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Capsulation in *Rhizobium* Species

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Early reports of capsulation in *Rhizobium* spp. (F. C. Harrison and B. Barlow, Zentr. Bakteriol. Parasitenk. Abt. II **19**:426, 1907; R. Greig-Smith, Zentr. Bakteriol. Parasitenk. Abt. II, **30**:552, 1911; F. Löhnis and R. Hansen, J. Agr. Res. **20**:543, 1921) have never been substantiated and appear to have been forgotten by more recent investigators. References to capsules of *Rhizobium* have appeared in some subsequent papers (O. A. Bushnell and W. B. Sarles, J. Bacteriol. **38**:401, 1939; D. H. Hubbell and G. H. Elkan, Can. J. Microbiol. **13**:235, 1967), but the term is used loosely to mean extracellular polysaccharide. Because of the importance of capsules in many groups of bacteria, it was necessary, as part of a study of the antigens of *Rhizobium* spp., to examine their capsulation more carefully.

A total of 23 *Rhizobium* strains from all cross-inoculation groups were grown on yeast-extract mannitol agar for 7 days and examined for capsules by the wet-film India ink method (J. P. Dugu'd, J. Pathol. Bacteriol. **63**:673, 1951). *R. meliloti* (three strains), *R. japonicum* (one strain), and the cow-pea organisms (two strains) had grown well and produced mucoid gummy cultures but were noncapsulated. Their microscopic appearance and the solubility of their polysaccharides varied from strain to strain. The cultures of *R. meliloti* dispersed easily when suspended in saline, and the cells were seen to be free of adherent polysaccharide. The cultures of *R. japonicum* and the cow-pea organisms were hard to disperse and their polysaccharide much less soluble; the cells remained embedded in firm adherent masses of amorphous polysaccharide.

The 17 strains of *R. trifolii*, and the single strains of *R. leguminosarum*, *R. lupini*, and *R. phaseoli* that were examined, contained both truly capsulated and bare cells, with the latter predominating. The presence of capsules in these strains was clearly observed by wet-film staining with nigrosin (10%) and methylene blue (1%). The latter dye revealed the capsules as distinct glassy layers, with definite edges, around the cells. The antigenic nature of the capsules was demonstrated in five strains of *R. trifolii*, which reacted with their homologous antisera to give positive quellung and indirect immunofluorescence reactions.

TABLE 1. *Capsulation and polysaccharide production in Rhizobium trifolii cultures*

R. trifolii strains	Capsulation[a]							Polysaccharide[b]	
	Yeast mannitol agar medium		Defined agar medium		Defined liquid medium[c]			Defined liquid medium	
					Shaken		Shaken/static	Shaken	
	3 days	28 days	4 days	24 days	4 days	10 days	10 days[d]	4 days	10 days
	%	%	%	%	%	%	%	*mg/ml*	*mg/ml*
TA1	7	30	5	25	7	20	18	0.67	2.35
UNZ29	3	4	0.5	1	0	5	4	0.72	1.93
WA67	3	7	0.3	36	0.2	0	0	0.34	1.62
CC10	3	15	0.1	2	0.1	12	5	0.18	2.38
2480*a*	14	12	3	5	1	8	6	0.38	1.62

[a] Percentage of capsulated cells, counted by the wet-film India ink method.
[b] Determined by the anthrone method on samples of the whole culture, expressed as "glucose."
[c] Mannitol, 10 g; sodium glutamate, 1 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; trace element solution (W. F. Dudman, J. Gen. Microbiol. **21**:327, 1959), 2 ml; agar (when required), 20 g; water, 1 liter. After sterilization, $FeCl_3$, 0.02 g; $CaCl_2$, 0.04 g; thiamine, 100 μg; biotin, 250 μg; calcium pantothenate, 100 μg; all added as sterile solutions.
[d] Shaken 4 days, static 6 days.

The influence of cultural conditions and age on the relative number of capsulated cells was examined in five strains of *R. trifolii* by direct microscopic counts of India ink films in a counting chamber. Only strain TA1 produced capsules consistently under all conditions (Table 1). Capsulation in the other strains varied between wide limits with the nature of the medium and with shaking or static conditions. The results with strain WA67 in shaken defined medium indicate clearly that capsulation is not correlated with polysaccharide production.

When plated onto agar medium, some *R. trifolii* strains yielded colonial variants, recognized by unusually small and dense colonial morphology. The variants were established by immunodiffusion to be antigenically identical with the parent strains; they were found, however, to contain higher proportions of capsulated cells than the parent strains. For example, the variant colonies of strain UNZ29 contained 32% capsulated cells in contrast to 3% in the normal colonies.

Apart from their importance as antigens, capsules may also be significant in affecting the ecological behavior of *Rhizobium* spp. In a field trial with subterranean clover, 80% of the nodules examined were found to contain strain TA1 when the seed had been inoculated with a centrifuged culture fraction containing only capsulated TA1 cells, in contrast to 50% of the nodules when a normal TA1 culture was used. This suggests that, in the preparation of *Rhizobium* cultures for legume inoculation, enhanced nodulation may possibly be attained by attention to increased capsulation through the use of more heavily capsulated strains or the use of cultural conditions favoring capsule production. It must be emphasized, however, that capsulation in itself does not give any indication of the symbiotic behavior of *Rhizobium* spp.

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