of endospores in *Rhizobium* was at one time the subject of considerable controversy, but it has been almost entirely forgotten of recent years (Lewis, 1938). The present attitude is well represented by Nutman (1952), who expressed surprise at the powers of survival of lucerne *Rhizobium*, 'since *Rhizobium* does not form spores'. It is my experience that precisely this strain of *Rhizobium* forms spores very readily, which serves to explain its resistance. Here, and in a previous paper (Bisset, 1952*a*), evidence is presented to show that some, but not all, strains produce resistant endospores, and have, at certain stages of their life cycle, morphological characters which suggest a relationship with the true sporing bacilli.

METHODS

Thirty-four strains of *Rhizobium* were examined. Twenty-nine were newly isolated from the root nodules of pea, bean, everlasting sweet-pea, wild vetch, lupin, broom, tree-lupin and various species of clover. Five were laboratory cultures, of which all but one were of classical morphology.

Initial cultures were made by sterilizing nodules externally with mercuric chloride, crushing between sterile slides, and inoculating upon nutrient agar containing 0.3% Yeastrel. Subcultures were made upon this medium, or, for the production of 'bacteroids' such as appear in the root nodules, in a simple infusion of sweet-pea foliage. Strains which produced variants of unusual appearance were repeatedly plated for purity.

Preliminary observations

Because the evidence and conclusions presented in this paper are contrary to long-established belief upon the nature and morphology of *Rhizobium*, I think it best to describe the observations from which they arose, in the order and manner in which they occurred.

A large number of strains of *Rhizobium* was isolated in this laboratory in the course of an investigation upon the production of swarmers (Bisset & Hale, 1951). While this work was in progress it was observed that the morphology and cultural characters of many isolates were not in accordance with previous descriptions of the genus. Many were Gram-positive, especially in very young cultures. The degree of Gram-positivity varied, and most frequently consisted of little more than the appearance of a few granules, which disappeared as the culture aged. Some, however, were so strongly Gram-positive, and so closely resembled ordinary sporing bacilli that they were repeatedly discarded as contaminants until the failure to isolate any other bacteria from the same sources, together with the discovery of similar, *Bacillus*-like variants in stock cultures of unimpeachable authenticity and purity, led to their further investigation. It was then discovered that by every criterion of life cycle and plant symbiosis they were indeed *Rhizobium*. It was also apparent that, despite their resemblance to *Bacillus*, they had many characters quite unlike those of any known member of the genus; and that although they produced endospores capable of surviving boiling, these were formed in a manner which differed in detail from the cytological process found normally in *Bacillus* and *Clostridium*.

By the selection of heat-resistant strains, similar, partially Gram-positive, sporogenous strains were also obtained from isolates which were quite typical in appearance. These almost invariably lost their Gram-positivity and *Bacillus*-like morphology as the cultures aged, but in addition, reverse mutations to a typical form occurred in strongly Gram-positive sporogenous strains, giving rise once more to a strain which was Gram-negative and of characteristic *Rhizobium* morphology in all stages of culture.

The biochemical characters of the various strains were exceedingly variable, and frequently quite unlike the accepted reactions for *Rhizobium*, which is supposed to be incapable of producing detectable fermentation of carbohydrates in peptone water cultures. The reactions were equally unlike those of any species of *Bacillus* which might be expected to appear as accidental contaminants, in that both acid and gas were produced. Cultural characters are described in a later section; it suffices to record here that whereas certain strains, both *Bacillus*-like and typical, did in fact fail to ferment any of the

carbohydrates tested, others produced acid and gas, very slowly, in a varied range of substrates. The biochemical reactions of typical and sporogenous variants from any one strain were always identical, whereas those from different sources were very seldom alike. In sweet-pea infusion and similar dilute media of acid reaction, branched bacteroids, typical of *Rhizobium*, were produced by strains of all types. Species of *Bacillus* did not produce these appearances. Thus all available evidence pointed to the identity of the *Bacillus*-like and typical *Rhizobium* strains.

In some strains, including the majority of those obtained from cultivated crops such as clover and peas, the small typical forms alone were found. Spores and true swarmers were both very rare. This is a highly significant finding, as it is exactly these types of *Rhizobium* which have been most fully studied in the past, and upon which the accepted descriptions are based. Strains of atypical morphology and behaviour were commonest in the nodules of wild legumes or garden flowers, which in the past appear to have been investigated very rarely. It is also highly probable, from my experiences in this work and in examination of practical exercises performed by students, that sporogenous strains have many times been isolated and discarded in the erroneous belief that they were contaminants. There is more than one hint of this in the literature (cf. the review by Allen & Allen, 1951), and occasional reports of Gram-positive variants verified to be *Rhizobium* (Kleczkowska, 1950).

Sporogenous strains of *Bacillus*-like morphology, and with a greater or less tendency to Gram-positivity, were isolated from lupin, everlasting sweet-pea, tree-lupin and broom, and from a stock culture derived from lucerne. The latter will be discussed separately. Strains which were morphologically typical, small and Gram-negative, but which produced visible spores within the cell, as observed by routine spore stains, and which survived boiling, were regularly isolated from vetch, and occasionally from clover.

Morphology

The cytology of endospore formation in *Rhizobium* is described in a later section. The large, 'barred' cells, from which swarmers were liberated, have frequently been described, and an account has been given of them elsewhere (Bisset & Hale, 1951). These were never Gram-positive, but although considerably larger in size they nevertheless strongly resembled *Bacillus* and the vegetative cells of the *Bacillus*-like variants of *Rhizobium* in the details of their cytology. The transverse bars, so often described in the past, are in fact cross-walls closely resembling those of multicellular species of *Bacillus* (Bisset, 1950, 1952*b*), but combined with a thick layer of basophilic material which thus lines the cells of which the bacillus is composed and is presumably concerned with the formation of the swarmers (Bisset & Hale, 1951). These cross-walls were absent from *Rhizobium* of typical morphology, the cytological structure of which resembled that of *Bacterium* (Bisset, 1950, 1952*b*), but were exceptionally well-marked in the sporogenous strains, whether strongly or weakly Gram-positive, or completely Gram-negative (Pl. 1, fig. 1). Paired nucleoids were seen in each of the four, six or more cells into which the bacillus

was thus divided, exactly as in rough forms of *Bacillus* (Pl. 1, figs. 2, 3). Vegetative cells had a small number of peritrichous flagella; swimmers, one or two polar flagella (Bisset & Hale, 1951).

The morphology of the bacteroids in plant nodules or in sweet-pea infusion was distinctly different in strains which freely produced true swimmers and endospores from septate bacilli, from that of those strains in which typical forms alone were found. In the former the bacteroids also were large and septate and branched freely (Fig. 1A); in the latter they were unicellular or

Fig. 1. Drawings of tannic-acid-violet preparations of bacteroids from root-nodules of various plants. A: from lupin; branched, multicellular type. B: from vetch; intermediate type. C: from cultivated bean; intermediate type. D: From cultivated pea; irregular, mainly unicellular, degenerate type.

composed of one large, irregularly shaped cell, with a very small branch (Fig. 1D). Strains of intermediate characters, such as those from vetch, which produced endospores but not swimmers from typical, small, Gram-negative bacilli, were intermediate in this respect also and formed bacteroids with three or four cells and moderately frequent branches (Fig. 1B, C). Because of the amount of slime and plant debris in preparations of bacteroids good photomicrographs were difficult to make, and they have been illustrated only by drawings.

Even the most *Bacillus*-like strains were immediately transformed into bacteroid morphology when subcultured in sweet-pea infusion. Their appearance in the nodule and in the culture was always the same for any one strain of *Rhizobium* and characteristic of the types described above. Cultures of a variety of species of *Bacillus* were grown in this medium but either retained their original morphology or failed to grow. These included *B. subtilis*, *B. megaterium*, and several strains each of *B. polymyxa*, *B. cereus* and *B. mycoides*. This provides additional proof that the sporogenous strains of *Rhizobium* are not contaminants.

Plant inoculation

In order still further to confirm the identity of the sporogenous strains plant inoculation experiments were performed. Cultures of an isolate from lupin were heated for 10 min. at 100°. Subcultures were made, from which

suspensions were inoculated on lupin seeds which had previously been sterilized externally with 1/1000 mercuric chloride, and washed in sterile water. Twelve seeds were sown in autoclaved soil and watered with autoclaved water. Twelve control seeds were similarly treated but were not inoculated. When the seedlings were about 3 in. high the roots were washed and examined. The test seedlings had numerous well-formed nodules on the roots; the control seedlings had none. From the nodules of the test seedlings a strain of *Rhizobium* was isolated which again survived 10 min. at 100°, and had the same biochemical characters (see below) as the strain with which they had been inoculated. The recovered strain, at first, although sporogenous was Gram-negative and typical in appearance, like those recorded above from vetch and clover. It also resembled vetch strains in its tendency to produce transient orange pigmentation of the colony after heat-testing, a phenomenon discussed in the section on culture. This experiment proves that *Rhizobium* may pass through a heat-resistant stage while retaining the power of entering into symbiosis with the host plant.

Parallel experiments were made with lupin seeds sown upon nutrient salt agar slopes, in which the early stages of seedling growth and infection were observed. The original inoculum consisted entirely of Gram-positive, Bacillus-like forms, but after contact with the roots typical forms became more common, and these alone were found in crushed preparations of the roots.

A similar effect was observed in an experiment made with a strain of *Rhizobium* derived from a stock culture from lucerne. The original heat-resistance test produced a Bacillus-like strain in which, at first, no Gram-negative forms could be seen. After several weeks it again dissociated, giving rise to typical Gram-negative strains which did not survive boiling and which had completely changed their antigenic structure (antisera against the original strain were available for agglutination tests). The sporogenous strain from which they were derived was also antigenically different from the original and was not infective for lucerne in that condition. The newly dissociated typical variants, however, were fully infective for lucerne, thus confirming that normally infective *Rhizobium* may pass through a heat-resistant phase.

In a further experiment inoculation of lucerne seedlings, grown on agar slopes, with suspensions which had been boiled for 5 min. immediately before inoculation, produced poor, atypical nodules on the side-roots, from which a very slow-growing strain of *Rhizobium* was recovered. All these experiments appear to indicate that infection of the host plant is best achieved by bacteria of the typical Gram-negative, Bacterium-like morphology, and that reversion to this form may occur with varying readiness, either spontaneously or when sporogenous strains are brought into contact with susceptible plants. Infection by means of swarmers, which has been the subject of much comment by previous writers, was not specifically studied. It is, however, by no means the only mode of infection in *Rhizobium*.

Heat resistance

The character of heat resistance was very variable in the *Rhizobium* strains examined. As has been explained, some variants formed spores freely, some rarely, some never. The resistance of the spores themselves was also very variable, as was the after-effect of boiling on those strains which survived it. Some grew very slowly on first subculture after boiling, taking as long as 8 weeks or more to produce recognizable colonies. The colonies of isolates from vetch and lucerne, which normally were grey in colour, were bright orange on first subculture after boiling, but reverted to normal colour after two or three further subcultures. This also occurred, as described above, in the case of the lupin strain, re-isolated from experimental plant nodules. No explanation of this phenomenon can be offered.

The most resistant strains were those from lupin, vetch and everlasting sweet-pea, which survived boiling for 10 min. The standard test which eventually was used as a criterion of heat resistance consisted in heating a broth culture in a test-tube for 30 min. in a water-bath at 80°. At the end of this period the temperature was raised to 100° and maintained there for 5 min.

The variant from the stock culture of lucerne *Rhizobium* with which some of the plant inoculation experiments were performed was a source of much confusion because of its unpredictable heat resistance. It was the only stock culture from which resistant variants were isolated, and had been maintained in the laboratory for more than 20 years in artificial culture. The subcultures from the first heat test were discarded after showing no signs of growth in more than 2 weeks. A second test was made and in this instance the subcultures were left by chance for approximately 5 weeks before examination. On this occasion they were found to have grown. The sporogenous variant which arose survived boiling and typical variants from it were infective for lucerne, but the original strain invariably took an excessively long time to recover from boiling, which more often than not it failed to survive. Subcultures which did survive were among those which at first produced orange-pigmented growth, but which rapidly lost this character on further subculture.

Heat-resistance tests performed on this stock culture, at the institute from which it was obtained, consistently failed to produce resistant variants, although these were obtained in this laboratory on several occasions. The eccentric behaviour of this stock culture may be explicable by the long period since its first isolation, whereas the other sporogenous strains were all freshly isolated.

Cultural characters

In culture *Rhizobium* characteristically produces a voluminous pale-grey mucoid growth. Some of our cultures showed this appearance at all stages; in others the growth at first isolation was flat and granular, but changed after 2 or 3 days to the typical mucoid appearance. Granular colonies sometimes became surrounded with mucoid colonies, apparently derived from swarmers. Orange-pigmented colonies were occasionally formed by heat-

resistant strains on first subcultures after boiling. Others grew very slowly after boiling.

Although the standard descriptions suggest that *Rhizobium* is incapable of producing recognizable fermentation of carbohydrates in peptone water, it is my experience that strains vary very much in this respect, although invariably slow in their reactions. Some, in fact, fermented nothing, but many produced acid and gas, under aerobic conditions, so that the fluid in the Durham tube remained unaltered long after the remainder had changed. Usually only a small range of substrates was affected. Acid reactions appeared in 2 or 3 days at 20°, but gas began to collect only after a week or more. As there is little or no correlation between the results given by different isolates and their morphological and other characters, and as these biochemical characters were extraordinarily variable, little purpose would be served by tabulating a large number. One important point, however, is that despite this variability, morphologically typical and atypical strains from the same isolate were always alike, e.g. the original and re-isolated strains from the lupin inoculation experiment were alike in fermenting only glucose; the sporogenous non-infective, the asporogenous infective, and the original, antigenically distinct, stock cultures from lucerne all failed to ferment any carbohydrate. Isolates from broom and lupin fermented only glucose; those from sweet-pea fermented glucose, sucrose and maltose; one strain from clover fermented glucose, lactose, mannitol, sucrose, rhamnose, aesculin, xylose, arabinose, maltose and salicin, but not dulcitol or inositol.

I am unable to account for the discrepancy between these results and the standard descriptions of the genus. These tests were made with strains which had been repeatedly plated for purity. Whereas the power to ferment carbohydrates was by no means confined to the morphologically atypical strains, but was especially remarkable in the typical strains from clover, nevertheless the five stock cultures were, without exception, completely or almost completely devoid of fermentative ability. It may thus be that this organism rapidly loses this power in artificial culture, and it is this which has been responsible for the existing descriptions.

The production of gas in fermentation, or total failure to ferment carbohydrates, are both very unusual characters in sporing bacilli. This decreases still further the possibility that the sporogenous strains were accidental contaminants. Gas production, however, is found in *B. polymyxa*, which also has a plant dwelling habit and is especially liable to the production of Gram-negative asporogenous variants. This possible relationship is referred to in the Discussion, but it is perhaps well to emphasize that the sporogenous *Rhizobium* strains are not identical with *B. polymyxa*, which ferments a standard series of substrates, does not form swarmers or bacteroids, and produces its spores in a cytologically distinct fashion.

Endospore formation

In those strains which exhibited the complete life cycle, spore-like resting cells and tiny coccal swarmers were frequently found to be formed in the same

culture. The mother cells were always the large multicellular *Bacillus*-like stages (Pl. 1, figs. 1-3). In typical members of the genus *Bacillus* a single spore is formed, as a rule, in a four-celled bacillus. In the sporogenous strains of *Rhizobium*, however, it is not unusual for each cell to produce a spore. These remain enclosed within the cell wall of the sporangium until released by its rupture, and thus appear in long chains (Pl. 1, fig. 4-6). Apart from these points, the cytology of spore formation is similar to that seen in other sporogenous genera. A rod-shaped fusion-nucleus is formed (Pl. 1, fig. 7), and maturation is preceded by a reduction process, in which one nuclear element is rejected (Pl. 1, figs. 8, 9). After release from the sporangium, the spores germinate by the rupture and rejection of the spore coat (Pl. 1, fig. 10).

DISCUSSION

Strains of *Rhizobium* capable of surviving boiling by virtue of the production of resistant resting-cells closely resembling endospores, have been isolated from root-nodules in leguminous plants. Some, but not all, of these passed through a phase in which they resembled an ordinary Gram-positive sporing *Bacillus*, but differed from most of these in many respects, notably in their metabolism and in the mode of formation of the spore. They also possessed, even in this atypical phase, the typical *Rhizobium* characters of producing swarmers from barred cells and bacteroids in appropriate culture media which had the distinctive appearance of the bacteroids in the root-nodules from which they were isolated. True *Bacillus* species did none of these things. The identity of the *Bacillus*-like strains with *Rhizobium* is further proved by their ability after having survived boiling to infect the appropriate species of host plant (in which respect *Rhizobium* is highly specific) and to produce nodules in the roots. As a rule infection occurred only through the agency of variants of typical morphology; these were derived from atypical strains, either spontaneously or by the influence of contact with seedling plants. The identity of the typical and atypical variants was again confirmed by the fact that, despite the exceedingly variable biochemical reactions within the genus, those of variants from the same isolate were always alike. The classical description of the genus, as incapable of fermenting carbohydrates, is true of some strains, and especially of stock cultures, but it is not confirmed in the majority, irrespective of their morphological appearance. Some produced acid and gas in a small range, others in a much wider range of substrates, but all were very slow to do so.

Hints and suggestions in the literature (cf. Allen & Allen, 1951) make it apparent that such peculiarities in the morphology and cultural characters of *Rhizobium*, especially when derived from unusual host plants, are by no means unknown to workers in this field, but little beyond these hints has so far been ventured in print, although recently a Gram-positive variant of *Rhizobium* was reported by Kleczkowska (1950).

In effect therefore the strains were found to be of two distinct kinds with a number of intermediate forms. They may be described as having complete and reduced life cycles respectively. The strains with a complete life cycle

(Fig. 2) notably those from lupin, sweet-pea and tree-lupin, produced swimmers very freely from large barred (i.e. multicellular) bacilli from which were also produced resistant resting cells resembling endospores. The bacteroid stages, whether in nodules or in appropriate culture media, were also multicellular and freely branched. The reduced life cycle was found in varying degrees in most of the remaining strains, and was well seen in those from cultivated peas and most species of clover. The bacteroids were roughly spherical, triangular

Fig. 2. Complete type of life cycle in *Rhizobium*. N: in the nodule, small bacteria develop into branched, multicellular bacteroids; S: in the soil, reproduction may be by the formation of endospores or of swimmers from large *Bacillus*-like rods. The swimmers are transformed into small rods which may attack the root-hairs of susceptible plants or grow up into a new generation of large bacilli, either directly or via a *Bacterium*-like generation. (1) *Bacillus*-like mother cells; (2) formation of swimmers; (3) plant-infective cells; (4) bacteroids; (5, 6) *Bacterium*-like motile cells; (7, 8) spore formation.

or irregular in outline, and were either unicellular or consisted of one large cell with a side branch of a few relatively tiny cells. In normal culture the small bacteria alone were seen; often they were so small as to resemble swimmers, and presumably served as such (Bisset & Hale, 1951). The entire picture of the reduced cycle was suggestive of the degeneracy often associated with parasitism. A small proportion of strains was intermediate in morphology, and produced rare rather small endospores but seldom swimmers. The bacteroids were also intermediate in morphology in most cases.

The morphology of the various strains of *Rhizobium* which were examined, and the differences between them, suggest that the genus fundamentally resembles the sporing bacilli in almost all respects, but has suffered a varying degree of loss of these characters, so that Gram-positivity is greatly reduced in all strains, some sporulate very seldom or not at all, and some, by chance the

agriculturally important and best-known strains, have become so altered, presumably by prolonged parasitism, that the original morphology has been almost completely lost. It is also interesting to consider that the very typical *Rhizobium* character of reproduction by swarmers is closely paralleled by the mode of production of tiny, bacillary gonidia in many species of sporing bacillus (Bisset, 1950), although much less is known about these.

Apart from this phenomenon, *Rhizobium* appears to bear the closest resemblance to the *B. polymyxa* group, in habitat, biochemistry, tendency to loss of Gram-positivity, and even in such details as the morphology of the spore (Bisset, 1952*b*, p. 48), and the appearance of cultures.

This study may perhaps be claimed to be one of the first in which the principle of establishing the phylogeny of a genus by reference to the life cycle, so commonly employed in other biological fields, has been applied to bacteria.

REFERENCES

- ALLEN, E. K. & ALLEN, O. N. (1951). Biochemical and symbiotic properties of rhizobia. *Bact. Rev.* **14**, 273.
- BISSET, K. A. (1950). *The Cytology and Life-History of Bacteria*. Edinburgh: Livingstone.
- BISSET, K. A. (1952*a*). Observations on the morphology and natural relationships of *Rhizobium*. *J. gen. Microbiol.* **6**, i.
- BISSET, K. A. (1952*b*). *Bacteria*. Edinburgh: Livingstone.
- BISSET, K. A. & HALE, C. M. F. (1951). The production of swarmers in *Rhizobium*. *J. gen. Microbiol.* **5**, 592.
- KLECZKOWSKA, J. (1950). A study of phage-resistant mutants of *Rhizobium trifolii*. *J. gen. Microbiol.* **4**, 298.
- LEWIS, I. M. (1938). Cell inclusions and life-cycle of rhizobia. *J. Bact.* **35**, 573.
- NUTMAN, P. S. (1952). Root-nodules of leguminous plants. *J. gen. Microbiol.* **6**, i.

EXPLANATION OF PLATE

(All photomicrographs at $\times 3000$.)

- Fig. 1. *Rhizobium* sp. from lupin. Large Bacillus-like rods, showing numerous cross-walls. Tannic acid-violet.
- Figs. 2 and 3. As fig. 1; showing nuclear structures. Acid Giemsa.
- Figs. 4 and 5. As fig. 1: liberation of endospores by rupture of the cell wall. Cross-walls well seen. Tannic acid-violet.
- Fig. 6. As fig. 1: chains of endospores. Gram.
- Figs. 7–9. As fig. 1: stages in spore formation. In fig. 7 the rod-shaped fusion nuclei are seen; in fig. 9 the arrows indicate stages in the reduction process and elimination of the rejected nucleoid. Acid Giemsa.
- Fig. 10. Germination of spores.

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K. A. BISSET · LIFE CYCLES IN *RHIZOBIUM*. PLATE 1