BERGERSEN, F. J. (1955). *J. gen. Microbiol.* 13, 411–419

The Cytology of Bacteroids from Root Nodules of Subterranean Clover (*Trifolium subterraneum* L.)

By F. J. BERGERSEN

Division of Plant Industry, C.S.I.R.O., Canberra, A.C.T., Australia

SUMMARY: Bacteroids from effective nodules of subterranean clover were ovoid, pear-shaped, or slightly branched and measured $5-7 \times 4-6 \mu$. In the fully developed bacteroid the nuclear material was central, divided into discrete bodies and showed signs of degeneration. Surrounding the nucleus was a perinuclear region, probably derived from it, and this was surrounded by cytoplasm which contained several mitochondria-like granules. The development of bacteroids from rod-shaped cells is described. Bacteroids from the ineffective nodules studied possessed no perinuclear region, and, in contrast to those from effective nodules, accumulated varying amounts of glycogen, depending on the length of time for which the bacteroids survived in the host cell.

The symbiotic nitrogen-fixing nodule bacteria have been the subject of much research, and it is surprising that the structure of the organisms within the host cells has not received more attention. The term 'bacteroid' has been given to the swollen irregularly shaped bacteria found in the cytoplasm of infected host cells in nodules on legume roots. Morek (1891) made a careful study of bacteroids, and his drawings show cells with clear central areas surrounded by granular cytoplasm. Uher (1937) described spindle- and globiform-shaped nuclei in bacteroids from *Trifolium* species, and Schaede (1939, 1941) demonstrated a diffuse Feulgen-positive reaction in bacteroids from *Lupinus* and *Neptunia* nodules but not in those from *Vicia faba*. Baylor, Appleman, Sears & Clark (1945) described nuclear granules visible in electron micrographs of nodule bacteria from cultures and excised nodules, and Bisset (1952) included some observations on bacteroids in a paper on life cycles in *Rhizobium* species. The reviews of Fred, Baldwin & McCoy (1932) and of Allen & Allen (1950) give further references to previous work on this subject.

As with other legume species, root nodules on subterranean clover may not always benefit the host plant by fixing atmospheric nitrogen, in which case they are termed 'ineffective'. In this paper, work is described in which bacteroids from both 'effective' and 'ineffective' nodules were examined by a variety of cytological techniques.

METHODS

Organisms. Subterranean clover (*Trifolium subterraneum* L.) variety Mt. Barker was used throughout as host plant. Plants were grown in test tubes on a nitrogen-free agar medium according to the method of Thornton (1930). After germination each seedling was inoculated with one of the strains of *Rhizobium trifolii* listed in Table 1, by means of one drop of a suspension from

a yeast water agar culture. The plants were kept in a glasshouse and nodules were examined at various intervals. Both effective and ineffective plant/bacteria combinations were compared with uninoculated controls. All ineffective combinations grew as badly as the controls.

Table 1. *The strains of Rhizobium trifolii studied*

Strain	Type of symbiosis	Source
297/3	Effective	Crimson clover*
297/33	Effective	Crimson clover*
297/32	Ineffective	Crimson clover*
298/533	Effective	Crimson clover*
298/534	Effective	Crimson clover*
298/531	Ineffective	Crimson clover*
298/536	Ineffective	Crimson clover*
C1F	Ineffective	Red clover, Rothamsted, England
161	Ineffective	White clover, Glen Innes, N.S.W., Australia
157	Ineffective	White clover, Glen Innes, N.S.W., Australia

* See Vincent (1954) and Vincent & Waters (1954) for further information about the origin of these strains.

Staining procedures. Preparations of bacteria from nodules were obtained by pressing the surface of cut nodules lightly on to thin coverslips. Fixation, when used, was obtained by floating the coverslip preparations for $\frac{1}{2}$ –1 min. on a drop of 2% (w/v) osmic acid in M/15 phosphate buffer (pH 7.0) in a watch-glass. Phase-contrast microscopy of living and fixed material was carried out with a Zeiss Opton system and green light. Nuclear structures were examined by the Feulgen reaction, the HCl+Giemsa method (Robinow, 1942) and by the method of DeLamater (1951). Cell walls were stained with 1% (w/v) crystal violet after 20 min. treatment with 10% (w/v) tannic acid.

Supravital staining reactions were used as follows. Suspensions of the bacteroids were prepared from crushed nodules, the reagents were added and a drop of the mixture mounted for microscopic observation by phase-contrast and bright field illumination. The following cytochemical reagents were used: (1) Janus green B at a final concentration of 0.005% (w/v); (2) Nadi reagent, 1 drop each of 1% (w/v) α -naphthol in 95% (v/v) ethanol and 1% (w/v) aqueous dimethyl-*p*-phenylenediamine added to 1 ml. of a bacteroid suspension; (3) 2, 3, 5-triphenyltetrazolium chloride at a final concentration of 0.05% (w/v); (4) the Gomori method for alkaline phosphatase, adapted as follows. Nodules were crushed in M/15 phosphate buffer (pH 7.0) and the bacteroids centrifuged down. They were then resuspended in a mixture containing 25 ml. 2% (w/v) sodium glycerophosphate, 25 ml. 2% (w/v) sodium veronal, 50 ml. distilled water, 5 ml. 2% (w/v) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 2 ml. 2% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. After incubation for 1 hr. at 25° the mixture was centrifuged, the bacteroids rinsed in buffered distilled water (10 ml. M/15 phosphate buffer, pH 7.0; 90 ml. distilled water) and resuspended in 2% (w/v) $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$

solution for 5 min. The bacteroids were then washed in two changes of buffered distilled water and resuspended in 0.5% (w/v) sodium sulphide solution. With this method sites of alkaline phosphatase activity stained brownish black.

Carbohydrate deposits were examined by Lugol's iodine and the Bauer-Feulgen glycogen stain (Glick, 1949).

At all times the morphology and cytology of the bacteroids were compared with the forms seen in microtome sections of the nodules, stained with Heidenhain's haematoxylin.

RESULTS

The structure of effective and ineffective nodules

Stained sections of effective subterranean clover nodules showed an anatomical structure similar to that described for several legume species by Chen & Thornton (1940), Schaede (1932) and others. The meristem of growing nodules was at the outer tip, and next to this were cells which were being penetrated by infection threads. Next were cells in which bacteria, released from infection threads, were undergoing bacteroid formation within the host cell cytoplasm. The bulk of the bacteria-containing tissue of the nodule consisted of vacuolated host cells, whose cytoplasm was tightly packed with mature bacteroids. At the base of the nodule was a region of degeneration which was devoid of bacteroids and which became more extensive as the nodule aged.

The ineffective nodules on subterranean clover were usually smaller than the effective ones, but differed from the description of Chen & Thornton (1940) of such nodules on clover, pea and soya-bean roots, in that the bacterial tissue persisted for some time before the bacteroids disappeared. The period of persistence varied with the strain of ineffective *Rhizobium* used. The bacteroids in these nodules on subterranean clover were loosely packed in the host cytoplasm and were often irregularly distended. Pl. 1, figs. 1 and 2, show the general arrangement of the tissues in effective and ineffective subterranean clover nodules of similar ages.

Bacteroids from effective nodules

With phase-contrast microscopy considerable intracellular detail is shown. Young bacteroids appeared fairly opaque and to contain several small, dark cytoplasmic granules. In older bacteroids one to four pale central regions were surrounded by darker cytoplasm and several dark granules.

Haematoxylin-stained nodule sections suggested that the bacteroids possess nuclear material, and this was confirmed by the nuclear stains. In the Feulgen preparations a central portion of the bacteroids gave a diffuse positive reaction and contained more densely stained bodies within it. The HCl + Giemsa method showed a variety of basophilic structures. In some preparations there was an unstained central portion of the bacteroids which contained several basophilic bodies. In others the whole of the central portion stained purplish blue. Unhydrolysed bacteroids stained with Giemsa showed a purple central region

surrounded by several cytoplasmic granules. DeLamater's method revealed purple-stained chromatin elements within an unstained space surrounded by lightly stained cytoplasm. From this description it seems probable that the central 'perinuclear' region is of nuclear origin. Many different reagents were used in an attempt to determine the nature of the material which made up the perinuclear portion of the bacteroids, but apart from the basophilic properties shown in the unhydrolyzed preparations stained with Giemsa, nothing was found.

By comparing preparations from nodules of various ages, stained by all the methods, the following development of the nuclear structures was deduced. Bacteria released from an infection thread into the host cells contained a pair of nuclear bodies in a rod-shaped cell 2-3 μ . long and *c.* 0.5 μ . wide. Several divisions took place and then the rods began to swell. At this stage the nucleus consisted of a string of chromatinic material, axial to the organisms and divided in branching bacteroids. With continued enlargement the perinuclear regions began to appear and the amount of nuclear material seemed to be decreased as these perinuclear regions enlarged. Just before lysis, which was very rapid, no discrete chromatin was found (Fig. 1). Some bacteroids were seen to contain up to four of these perinuclear regions, each with its chromatinic element or set of elements, but in no case did the cell-wall stain reveal any septation. Stained walls showed variations in thickness which probably arose during the uneven growth which took place during bacteroid formation.

The supravital methods stained the granules which were seen in the cytoplasm of the bacteroids by phase-contrast microscopy. With Janus green B the cytoplasm was stained pale green, and the granules a dense green which passed through the characteristic changes green $\rightarrow$ pink $\rightarrow$ colourless which indicate reduction of the dye. The perinuclear region remained unstained by Janus green B. The tetrazolium compound stained the granules a bright red, leaving the rest of the bacteroid unstained. Nadi reagent stained the granules blue at pH 5.7 but only occasionally at higher pH values. The Gomori reaction of the granules was always strongly positive. These granules were very small, and although visible in microscope preparations no satisfactory photographs of them were obtained. From these results it was concluded that the cytoplasmic granules seen in the bacteroids from effective nodules on subterranean clover have most of the properties ascribed to mitochondria-like bodies in other species of bacteria (Mudd *et al.* 1951; Bergersen, 1953) in that they are centres of oxidation-reduction activity, possess cytochrome oxidase as shown by the Nadi reagent, and have a system capable of dephosphorylating glycerophosphate. Similar results were obtained with all the effective strains of bacteria studied, no differences in bacteroid morphology or cytology were seen (Pl. 1, figs. 3-5).

Bacteroids from ineffective nodules

Bacteroids from ineffective nodules differed from effective bacteroids in two major respects: no perinuclear regions developed in the ineffective bacteroids, they showed a considerable accumulation of glycogen. There was a variation

in shape as between different strains. Ineffective substrains of nodule bacteria isolated from the subterranean-crimson clover group (Vincent & Waters, 1954), produced bacteroids very similar in shape to the bacteroids produced by the effective parent strains. However, ineffective strains isolated from other *Trifolium* species gave bacteroids in subterranean clover nodules which were

Fig. 1. The development of effective bacteroids: 1, rod-shaped bacteria released from infection threads; 2, beginning of bacteroid formation; 3, beginning of the formation of perinuclear regions; 4, mature bacteroids which pack most of the bacterial tissue of active nodules; 5, old bacteroids before lysis. *m*=mitochondria-like granules; *n*=nuclear material; *c*=cytoplasm; *p*=perinuclear regions.

less swollen, more variable in shape, and often more branched; this was especially the case with strain C1F. The nuclear material in these ineffective bacteroids was very irregular and usually appeared as a bar of chromatin axial to the cell. With the distortion of the cytoplasm due to accumulation of inclusions, the nuclei became distorted and often were not revealed by the usual nuclear stains. The cytoplasmic mitochondria-like granules of ineffective bacteroids were larger and more prominent than those of effective bacteroids and fewer in number, but they gave the same reactions with the supravital stains.

The most noticeable feature of the ineffective bacteroids was the accumulation of glycogen. This occurred first on the outside of the mitochondria-like granules, as indicated by a red-brown staining reaction with Lugol's iodine. Inclusions were then formed which usually clustered around the granules as refractile globules in the cytoplasm. These refractile globules enlarged and increased in number as long as the bacteroid itself remained in the host cell;

Table 2. *The persistence of the bacteroid stage of Rhizobium trifolii (ineffective and effective strains) in subterranean clover nodules and the presence or absence of glycogen*

Ineffective strains of <i>Rhizobium</i> <i>trifolii</i>	Age of nodule (days)			
	20	30	40	50
C 1F	Bacteroids + + + Glycogen +	Bacteroids + + + Glycogen + +	Bacteroids + + + Glycogen + + +	Bacteroids + + + Glycogen + + +
297/32	Bacteroids + + Glycogen +	Bacteroids + + Glycogen + +	Bacteroids and glycogen absent	Bacteroids and glycogen absent
298/536	Bacteroids + + Glycogen +	Bacteroids + Glycogen +	Bacteroids and glycogen absent	Bacteroids and glycogen absent
161	Bacteroids + Glycogen +	Bacteroids and glycogen absent	Bacteroids and glycogen absent	Bacteroids and glycogen absent
298/531	Bacteroids + Glycogen +	Bacteroids and glycogen absent	Bacteroids and glycogen absent	Bacteroids and glycogen absent
157	Bacteroids ± Glycogen ±	Bacteroids and glycogen absent	Bacteroids and glycogen absent	Bacteroids and glycogen absent
All effective strains	Bacteroids + + + + Glycogen —	Bacteroids + + + + Glycogen —	Bacteroids + + + + Glycogen —	Bacteroids + + + + Glycogen —

they stained red-brown with Lugol's iodine and gave the characteristic red-violet colour with the Bauer-Feulgen technique. On the contrary, the mitochondria-like granules in effective bacteroids did not stain with Lugol's iodine, and there was no evidence of glycogen accumulation. The relationship between the abundance and degree of persistence of the bacteroids and the extent of glycogen accumulation is shown in Table 2. Most accumulation was found with the strain C1F which persisted as the bacteroid stage in the host cells for more than 50 days. At the end of this time the bacteroids were distended with globules of glycogen (Pl. 1, figs. 7, 10) while with strain 157, which barely persisted for 20 days, accumulation was slight, the only glycogen present being shown as iodine-stained mitochondria-like granules (Pl. 1, fig. 8). The simplified drawings of Fig. 2, made from actual preparations, show some of the types of bacteroids found in ineffective nodules.

Examination of frozen sections of fresh ineffective nodules mounted in Lugol's iodine indicated that there was an inverse relationship between the amount of starch in the host cells and the amount of glycogen accumulated by the bacteroids. Thus strain C1F nodules contained only small amounts of starch but strain 157 nodules contained a great deal which continued to accumulate after the bacteroids had disappeared from the host cell.

Fig. 2. Ineffective bacteroids: 1, from strains 161 and 157; 2, from strains 298/531 and 298/536; 3, from strain 297/32; 4, from strain C1F.

g = glycogen globules, other abbreviations as Fig. 1.

DISCUSSION

From the foregoing results it may be seen that the distinctive feature of bacteroids from nitrogen-fixing nodules on subterranean clover is the development of the perinuclear regions. Chemical tests did not give any clue as to the constitution of these regions of the organisms, and no theories about their role can be advanced. However, the constant occurrence of these regions in effective bacteroids and their absence in ineffective bacteroids seems significant and may indicate some relation with the nitrogen-fixing processes. The perinuclear regions are linked with the nuclear material which they contain and may be in fact derived from the nucleus. In this connexion it is interesting to observe that the nuclear material possessed no detectable phosphatase activity while the cytoplasmic granules did. This would seem to indicate a low degree of nuclear activity since in most animal and plant cells alkaline phosphatase activity in the nuclei seems to be associated with nuclear activity. This may have a bearing on the non-viability of the bacteroids (Almon, 1933) when transferred to nutrient media.

The general picture of nitrogen-deficient plants is one of carbohydrate excess which is shown by the accumulation of starch in most tissues. It is therefore

interesting, but not surprising, that in ineffective nodules in which the bacteroids persist, evidence of carbohydrate excess in the bacteria is shown by the accumulation of the characteristic bacterial reserve carbohydrate, glycogen. Bacteroids which disappear from the host cell at a fairly early stage accumulate little glycogen, but the host cell accumulates a considerable amount of starch.

Thanks are due to Dr P. S. Nutman for advice and criticism, and to Mr J. M. Vincent, University of Sydney, for supplying some of the cultures.

REFERENCES

- ALLEN, E. K. & ALLEN, O. M. (1950). Biochemical and symbiotic properties of the rhizobia. *Bact. Rev.* **14**, 273.
- ALMON, L. (1933). Concerning the reproduction of bacteroids. *Zbl. Bakt.* (2 Abt.), **87**, 289.
- BAYLOR, M. B., APPLEMAN, M. D., SEARS, O. H. & CLARK, G. L. (1945). Some morphological characteristics of nodule bacteria as shown by the electron microscope. *J. Bact.* **50**, 249.
- BERGERSEN, F. J. (1953). A probable growth cycle in *Bacillus megaterium*. *J. gen. Microbiol.* **9**, 26.
- BISSET, K. A. (1952). Complete and reduced life cycles in *Rhizobium*. *J. gen. Microbiol.* **7**, 233.
- BUCHANAN, K. E. (1909). The bacteroids of *Bacillus radicola*. *Zbl. Bakt.* (2. Abt.), **23**, 59.
- CHEN, H. K. & THORNTON, H. G. (1940). The structure of 'ineffective nodules' and its influence on N₂ fixation. *Proc. Roy. Soc. B*, **129**, 208.
- DELAMATER, E. D. (1951). A staining and dehydrating procedure for microorganisms. *Stain. Tech.* **26**, 199.
- FRED, E. B., BALDWIN, I. L. & MCCOY, E. (1932). *Root Nodule Bacteria and Leguminous Plants*. University of Wisconsin studies in Science, 5.
- GLICK, D. (1949). *Techniques of Histo- and Cytochemistry*. New York: Interscience Publishers.
- MORCK, D. (1891). Über die Formen der Bakteroiden bei den einzelnen Spezies der Leguminosen. Inaug. Diss. Leipzig.
- MUDD, S., BRODIE, A. F., WINTERSCHIED, L. C., HARTMAN, P. E., BEUTNER, E. H. & MCLEAN, A. A. (1951). Further evidence for mitochondria in bacteria. *J. Bact.* **62**, 729.
- ROBINOW, C. F. (1942). A study of the nuclear apparatus of bacteria. *Proc. Roy. Soc. B*, **130**, 299.
- SCHAEDE, R. (1932). Bakterien in den Knöllchen von *Lupinus albus*. *Zbl. Bakt.* (2 Abt.), **85**, 416.
- SCHAEDE, R. (1939). Zum Problem des Vorkommens von chromatischer Substanz bei Bakterien und Aktinomyceten. *Arch. Microbiol.* **10**, 473.
- SCHAEDE, R. (1941). Untersuchungen in den Wurzelknöllchen von *Vicia faba* und *Pisum sativum*. *Beitr. Biol. Pfl.* **27**, Zweites Heft., 165.
- THORNTON, H. G. (1930). The early development of the root nodules of lucerne. *Ann. Bot., Lond.*, **44**, 385.
- UHER, M. (1937). A contribution to the problem of the nucleus of bacteria. *Ann. Acad. tchécosl. Agric.* **12**, 474.
- VINCENT, J. M. (1954). The root nodule bacteria of pasture legumes. *Proc. Linn. Soc. N.S.W.* **79**, 4.
- VINCENT, J. M. & WATERS, L. M. (1954). Survival and success of inocula in laboratory trials. *Aust. J. agric. Res.* **5**, 61.

F. J. BERGERSEN—CYTOLOGY OF ROOT NODULE BACTERIODS. PLATE 1

(Facing p. 419)

EXPLANATION OF PLATE

- Fig. 1. Twenty-day effective nodule. Haematoxylin. $\times 160$. *a*=meristem; *b*=region of penetration and beginning of bacteroid formation; *c*=region of mature bacteroids; *d*=region of degeneration.
- Fig. 2. Twenty-day ineffective nodule showing persistent bacterial tissue (*c*). Haematoxylin. $\times 160$.
- Fig. 3. Effective bacteroids; stained by DeLamater's method. $\times 1600$.
- Fig. 4. Effective bacteroids; stained HCl+Giemsa. $\times 1600$.
- Fig. 5. Effective bacteroids; unfixed and seen by phase-contrast microscopy. $\times 1600$.
- Fig. 6. Ineffective bacteroids; strain 297/32; phase contrast. $\times 1600$.
- Fig. 7. Ineffective bacteroids; strain C1F; phase contrast. $\times 1600$.
- Fig. 8. Ineffective bacteroid; strain 157; phase contrast. $\times 1600$.
- Fig. 9. Ineffective bacteroids; strain 161; phase contrast. $\times 1600$.
- Fig. 10. Ineffective bacteroid (strain C1F) stained by the Bauer-Feulgen method and counterstained with light green. $\times 2400$.

(Received 8 March 1955)