Nodule Bacteria Associated with the Indigenous Leguminosae of South-Western Australia

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SUMMARY

Root-nodule bacteria (rhizobia) isolated in pure culture from legumes indigenous to south-western Australia were examined for cultural and infective characters. All isolated strains had cultural characters consistent with the slow-growing bacteria of the lupin or soybean type. None of the strains nodulated peas, clover or medic, but hosts from the other four recognized cross-inoculation groups were nodulated. *Lupinus digitatus*, *Glycine hispida*, *Vigna sinensis* and *Phaseolus vulgaris* formed a natural grouping on the basis of susceptibility to nodulation by the native strains. Within the narrower host range of the genus *Lupinus* it was found that *L. digitatus*, *L. albus* and *L. pilosus* grouped together on susceptibility to nodulation, whereas *L. luteus*, *L. angustifolius* and *Ornithopus sativus* were not nodulated by any of the sample strains. The use of this geographically isolated sample of rhizobia has further demonstrated the weakness of the present scheme of classification of the nodule bacteria, which is based on the infective character of the bacteria. A taxonomic system based on Adansonian principles could be applied to the classification of the rhizobia, and a procedure for attempting this is outlined.

INTRODUCTION

The genus *Rhizobium* comprises a group of organisms characterized by their ability to induce the formation of nodules on the roots of legumes. Allen & Allen (1950) stated '...the ability to invade the roots of leguminous plants and stimulate the production of nodules...is the sole criterion for the existence of the genus *Rhizobium*'. Bacteria thus grouped on the feature of legume root nodulation are subgrouped on the range and type of host plants infected. *Bergey's Manual* (1957) accords six of these subgroups specific status but appends a note that some of them may be related. While this classification is generally recognized as imperfect, it persists in the absence of a better system. The present paper reports some results from a survey of the rhizobia associated with indigenous legumes of the botanical South-West Province of Western Australia (Lange, 1960). The province itself is about 100,000 square miles in area. Its climate was discussed by Gentilli (1946), its geomorphology by Jutson (1950), soils by Teakle (1938) and the indigenous vegetation by Diels (1906) and Gardner (1942). Indigenous Leguminosae total over 400 species in about 35 genera of the Caesalpiniaceae, Mimosaceae and Papilionaceae. A great many of the species are endemic to the area. Nearly half are species of

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Acacia; the remainder are mostly papilionaceous, principally in genera of the tribe Podalyrieae. The incidence of nodulation in these indigenous legumes has already been tabulated (Lange, 1959).

METHODS

Over a 2-year period, 7000 miles of traverses were made throughout the study area and the vegetation examined at intervals, usually of only a few miles. Leguminous species were collected for identification and examined for root-nodulation. Species representative of the indigenous Leguminosae were selected from those found to be nodulated. Nodules were removed from these plants in the field, and desiccated in tubes over anhydrous CaCl_2 (Dr D. O. Norris, personal communication). In the laboratory the nodules were reconstituted by soaking in sterile water. They were then surface-disinfected by immersion for 15 min. in a mixture of equal volumes of H_2O_2 (100 vol.) and absolute ethanol, washed and macerated. Samples were streaked on plates of yeast extract + mannitol agar and incubated at 27° .

One hundred and sixty-three strains of presumptive rhizobia were isolated in pure culture. Eighty-five of these isolates from 83 host species in 24 legume genera were selected as a fair sample of the Rhizobium population associated with indigenous Leguminosae in the area (see Table 1).

Two series of tests were applied to the sample strains.

Tests of cultural characters

Colony characters. Visible characteristics of colonies on isolation plates and in pure culture were recorded. Strains of known performance from *Glycine*, *Lathyrus*, *Trifolium*, *Medicago*, *Phaseolus* and *Lupinus* were included in all tests to allow direct comparisons to be made.

Morphology and staining. Preparations from 6-day-old cultures were stained by Gram's method and with carbol rose bengal and examined.

Calcium glycerophosphate medium. Strains were streaked on slopes of calcium glycerophosphate agar (Hofer, 1941), incubated for 3 weeks at 27° and observed for browning of the medium.

Litmus milk. Strains were inoculated into sterile litmus milk, incubated for 4 weeks at 27° and observed for pH change and for serum zone formation.

Colony growth rate. Strains were dispersed in sterile water and dilution series made. Drops of appropriate dilutions were spread over yeast extract + mannitol agar plates to yield ten to thirty well-isolated colonies. Each plate was incubated at 27° , inspected daily, and the times for half of the colonies in sector areas to reach diameters of 1 mm. recorded.

Reactions on sugar. Cultures were inoculated on to nitrate agar medium (Baldwin & Fred, 1927) containing either arabinose, rhamnose, xylose, maltose or sucrose, and pH changes observed after incubation for 5 weeks at 27° .

Tests of infectiveness on legumes

Each of the 85 sample strains was further investigated in a series of glasshouse experiments to determine its infectiveness on the following legumes: *Pisum sativum* L., *Lathyrus cicera* L., *Lens esculenta* Moench., *Vicia sativa* L., *Lupinus angustifolius* L., *L. luteus*, L., *L. nanus* Dougl., *L. digitatus* Forsk., *L. albus* L., *L. pilosus* Murr.,

L. villosus Willd., *L. subcarnosus* Hook., *Ornithopus sativus* Brot., *O. compressus* L., *Medicago tribuloides* Desr., *Trifolium subterraneum* L., *Vigna sinensis* Engl., *Phaseolus vulgaris* L., *P. lathyroides* L., and *Glycine hispida* Maxim. These include hosts from the seven major cross-inoculation groups. The eight *Lupinus* species were included since it had been indicated earlier that lupins may be differentially susceptible to native rhizobia in the area (Adams & Riches, 1980; Lange & Parker, 1960). The following conditions were imposed during the course of the experiments.

(1) Strains known to be infective on the hosts were included. These invariably nodulated the host legume under the conditions of experiment. This ensured that experimental conditions did not restrict nodulation of the plant.

(2) Experiments were terminated when the control nodulation was fully established.

(3) Control uninoculated plants were included at a frequency of 20% of inoculated plants.

(4) Conditions for plant growth were kept as close to optimal as possible.

Experiments were conducted in closed glasshouse rooms with adequate sunlight and pressurized by air coolers delivering washed air. The rooms were sprayed down completely with insecticidal, fungicidal and bactericidal sprays, and with paraffin oil emulsion (25% paraffin oil). Dust immobilization was maintained by further spraying with paraffin oil emulsion containing antibacterial substances. Large plants were grown in open sand-filled porous drainage pots spaced on slatted bench tops over an area heavily coated with paraffin oil emulsion. Pots were dressed with a nitrogen-deficient nutrient solution providing major and minor elements. Superphosphate was mixed with the sand separately at the rate of 300 p.p.m. as the fertilizer. *Trifolium* and *Medicago* plants were grown in agar tubes after the method of Chen & Thornton (1940).

Pots were brought to field capacity, wrapped, stacked in a bin and subjected to a continuous flow of steam for 1 hr.; this raised the temperature of the sand to at least 90°. Seeds were disinfected by immersion for 10–20 min. in a mixture of equal volumes of 100 vol. H₂O₂ and absolute ethanol, followed by washing in sterile water. The seeds were then germinated and planted under sterile conditions. Inoculum was applied direct to the seed coat, and pots were watered with sterile water. The tests were completed in a total of twelve experiments, each of factorial design: host species × sample strains × a minimum of four replications.

Root systems were washed out usually about 28 days after germination and floated in water for examination. In one experiment nodulation occurred in some of the uninoculated control plants; this experiment was repeated. In the experiments reported here the control of nodulation was absolute.

RESULTS

Cultural characters

All 85 bacterial strains originated from within the tissues of sound legume root-nodules and grew as aerobic heterotrophs. Colonies after 8 days were punctiform, rarely exceeding 1 mm. diam., and were opaque or rarely translucent, whitish, and gummy. No isolated colony exceeded punctiform size within 5 days, and all strains were therefore slow-growing compared to the known strains of *Rhizobium meliloti*,

354 Table 1. *The infectiveness of 85 strains of nodule bacteria on 9 legume test hosts*

+, Nodulation on all plant replications; ±, nodulation on some but not all plants; —, complete absence of nodulation.

Host of isolation	Strain no.	Host nodulated								
		<i>Gly- cine hispida</i>	<i>Lupi- nus albus</i>	<i>L. digi- tatus</i>	<i>L. pilosus</i>	<i>L. subcar- nosus</i>	<i>L. vil- losus</i>	<i>Phase- olus lathy- roides</i>	<i>P. vul- garis</i>	<i>Vigna sin- ensis</i>
<i>Acacia acuminata</i> Benth.	3	±	±	±	—	—	—	+	±	+
<i>ericifolia</i> Benth.	54	—	—	+	—	—	—	+	±	+
<i>stenoptera</i> Benth.	65	+	±	+	±	—	—	+	+	+
<i>erinacea</i> Benth.	48	—	±	±	—	—	—	±	±	±
<i>extensa</i> Lindl.	121	±	±	+	±	—	—	+	±	+
<i>Drummondii</i> Lindl.	29	+	±	±	—	—	—	±	±	±
<i>myrtifolia</i> Willd.	113	—	—	+	—	—	—	+	±	+
<i>cyanophylla</i> Lindl.	20	—	±	+	+	—	—	±	±	+
<i>hastulata</i> Smith	114	+	±	+	—	—	—	±	—	+
<i>horridula</i> Meissn.	85	+	—	+	—	—	—	+	±	+
<i>nervosa</i> D.C.	115	—	±	+	±	—	—	+	+	+
<i>volubilis</i> F.v.M.	46	+	±	+	±	—	—	—	±	+
<i>pulchella</i> R.Br.	64	—	±	+	±	—	—	—	±	+
<i>diptera</i> Lindl.	14	+	±	+	—	—	—	+	±	+
<i>Albizzia distachya</i> (Vent.) Macbride	97	+	—	+	—	—	—	+	±	+
<i>Aotus villosa</i> Sm.	134	—	±	+	—	—	±	+	±	+
<i>Preissii</i> Meissn.	126	+	±	+	±	—	—	+	±	+
<i>Tietkinsii</i> F.v.M.	132	—	±	—	±	—	—	—	±	—
<i>Bossiaea Presii</i> Meissn.	92	+	—	+	±	—	—	+	±	+
<i>Laidlawiana</i> Tovey et Morris	96	—	±	—	—	—	—	—	—	—
<i>Webbii</i> F.v.M.	110	—	±	+	±	—	±	±	±	+
<i>dentata</i> Benth.	107	±	±	±	—	—	—	±	±	+
<i>aquifolium</i> Benth.	95	±	—	—	—	—	—	+	±	+
<i>eriocarpa</i> (R.Br.) Benth.	88	—	—	+	—	—	—	±	+	+
<i>linophylla</i> R.Br.	99	—	—	—	±	—	—	±	±	+
<i>Brachysema lanceolatum</i> Meissn.	91	±	±	+	±	±	—	+	—	+
<i>sericeum</i> (Sm.) Domin.	117	+	—	—	—	—	—	+	±	+
<i>praemorsum</i> Meissn.	124	—	—	—	—	—	—	—	—	+
<i>Chorizema Dicksonii</i> Grah.	90	—	±	+	±	—	—	+	+	+
<i>ilicifolium</i> Labill.	123	—	—	±	—	—	±	—	±	+
<i>reticulatum</i> Meissn.	122	+	+	+	—	—	—	+	±	+
<i>aciculare</i> (D.C.) C.A. Gardn.	120	+	—	±	+	—	—	—	+	+
<i>cytisoides</i> Turcz.	119	+	—	+	—	—	—	+	±	+
<i>ericifolium</i> Meissn.	52	+	+	+	±	—	—	+	+	+
<i>Davesia hakeoides</i> Meissn.	57	±	—	±	—	—	—	—	±	—
<i>brevifolia</i> Lindl.	101	±	—	±	—	—	—	—	+	+
<i>pectinata</i> Lindl.	68	—	±	—	±	—	—	—	±	—
<i>sphylla</i> (F.v.M.) Benth.	112	—	+	—	+	—	—	—	—	—
<i>incrassata</i> Sm.	163	—	—	—	—	—	—	±	+	±
<i>Dillwynia uncinata</i> (Turcz.) C.A. Gardn.	98	±	±	±	±	—	±	±	—	+
<i>Euchilopsis linearis</i> (Benth.) F.v.M.	62	—	—	+	—	—	—	—	+	+

Host of isolation	Strain no.	Host nodulated								
		<i>Gly- cine hispida</i>	<i>Lupi- nus albus</i>	<i>L. digi- tatus</i>	<i>L. pilosus</i>	<i>L. subcar- nosus</i>	<i>L. vil- losus</i>	<i>Phase- olus lathy- roides</i>	<i>P. vul- garis</i>	<i>Vigna sin- ensis</i>
<i>Eutaxia epacridioides</i> Meissn.	87	+	—	+	—	—	—	+	+	+
<i>virgata</i> Benth.	105	—	±	+	—	—	—	+	+	+
<i>densifolia</i> Turcz.	137	—	—	—	±	—	—	±	+	±
<i>Gastrolobium villosum</i> Benth.	106	—	—	+	—	—	—	±	±	+
<i>spinosum</i> Benth.	61	—	+	+	±	—	—	+	—	+
<i>obovatum</i> Benth.	32	—	±	±	—	—	—	±	±	+
<i>trilobum</i> Benth.	100	+	—	+	—	—	—	+	+	+
<i>Gompholobium marginatum</i> R.Br.	60	+	—	+	—	—	—	±	±	+
<i>venustum</i> R.Br.	66	—	—	+	—	—	—	—	—	+
<i>polymorphum</i> R.Br.	111	+	—	+	—	—	—	+	±	+
<i>Knightianum</i> Lindl.	108	±	+	+	+	—	—	—	+	—
<i>tomentosum</i> Labill.	104	±	±	+	—	—	—	+	±	+
<i>Hardenbergia Comptoniana</i> (Andr.) Benth.	19	+	±	+	±	—	—	—	+	+
<i>Hovea elliptica</i> D.C.	109	—	—	±	—	—	—	±	±	+
<i>trisperma</i> Benth.	15	+	±	+	—	—	—	+	+	+
<i>chorizemifolia</i> D.C.	26	+	±	+	±	—	—	—	—	+
<i>pungens</i> Benth.	118	+	±	+	±	—	—	+	±	+
<i>Isotropis cuneifolia</i> (Sm.) Domin.	16	+	±	+	±	—	—	—	+	+
<i>Jacksonia hakeoides</i> Meissn.	69	+	—	±	—	—	±	±	±	+
<i>spinosa</i> (Labill.) R.Br.	102	+	±	+	—	—	—	+	±	+
<i>horrida</i> D.C.	33	—	±	±	—	—	—	±	±	±
<i>furcellata</i> (Bonpl.) D.C.	125	+	±	+	±	—	—	+	±	+
<i>Kennedyia eximia</i> Lindl.	73	+	—	+	—	—	—	+	+	+
<i>coccinea</i> Vent.	77	+	±	+	—	—	—	+	+	+
<i>Stirlingii</i> Lindl.	78	+	±	+	±	—	—	+	±	+
<i>prostrata</i> R.Br.	2	+	±	+	—	—	—	+	±	+
<i>Stirlingii</i> Lindl.	76	+	—	+	—	—	—	+	+	+
<i>Latrobea hirtella</i> (Turcz.)	133	+	±	±	—	—	—	+	±	+
<i>Mirbelia floribunda</i> Benth.	89	±	±	+	—	—	—	+	±	+
<i>spinosa</i> Benth.	127	+	±	±	±	—	—	+	±	+
<i>dilatata</i> R.Br.	86	—	—	±	—	—	—	+	±	+
<i>Oxylobium parviflorum</i> Benth.	27	+	±	+	—	—	—	+	±	+
<i>lanceolatum</i> Benth.	130	±	—	+	—	—	—	+	+	+
<i>capitatum</i> Benth.	17	+	—	+	±	—	—	+	+	+
<i>atropurpureum</i> Turcz.	141	±	—	+	—	—	—	—	+	+
<i>reticulatum</i> Meissn.	81	—	—	+	—	—	—	+	±	+
<i>Pultenaea reticulata</i> (Sm.) Benth.	84	±	—	+	—	—	—	+	+	+
<i>strobilifera</i> Meissn.	85	+	—	—	±	—	—	+	+	+
<i>ochreate</i> Meissn.	94	+	±	—	—	—	—	±	±	±
<i>Sphaerolobium alatum</i> Benth.	59	—	—	+	—	—	—	+	+	+
<i>medium</i> R.Br.	128	+	±	—	—	—	—	+	±	+
<i>grandiflorum</i> R.Br.	116	—	—	—	—	—	—	±	±	+
<i>Templetonia retusa</i> R.Br.	21	—	—	+	+	—	—	+	±	+
<i>Viminaria denudata</i> (Sm.)	93	+	+	+	—	—	—	+	±	+

R. trifolii and *R. leguminosarum* which were included. All strains were Gram-negative. None exhibited spores or morphologically distinctive forms, but ranged from coccobacilli to more or less straight bacilli from 0.5 to 4.5 μ by 0.5 μ . On calcium glycerophosphate agar all failed to cause browning. In litmus milk, all strains produced an alkaline reaction without appreciable dye reduction. None produced a serum zone. All 85 strains produced acid on arabinose and xylose, an acid reaction or no change of pH value on rhamnose, and alkaline reactions on maltose and sucrose.

Infectiveness

Under the conditions of the experiments, none of the 85 strains nodulated *Pisum sativum*, *Lathyrus cicera*, *Lens esculenta*, *Vicia sativa*, *Lupinus angustifolius*, *L. luteus*, *L. nanus*, *Ornithopus sativus*, *O. compressus*, *Medicago tribuloides*, or *Trifolium subterraneum*. The following plant species were nodulated by sample strains: *Vigna sinensis* (79 strains); *Phaseolus vulgaris* (76); *Lupinus digitatus* (71); *Phaseolus lathyroides* (67); *Glycine hispida* (53); *Lupinus albus* (47); *L. pilosus* (31); *L. villosus* (5) and *L. subcarnosus* (1). The details of their infective performance on these species are presented in Table 1.

DISCUSSION

Breaches of the cross-inoculation boundaries between the soybean and the cowpea (Leonard, 1923), and between lupins, soybeans and cowpea (Bushnell & Sarles, 1937) have been reported before. The results submitted here further illustrate the deficiencies of the cross-inoculation-group concept when used to delineate species of *Rhizobium*. The bacterial strains used in this study were selected as representative of the microsymbionts from indigenous legumes in the study area. When compared with recognized *Rhizobium* species on the basis of their *in vitro* characters, all of them showed the characteristics of the slow-growing species *Rhizobium japonicum*, or of the cowpea group of rhizobia.

The range of infective performance exhibited by the sample strains extended to hosts from four of the recognized cross-inoculation groups, namely, the cowpea, lupin, soybean and bean groups. However, infection across the boundaries of these four groups was prevalent to the extent of being typical rather than exceptional. Thus of the 85 strains examined

- 45 strains nodulated hosts in 4 cross-inoculation groups;
- 31 strains nodulated hosts in 3 cross-inoculation groups;
- 6 strains nodulated hosts in 2 cross-inoculation groups;
- 3 strains nodulated hosts in 1 cross-inoculation group.

On the evidence it is impossible to place these strains in specific cross-inoculation groups, or to assign species designations to them on the basis of the accepted system. Distinctly different host groupings are apparent. For example, *Lupinus digitatus*, *Glycine hispida*, *Vigna sinensis* and *Phaseolus vulgaris* group together on susceptibility to infection by the sample strains, even though these hosts are from four different cross-inoculation groups. Similarly, *L. digitatus*, *L. albus* and *L. pilosus* group with each other on susceptibility, but do not group at all with their accepted cross-inoculation associates *L. angustifolius*, *L. luteus* and *Ornithopus sativus*.

Three *Rhizobium* species were not involved at all. None of the data implicated the clover, the medic or the pea cross-inoculation groups in any way, although

evidence from other sources (Kleczkowska, Nutman & Bond, 1944; Norris, 1959) shows that the pea and clover groups are not as discrete as they were once regarded.

Past studies of root-nodule bacteria show preoccupation with symbiont infectiveness patterns because of the economic importance attached to them. For the same reason, there has been a further preoccupation with agronomic legumes. This bias has dominated microsymbiont classification. In the period around 1920 there were two conflicting approaches. On the one hand, an expedient classification of the bacteria was derived based solely on their segregations in symbiotic infectiveness patterns (Garman & Didlake, 1914). On the other hand, the practice of classifying strains on the basis of symbiotic performance alone was rejected by Löhnis & Hansen (1921). Literature published over the succeeding period recorded the continued dominance and development of the former approach (Fred, Baldwin & McCoy, 1932). A species of *Rhizobium* was recognized as containing those microsymbionts which would nodulate a particular group of legumes. Other characters of the bacteria were not accorded equivalent taxonomic weight. Subsequently, patterns of symbiotic infectiveness were demonstrated to be less discrete (Leonard, 1923; Sears & Clark, 1930; Raju, 1936; Bushnell & Sarles, 1937). Interrelationships led some authors to suggest consolidations of groups and of species (Walker & Brown, 1935), whilst others suggested retention of the old groups with provision for special cases (Allen & Allen, 1936).

A second and very emphatic rejection of the cross-inoculation basis of strain classification was made by Wilson (1939) who produced substantial data to support his contentions. Further demonstrations of the inadequacies of the cross-inoculation groups continued (Appleman & Sears, 1942; Johnson & Allen, 1952; Ishizawa, 1954; Bowen, 1960). Recently a third rejection of the cross-inoculation-group concept was made. Norris (1956) considered that there was no satisfactory known basis for the subdivision of the rhizobia into species. He suggested the use of a symbiotic rating which would indicate the relative affinities of a particular strain for hosts within the three subfamilies of the Leguminosae.

The suggested modifications all continue to classify the nodule bacteria on the basis of their infective performance, excluding comparisons with bacteria which cannot produce nodules on the roots of a legume. This is clearly unsatisfactory. To be effective as a classification of bacteria, a system should be erected on non-biased over-all similarities. The Adansonian classification proposed by Sneath (1957) fulfils these requirements. The classification of the root nodule bacteria might thus be resolved by research within the following stages:

- (1) Collection of types (or neotypes) of all recognized *Rhizobium* species.
- (2) Collection from botanically representative and geographically diverse sources the widest sample of legume root-nodule bacteria possible within research resources.
- (3) Collection of types and representatives of possibly related bacteria, e.g. *Agrobacterium* (Hofer, 1941; Bonnier, 1953); *Bacillus* (Bisset, 1952, 1959); *Beijerinckia* (Derx, 1953).
- (4) Derivation of a range of tests by which all these bacteria may be described.
- (5) Application of the tests, and classification of all strains on the results, by Sneath's (1957) method.

By thus relating a full range of root-nodule bacteria to each other and to other bacteria, their over-all similarities and groupings should become evident, and the

genus *Rhizobium* with its component species could be retained or rejected. Application of an Adansonian classification to the strains used in this study would be premature, since both the strain sample and the test range are not adequate for the derivation of a generalized classification.

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