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ISOLATION AND CHARACTERIZATION OF SYMBIOTICALLY DEFECTIVE MUTANT STRAINS OF *RHIZOBIUM TRIFOLII* AND *RHIZOBIUM MELILOTI*

By

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF SYMBIOTICALLY DEFECTIVE MUTANT STRAINS OF RHIZOBIUM TRIFOLII AND RHIZOBIUM MELILOTI

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Three classes of mutants were obtained after Tn5 mutagenesis of Rhizobium trifolii 0403 rif. Strains 738 and 755 (defective in nitrogen fixation) and 251 (partially defective in nitrogen fixation) produced the same plasmid pattern as wild type upon electrophoresis whereas 43 and 308 (unable to nodulate) contained a deletion in the sym plasmid. 251 attached better and 43 and 308 attached less to clover root hairs than did the wild type. Strain 251 with a single Tn5 insertion in the sym plasmid was agglutinated better and 43 and 308 less by trifoliin A than wild type. Strain 251 capsular polysaccharide (CPS) differed in depolymerization rate and non-carbohydrate substitutions from wild type CPS. These results indicate that CPS non-carbohydrate substitutions are important in R. trifolii 0403 rif attachment to clover root hairs and lectin-binding ability.

CPS was isolated from R. trifolii 843 and Tn5 mutants in nodulation genes of the symbiotic plasmid. CPS from mutants in region I(Hac) differed from wild type CPS in depolymerization rates and levels of pyruvic and acetic acid

substitutions and mutant strains had a significantly lower ability to bind the clover lectin.

An *in vitro* assay to measure CPS pyruvyl transferase activity (CPT) in *R. trifolii* was developed. Pyruvylation occurred at the lipid-bound oligosaccharide intermediate stage. CPT was measured for two wild type and symbiotically defective mutant strains. CPT was affected by Tn5 mutations in the *sym* plasmid and by clover root exudate suggesting that functions related to CPS pyruvylation may be encoded in this plasmid.

The effect of succinate metabolism on growth and bacteroid differentiation of *Rhizobium meliloti* was investigated with wild type (L5-30) and succinate dehydrogenase mutant (UR6). UR6 was defective in bacteroid differentiation (Bad^-) *in vivo*. *In vitro* succinate effects were concentration dependent. At low concentration, succinate was utilized preferentially before glucose. At higher concentration, succinate decreased growth yield and induced bacteroid-like cell morphology in 15% of the cell population. These effects were observed for L5-30 but not for UR6 strain, suggesting that a functional TCA cycle is necessary for these *in vivo* and *in vitro* succinate effects.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Stages in the symbiotic process

Rhizobium is a genus of gram-negative soil bacteria that can infect and nodulate legumes. The establishment of a functional symbiosis is a multi-step process. Rhizobium motility and chemotaxis may be important in interstrain competition for nodule sites on the root (5). Plant root hairs are deformed early in the infection process by unknown substances made by the bacteria (110). After recognition and attachment of the rhizobial symbiont to root hairs (28,26) the bacteria specifically infect the host cells, presumably by enzymatic degradation of the root hair cell wall (19). A tubular infection thread confines the invading bacteria as they enter the host cell (24). The infectibility of legume root hairs is transient and maturing root hairs are important infection points in clover while in soybean, cowpea or alfalfa infections occur more frequently in newly emergent root hairs (13). The infection thread branches at the base of the root hair, penetrates the outer cortex of the root and stimulate the proliferation and enlargement of the inner cortex cells (66). This results in the formation of differentiated nodule tissue infected intracellularly by rhizobia released from the infection threads and surrounded by a peribacteroid membrane of plant origin. The bacteria

then differentiate into bacteroids and fix nitrogen (transform atmospheric nitrogen into ammonia) which is excreted into the cytosol of the host cells to be assimilated by the plant. In this symbiosis the bacteria receives photosyntates from the plant (24). The phenotypic code used for the different stages of this developmental process are: root colonization (Roc), attachment to root (Roa), root hair curling (Hac), infection thread formation (Inf), nodule initiation (Noi), bacterial release (Bar), bacteroid differentiation (Bad), nitrogen fixation (Nif), and nodule persistence (Nop) (108).

Rhizobium - lectin interaction

Lectins are sugar-binding proteins or glycoproteins of non-immune origin which agglutinate cells and/or precipitate glycoconjugates (51). It has been hypothesized that lectins are regulatory molecules, being receptors for a signal molecule that then elicits a response in different systems (28,73,76,79,82). Trifoliin A is a clover lectin which specifically binds to Rhizobium trifolii (33). This glycoprotein contains approximately 6 mol reducing sugar/mg protein, has a subunit molecular weight of 53,000 and an isoelectric point of 7.3 (33). The interaction between lectin and sugars has been studied by different methods including agglutination of bacteria (90), affinity chromatography (78), and binding to radioactively labeled saccharides (74). Trifoliin A agglutinates R. trifolii by interacting with the capsular polysaccharide (CPS) (25) and

lipopolysaccharide (LPS) (58). 2-deoxy-D-glucose and the - isomer of quinovosamine are haptens (27,58) of this lectin.

Rhizobium polysaccharides that bind the clover lectin

Isolation, purification and characterization of Rhizobium polysaccharides have recently been reviewed (20,42). There are several published structures of acidic heteropolysaccharides and homoglucans secreted by strains of Rhizobium trifolii and other rhizobia. Complete structures of Rhizobium lipopolysaccharies are not yet known, mostly because of their exceedingly complex sugar composition. The first structure proposed for a rhizobial acidic extracellular polysaccharide (EPS) was a repeating octasaccharide of R. meliloti (60). Subsequently, saccharide structures for EPS of R. trifolii U226 and R. trifolii 0403 were proposed (61,81). The same saccharide structure was also proposed for an EPS from two strains of R. leguminosarum and R. trifolii NA-30 (81), and also for one strain of R. phaseoli (43). A slightly different saccharide structure has been proposed for an EPS of R. trifolii 4S (4). Glycosyl composition and non-carbohydrate substitutions of R. trifolii 0403 oligosaccharide repeating unit obtained by cleavage of CPS with a polysaccharide depolymerase system has recently been reported (56, R. Hollingsworth, manuscript in preparation). CPS and EPS of plate-grown cultures of R. trifolii 0403 are not identical since they differ in degree of non-carbohydrate substitution (2).

Rhizobium polysaccharides have different non-carbohydrate substitutions including pyruvate (41), acetate (41), 3-hydroxybutanoate (55), methyl (74), and succinate (54). These substitutions can change with culture age thereby changing the ability to be bound by lectins. The methylation of galactosyl residues of *R. japonicum* CPS during stationary phase of growth results in a reduction of its interaction with the soybean lectin (74). The levels of non-carbohydrate substitutions of the CPS of *R. trifolii* 0403 change with culture age and the lectin binding ability corresponds to these changes (2,91). The neutral glycosyl composition does not vary with culture age (91). Binding of trifoliin A to *R. trifolii* LPS is also culture age-dependent, being optimal in early stationary phase (58). A compound which increased when the LPS was able to bind trifoliin A was 2-amino-2,6-dideoxyglycose (quinovosamine) (58) . Pyruvate and acetate substitutions in the EPS of *R. trifolii* L158 and other fast-growing rhizobia have also been shown to change with culture age (18). These culture age-dependent changes in the ability to bind the host lectin have been shown for *R. japonicum* (74), *R. leguminosarum* (107), and *R. trifolii* (32,58).

The *Rhizobium*-lectin interaction can also be affected at the plant level (by addition of fixed nitrogen to the plant growth media), and by interactions between the plant and bacteria (12,31).

LPS of R. trifolii 0403 (58) activates a host response in white clover seedlings which triggers root hair infection (29). At 1 μg/seedling, these lectin-binding polymers bind to clover root hair tips and significantly enhance infection thread formation by R. trifolii 0403 when applied to seedlings. At 10 μg/seedling, the LPS suppressed root hair infection (29). LPS from a serologically unrelated strain of R. trifolii (strain 2S-2) also stimulated root hair infection of white clover by R. trifolii 0403 at 1 μg/seedling, whereas no stimulation of infection was obtained by pretreatment with LPS from R. meliloti F28, or from E. coli. CPS from R. trifolii 0403 at 2.5 μg/seedling stimulated the formation of infection threads in white clover using a similar assay, and inhibited formation of infection threads in clover root hairs at higher concentrations (2). The EPS from the same culture of R. trifolii 0403 which was unable to bind the lectin did not display this biological activity on the infection process (2). Cyclic β(1-2)glucan from the periplasmic space of R. trifolii 4S also was able to increase the number of infections (1).

Attachment to clover root hairs

The time-course and the orientation of attachment of R. trifolii to root hairs on white clover seedlings inoculated with encapsulated bacteria which bind trifoliin A uniformly has been examined. Specific reversible interactions involving trifoliin A (Phase I) were followed by

irreversible interactions involving extracellular microfibrils (Phase II). A similar sequence of reversible and irreversible phases of attachment of Agrobacterium tumefaciens to plant cells has been proposed (70).

Phase I attachment can be subdivided into 3 steps (30). Phase 1A is the clumping of cells in random orientation at root hair tips. Phase 1B involves erosion of the capsule of unattached cells by enzymes in root exudate. 1C attachment is initially randomly oriented and then predominantly polar. Granular, electron-dense aggregates and trifoliin A can be detected at the interface between the bacteria and the root hair surface in 1A and 1C attachments.

The enzyme(s) in clover root exudate which alter the capsular polysaccharide of R. trifolii during Phase 1B are antigenically unrelated to trifoliin A (31). Immunoelectrophoresis studies (31) suggest that the capsular polysaccharides of R. trifolii are cleaved into smaller, dialyzable fragments. Bhuvaneswari and Solheim (14) have proposed that these enzyme-mediated cleavages result in oligosaccharide products which induce clover root hair branching.

Within hours R. trifolii displays a pattern of attachment combining 1A + 1C attachments on the same root hair. This attachment pattern is symbiont-specific and 2-deoxy-D-glucose inhibitable, and is present on approximately 93% of the infected root hairs examined 4 days after inoculation with R. trifolii 0403 (30). This orientation may

provide the optimal distribution of bacteria for marked curling and successful infection of the root hair (44).

The hapten 2-deoxy-D-glucose can inhibit the attachment of trifoliin A-binding R. trifolii cells to clover root hairs (27,30,112). Cells remain attached to clover root hairs when exposed to the shear forces of high-speed vortexing after 12h of incubation, what suggested a Phase II of firm attachment. At Phase II, extracellular microfibrils associated with the bacteria attached to the root hair surface can be seen by SEM. These are probably bundles of cellulose and/or fimbriae. Host-specific attachment has been demonstrated in R. trifolii - clover, R. japonicum - soybean, and R. leguminosarum - pea root systems reviewed in (28).

Rhizobium symbiotic genes

The development of a nitrogen-fixing Rhizobium-legume symbiosis is a multi-step process that requires bacterial and plant gene products. Early genetic evidence showed that the chromosomes of R. trifolii, R. leguminosarum, and R. phaseoli were essentially identical when in a series of genetic crosses the chromosome of R. leguminosarum was replaced section by section by the corresponding part of the genome from either R. phaseoli or R. trifolii (16) and in no case was any of the interspecific recombinants altered in its host range. Several reports have shown that many of the bacterial symbiotic genes of fast-growing Rhizobium species are plasmid-borne and are located in one plasmid called for

that reason sym plasmid which spans approximately 30 kilobase pairs (kb)(69). Root hair curling (hac), nodulation (nod) (7,11,15,21,38,39,40,52,53,62,64,69,84,88,113) and nitrogen fixation (nif and fix) genes (17,22,48,72,77,80,86,97,111) have been located in this megaplasmid.

Roa genes for root hair attachment of R. trifolii to clover root hairs are also encoded on the sym plasmid (112) and the genetic information required for trifoliin A-binding, "Phase I" attachment, marked root hair curling (Shepherd crooks), root hair penetration and infection thread formation was expressed in pTi-cured A. tumefaciens containing the sym plasmid of R. trifolii 5035 (57, F. B. Dazzo, G. L. Truchet, and P. J. Hooykaas, Abstr. Annu Meet. Am. Soc. Microbiol. 1983, K9, p. 178).

In R. meliloti, nod genes are organized in two clusters. One appears to encode for conserved nodulation functions and the other affects host specificity (64). Other genetic models of nodulation also proposed that in R. leguminosarum, there is a core of conserved nodulation-specific genes and that the host range is determined by ancillary genes which are very closely linked (39). Three nodulation regions have been identified within a 14 kb Hind III fragment of the R. trifolii sym plasmid. Tn5 mutants in region I (Hac) are $Hac^{-}Nod^{-}$ whereas mutants in region II (Superhac) are Hac^{++} and induce delayed and fewer nodules. Mutants in region III (Hsp) acquired the ability to nodulate

peas and lost the ability to nodulate white clover efficiently (M. A. Djordjevic et al., Mol. Gen. Genet., in press). Functional conservation of nod genes involved in root hair curling has been demonstrated in several species (37,64) and similarity at the DNA sequence level has also been shown (85,100). DNA sequence and complementation data have allowed the identification of four common nod genes (nod A, B, C, and D) in R. meliloti, R. leguminosarum and R. trifolii (37,39,64,85,100, and T. T. Egelhoff et al., in press).

Mutant strains altered in the levels of polysaccharide synthesis and defective in nitrogen fixation have also been described (7,89). The reduction in EPS synthesis by mutants of R. japonicum (68) and R. leguminosarum (75) resulted in decreased infectibility and nodulation of their respective host plants. Genes necessary for EPS synthesis in R. trifolii have been cloned and the cloned DNA was able to restore both the ability to fix nitrogen and to synthesize EPS to a Fix^- EPS^- mutant strain (89). It was not reported at which level the polysaccharide synthesis was blocked nor the location of the genes involved. Recently, EPS^- mutant strains of R. meliloti have been described in which the ability to induce nodules and infection thread formation was uncoupled (45). The nature of the mutation causing this phenotype was unknown and so was the location of the affected genes. In order to test if there is a direct or indirect relationship between the mutations and the

symbiotic phenotypes, more knowledge is necessary about the biosynthetic pathway of these polymers. One must establish at which step the polysaccharide synthesis is affected in the different mutant strains as well as on the identification of the genes coding for these functions.

Rhizobium mutant strains defective in organic acids uptake and metabolism are ineffective in nitrogen-fixing symbiosis with the host plant (47,49,83). Succinate and malate are abundant organic acids within legume root nodules (35,63,94) and succinate is transported and metabolized by free-living bacteria (46,50,71,83) and bacteroids (9,50,87,93) of different Rhizobium species. Organic acids support the highest rate of oxygen respiration by bacteroid suspensions (103) and succinate is a very effective substrate for supporting nitrogen fixation by both free-living Rhizobium species (10,109) and bacteroids (101). Utilization of tricarboxylic acid intermediates are related to symbiotic effectiveness (6). "In vitro" induction of bacteroid - like cell morphology by succinate in R. trifolii (104,105,106) and induction of sphere to rod morphogenesis in Arthrobacter crystallopoietes (65) by succinate have been described.

Polysaccharide synthesis

Microbial exopolysaccharide synthesis has recently been reviewed (95). Certain physiological conditions favor exopolysaccharide synthesis. For many microorganims, nutrient imbalance in the presence of large amounts of

utilizable carbohydrate leads to increased polysaccharide production. Suboptimal incubation temperatures may also promote polysaccharide synthesis.

In synthesizing exopolysaccharide, the water-soluble monosaccharide units have to be converted into high energy molecules and then must be passed through the lipophilic cell membrane and assembled into polysaccharide chains. These chains will form a capsule or slime at the cell surface. Monosaccharides are activated through their incorporation into nucleoside diphosphate sugars and are transferred to lipid-linked derivatives, a process that leads to the assembly of oligosaccharides in a form soluble in organic solvent. Membrane-bound enzymes transfer a sugar 1-phosphate to the isoprenoid lipid phosphate acceptor. The key lipid carrier is a C55-polyisoprenoid called bactoprenol (67). Subsequently, further sugars are transferred from sugar nucleotides to form the oligosaccharide repeating units of the polymer. The lipid-linked oligosaccharides are polymerized in a block fashion to form the polysaccharide. Eventually, the polysaccharide is released from the bactoprenol and the lipid pyrophosphate converted back to the lipid phosphate. The biosynthesis of exopolysaccharide or capsular polysaccharide of different genera of bacteria including Klebsiella aerogenes (96), Xanthomonas campestris (59), Acetobacter xylinum (23), and Aerobacter aerogenes (102) have been studied and shown to involve lipid-bound oligosaccharide intermediates as precursors of the

polysaccharide. Synthesis of (1->2) β-glucan from UDP-[^{14}C] glucose by enzyme preparations of Rhizobium japonicum (34) and Rhizobium phaseoli (3) have been reported. Lipid-bound saccharides formed by incubation of UDP-glucose with a particulate enzyme of Rhizobium meliloti were also studied (98,99).

Pyruvic acid ketal residues are present in many bacterial polysaccharides (95). However, there are very few reports (59) on the in vitro pyruvylation of the polysaccharides, and none for Rhizobium polysaccharides.

LIST OF REFERENCES

1. Abe, M. A. Amemura, and S. Higashi. 1982. Plant and Soil 64:315-324.

2. Abe, M., J. E. Sherwood, R. I. Hollingsworth, and F. B. Dazzo. 1984. J. Bacteriol. 160:517-520.

3. Amemura, A. 1984. Agric. Biol. Chem. 48(7): 1809-1817.

4. Amemura, A., T. Harada, M. Abe, and S. Higashi. 1983. Carbohydr. Res. 115:165-174.

5. Ames, P., and K. Bergman, 1981. J. Bacteriol. 148:728-729.

6. Antoun, H., L. M. Bordeleau, and R. Sauvageau. 1984. Plant and Soil 77:29-38.

7. Arias, A., A. Gardiol, and G. Martinez-Drets. 1982. J. Bacteriol. 151:1069-1072.

8. Banfalvi, Z., Sakanyan, C. Koncz, A. Kiss, I. Dusha, and A. Kondorosi. 1981. Mol. Gen. Genet. 184:318-325.

9. Bergersen, F. J. 1977. p. 519-555. In R. W. F. Hardy and W. S. Silver (ed.), A treatise on dinitrogen fixation, section III. John Wiley and Sons, New York.

10. Bergersen, F. J., G. L. Turner, A. H. Gibson, and W. F. Dudman. 1976. Biochim. Biophys. Acta 444:164-174.

11. Beynon, J. L., Beringer, J. E., and Johnston, A. W. B. 1980. J. Gen. Microbiol. 120:421-429.

12. Bhuvaneswari, T. V., and W. D. Bauer. 1978. Plant Physiol. 62:71-74.

13. Bhuvaneswari, T. V., A. A. Bhagwat, and W. D. Bauer. 1981. Plant Physiol. 68:114-1149.

14. Bhuvaneswari T. V. and B. Solheim. 1985. Physiol. Plant. 63, 25-34.

15. Brewin, N. J., Beringer, J. E., Buchanan - Wollaston, A. V., Johnston, A. W. B., and Hirsch, P. R. 1980. J. Gen. Microbiol. 116:261-270.

16. Brewin, N. J., J. L. Beynon, and A. W. B. Johnston. 1981. p. 65-77. In J. M. Lyons, R. C. Valentine, D. A. Phillips, D. W. Rains and R. C. Huftaker (ed.), Genetic Engineering of Symbiotic Nitrogen Fixation and Conservation of Fixed Nitrogen. Plenum Press. New York and London.

17. Buikema, W. J., S. R. Long, S. E. Brown, R. C. van den Bos, C. Earl, and F. M. Ausubel. 1983. J. Mol. Appl. Genet. 2:249-260.

18. Cadmus, M. C., K. A. Burton, and M. E. Slodki. 1982. Appl. Environ. Microbiol. 44:242-245.

19. Callaham, D. A. and J. G. Torrey. 1981. Can. J. Bot. 59:1647-1664.

20. Carlson, R. W. 1982. In W. Broughton (ed), Nitrogen Fixation. II Rhizobium, Oxford University Press, UK.

21. Christensen, A. H., and K. R. Schubert. 1983. J. Bacteriol. 156:592-599.

22. Corbin, D., L. Barran, and G. Ditta. 1983. Proc. Natl. Acad. Sci. U.S.A. 80:3005-3007.

23. Couso, R. O., L. Ielpi, R. C. Garcia, and M. A. Dankert. Eur. J. Biochem. 1982. 123:617-627.

24. Dart, P. 1977. pp. 367-472. In A treatise on nitrogen fixation. R. W. F. Hardy and W. S. Silver, eds. J. Wiley and Sons, New York.

25. Dazzo, F. B. 1976. Appl. Environ. Microbiol. 32, 166-177.

26. Dazzo, F. B. 1984. p. 85-93. In K. C. Marshall (ed.), Microbial Adhesion and Aggregation. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.

27. Dazzo, F. B., C. A. Napoli, and D. H. Hubbell. 1976. Appl. Environ. Microbiol. 32:166-171.

28. Dazzo, F. B., and G. L. Truchet. 1983. J. Membrane Biol. 73:1-16.

29. Dazzo, F. B., G. Truchet, and E. Hrabak. 1984. In C. Veeger and W. E. Newton, (eds.), Adv. in Nitrogen Fixation Res., Martinus Nijhoff/Dr W. Junk Pub., The Hague, p. 413.

30. Dazzo, F. B., G. L. Truchet, J. E. Sherwood, E. M. Hrabak, M. Abe, and S. H. Pankratz. 1984. Appl. Environ. Microbiol. 48:1140-1150.

31. Dazzo, F. B., G. L. Truchet, J. E. Sherwood, E. M. Hrabak, and A. E. Gardiol. 1982. Appl. Environ. Microbiol. 44:478-490.

32. Dazzo, F. B., M. R. Urbano, and W. J. Brill. 1979. Curr. Microbiol. 2:15-20.

33. Dazzo, F. B., W. E. Yanke, and W. J. Brill. 1978. Biochim. Biophys. Acta 539:276-286.

34. Dedonder,R. A. and W. Z. Hassid, 1964. Biochim. Biophys. Acta 90:239-248.

35. de Vries, G. E., P. In´t Veld, and J. W. Kijne. 1980. Plant Sci. Lett. 20:115-123.

36. de Vries G. E., A. A. N. van Brussel, and A. Quispel. 1982. J. Bacteriol. 149:872-879.

37. Djordjevic, M.A., P.R. Schofield, R. W. Ridge, N. A. Morrison, B. J. Bassam, J. Plazinski, J. M. Watson, and B. G. Rolfe. 1985. Plant Molec. Biol. 4:147-160.

38. Downie, J. A., G. Hombrecher, Q-S. Ma, C. D. Knight, B. Wells, and A. W. B. Johnston. 1983. Mol Gen. Genet. 190:359-365.

39. Downie, J. A., C. D. Knight, A. W. B. Johnston, and L. Rossen. 1985. Mol. Gen. Genet. 198: 255-262.

40. Downie, J. A., Q.- S. Ma, C. D. Knight, G. Hombrecher, and A. W. B. Johnston. 1983. EMBO J. 2:947-952.

41. Dudman, W. F., and M.Heidelberger. 1969.Science 164:954-955.

42. Dudman, W. 1984. pp. 397-404. In C. Veegers & W. Newton (eds.), Advances in Nitrogen Fixation Research, Martinus Nijhoff/Junk, The Hague, Netherlands.

43. Dudman, W., L. Franzen, J. Darvill, M. Mcneil, A. Darvill, and P. Albersheim. 1983. Carbohydr. Res. 117:141-156.

44. Ervin, S. E. and D. H. Hubbell. 1985. Appl. Environ. Microbiol. 49,61-68.

45. Finan, T. M., A. M. Hirsch, J. A. Leigh, e. Johansen, G. Kuldau, S. Deegan, G. C. Walker, and E. R. Signer. 1985. Cell 40:869-877.

46. Finan, T. M., J. M. Wood, and D. C. Jordan. 1981. J. Bacteriol. 148:193-202.

47. Finan, T. M., J. M. Wood, and D. C. Jordan. 1983. J. Bacteriol. 154:1403-1413.

48. Forrai, T., E. Vincze, Z. Banfalvi, G. B. Kiss, G. S. Randhawa,and A. Kondorosi. 1983. J. Bacteriol. 153:635-643.

49. Gardiol, A., A. Arias, C. Cervenansky, and G. Martinez-Drets. 1982. J. Bacteriol. 151:1621-1623.

50. Glenn, A. R., P. S. Poole, and J. F. Hudman. 1980. J. Gen. Microbiol. 119:267-272.

51. Goldstein, I. J., R. C. Hughes, M. Monsigny, T. Osawa, and N. Sharon. 1980. Nature 285:66.

52. Higashi, S. 1967. J. Gen. Appl. Microbiol. 13:391-403.

53. Hirsch, P. R., van Montagu, M., Johnston, A. W. B., Brewin, N. J. and Schell, J. 1980. J. Gen. Microbiol. 120:403-412.

54. Hisamatsu, M., J. Abe, A. Amemura, and T. Harada. 1980. Agric. Biol. Chem. 44:1049-1055.

55. Hollingsworth, R. I., M. Abe, and F. B. Dazzo. 1984. Carbohydr. Res. 133:C7-C11.

56. Hollingsworth, R. I., M Abe, J. E. Sherwood, and F. B. Dazzo. 1984. J. Bacteriol. 160:510-516.

57. Hooykaas, P. J., A. A. N. van Brussel, H. den Hulk-Ras, G. M. S. van Slogteren, and R. A. Schilperoort. 1981. Nature.(London) 291:357-353.

58. Hrabak, E. M., M. R. Urbano, and F. B. Dazzo. 1981. J. Bacteriol. 148:697-711.

59. Ielpi, L., R. O. Couso, and M. A. Dankert. 1981. Biochem. Biophys. Res. Commun. 102:1400-1408.

60. Jansson, P., L. Kenne, B. Lindberg, I. Lonngren, V. Ruden, and S. Svensson. 1977. J. Amer. Chem. Soc. 99:3812-3815.

61. Jansson, P., B. Lindberg, H. Ljunngren. 1979. Carbohydr. Res. 75:207-220.

62. Johnston, A. W. B., Beynon, J. L., Buchanan - Wollaston, A. V., Setchell, S. M., Hirsch, P. R. and Beringer J. E. 1978. Nature 276:634-636.

63. Kawai, S., and S. Mori. 1984. Soil Sci. Plant Nutr. 30:261-266.

64. Kondorosi, E., Z. Banfalvi, and A. Kondorosi. 1984. Mol. Gen. Genet. 193:445-452.

65. Krulwich, T. A., and J. C. Ensign. 1969. J. Bacteriol. 97:526-534.

66. Law, I. J., Y. Yamamoto, A. J. Mort, and W. D. Bauer. 1982. Planta 154:100-109.

67. Lennarz, W. J. and H. G. Scher. 1972. Biochim. Biophys. Acta 265:417-441.

68. Libbenga, K. R., and P. A. A. Harkes, 1973. Planta 114:17-21.

69. Long, S. R., W. J. Buikema, and F. M. Ausubel. 1982. Nature (London) 298:485-488.

70. Matthysse, A. G. 1981. J. Bacteriol. 145, 583-595.

71. McAllister, C. F., and J. E. Lepo. 1983. J. Bacteriol. 153:1155-1162.

72. Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel. 1982. J. Bacteriol. 149:114-122.

73. Mellor, R. B., G. M. Gadd, P. Rowell, and W. D. P. Stewart. 1981. Biochem. Biophys. Res. Commun. 99:1348-1353.

74. Mort, A. J. and W. D. Bauer. 1980. Plant Physiol. 66: 158-163.

75. Napoli, C. and P. Albersheim. 1980. J. Bacteriol. 141:1454-1456.

76. Nordbring-Hertz, B., E. Friman, P. A. Johansson, and B. Mattiasson. 1981. pp. 43-50. In Lectins-biology, biochemistry, clinical biochemistry, Vol. 1. T. C. Bog-Hansen, ed. W. de Gruyter, Berlin.

77. Nuti, M. P., A. A. Lepidi,R. K. Prakash, R. A. Schilperoort, and F. C. Cannon, 1979. Nature. 282:533-535.

78. Oda, Y., K-L Kassai, and S-L Ishii. 1981. J. Biochem. 89:285-296.

79. Pistole, T. G. 1981. Ann. Rev. Microbiol. 35:85-112.

80. Prakash, R. K., R. A. Schilperoort, and M. P. Nuti, 1981. J. Bacteriol. 145:1129-1136.

81. Robertsen, B., P. Aman, A. Darvill, M. Mcneil, and P. Albersheim. 1981. Plant Physiol. 67:389-400.

82. Romeiro, R., A. Karr, and R. Goodman. 1981. Plant Physiol. 68:772-777.

83. Ronson, C. W., P. Lyttleton, and J. G. Robertson. 1981. Proc. Natl. Acad. Sci. USA. 78:4284-4288.

84. Rosenberg, C., P. Boistard, J. Denarie, and F. Casse-Delbart. 1981. Mol. Gen. Genet. 184:325-333.

85. Rossen, L., A. W. B. Johnston, J. A. Downie. 1984 Nucl. Acids Res. 12:9497-9508.

86. Ruvkun, G. B., and Ausubel, F. M. 1980. Proc. Nat. Acad. Sci. U.S.A. 77:191-195.

87. Saroso, S., A. R. Glenn, and M. J. Dilworth. 1984. J. Gen Microbiol. 130:1809-1814.

88. Schofield, P. R., M. A. Djordjevic, B. G. Rolfe, and J. Shine. 1983. Mol. Gen. Genet. 192:459-465.

89. Scott, K. F., J. E. Hughes, P. M. Gresshoff, J. E. Beringer, B. G. Rolfe, and J. Shine. 1982. J. Mol. Appl. Genet. 1:315-326.

90. Sequiera, L. and T. C. Graham. 1977. Physiol. Plant Pathol. 11:43-54.

91. Sherwood, J. E., J. M. Vasse, F. B. Dazzo, and G. L. Truchet. 1984. J. Bacteriol. 159:145-152.

92. Solheim, B. J. and K. E. Fjelheim. 1984. Physiol. Plant. 62, 11-17.

93. Stovall, I., and M. Cole. 1978. Plant Physiol. 61:787-790.

94. Stumpf, D. K., and R. H. Burris. 1979. Anal. Biochem. 95:311-315.

95. Sutherland, I. W. 1979. Trends Biochem. Sci. 4:55-59.

96. Sutherland, I. W., and Norval, M. (1970). Biochemical Journal 120:567-576.

97. Szeto, W. W., J. L. Zimmerman, V. Sundaresan, and F. M. Ausubel. 1984. Cell 36:1035-1043.

98. Tolmasky, M. E., R. J. Staneloni, and L. F. Leloir, 1982. J. Biol. Chem. 257:6751-6757.

99. Tolmasky, M. E., R. J. Staneloni, R. A. Ugalde, and L. F. Leloir. 1980. Arch. Biochem. Biophys. 203:358-364.

100. Torok, I., E. Kondorosi, T. Stepkowski, J. Posfai, and A. Kondorosi. 1984. Nucl. Acids Res. 12:9509-9524.

101. Trinchant, J. C., and J. Rigaud. 1979. L. Physiol. Veg. 17:547-556.

102. Troy, F. A., F. E. Frerman, and E. C. Heath. 1971. J. Biol. Chem. 246:118-133.

103. Tuzimura, K. and H. Meguro. 1960. J. Biochem. 47: 391-397.

104. Urban, J. E. 1979.. Appl. Environ. Microbiol. 38:1173-1178.

105. Urban, J. E., and D. B. Bechtel. 1984. Appl. Environ. Microbiol. 47:1178-1181.

106. Urban, J. E., and F. B. Dazzo. 1982. Appl. Environ. Microbiol. 44:219-226.

107. van der Schaal, I., J. Kijne, C. Diaz, and F. van Iren. 1984. p. 531-538. In Lectins, Vol III. T. C. Bog-Hansen and G. A. Spengler, eds., W. de Gruyter and Co., Berlin.

108. Vincent, J. M. 1980. p. 103-129. In W. E. Newton and W. H. Orme-Johnson (ed.), Nitrogen fixation, vol.2. Symbiotic associations and cyanobacteria. University Park Press, Baltimore.

109. Wilcockson, J., and D. Werner. 1979. Arch. Microbiol. 122:153-160.

110. Yao, P. Y. and J. M. Vincent. 1976. Plant and Soil 45:1-16.

111. Zimmerman, J. L., W. W. Szeto, and F. M. Ausubel. 1983. J. Bacteriol. 156:1025-1034.

112. Zurkowski, W. 1980. Microbios 7:27-32.

113. Zurkowski, W. and Lorkiewicz. 1979. Arch. Microbiol. 123:195-201.

CHAPTER I

SURFACE PROPERTIES WERE ALTERED IN R. TRIFOLII 0403 RIF WITH A TN5 INSERTION IN THE SYM PLASMID.

ABSTRACT

Rhizobium trifolii 0403 rif was mutagenized by transposon (Tn5) mutagenesis and the mutagenized pool was screened for mutant strains defective in root-nodule symbiosis with white clover. Eight hundred Kan^r Rif^r isolates were analyzed on the white clover host plant. Thirteen of these (1.6%) were symbiotically defective mutant strains. Three classes of mutant strains were obtained based on their symbiotic phenotype: unable to nodulate (Nod^-), defective in nitrogen fixation (Fix^-), and partially defective in nitrogen fixation ($Fix^{\pm}$).

Five strains were further studied: R. trifolii 43 Nod^-, R. trifolii 308 Nod^-, R. trifolii 738 Fix^-, R. trifolii 755 Fix^- and R. trifolii 251 $Fix^{\pm}$. Hybridization of total DNA restriction fragments with labeled Tn5 demonstrated the presence of a single Tn5 insertion in all the strains. Strains 251, 738 and 755 showed the same plasmid pattern as

the wild type strain, whereas strains 43 and 308 showed a deletion in the sym plasmid. Microscopic studies showed that strain 251 attached better and strains 43 and 308 attached less to clover root hairs than did the wild type strain. Strain 251 induced a similar number of clover root hair deformations and infection threads as did the wild type strain, whereas strains 308 and 43 were unable to deform or infect white clover root hairs. Quantitative agglutination assays with trifoliin A showed that strain 251 had a higher titer and strains 308 and 43 had lower titers than the wild type strain. Tn5 was located in the sym plasmid of strain 251 and presence of Mu DNA was not detected in this strain. Depolymerization of 251 CPS by a β-lyase enzyme was slower than that of wild type CPS, and isolated oligosaccharide products from strain 251 CPS had less acetic and more pyruvic acid substitutions than wild type oligosaccharides.

These results indicate that CPS non-carbohydrate substitutions are important in R. trifolii 0403 rif attachment to clover root hairs and lectin binding ability.

INTRODUCTION

The *Rhizobium trifolii* - clover symbiosis involves a complex sequence of interactions (54) leading to the formation of a root nodule that fixes nitrogen. Several reports have shown that many of the bacterial symbiotic genes of fast-growing *Rhizobium* species are plasmid-borne and are located in a region of the *sym* plasmid which spans approximately 30 kilobase pairs (kb) (41). Genes encoding root hair curling (*hac*), nodulation (*nod*) (2, 6, 7, 10, 19, 20, 21, 28, 30, 35, 39, 40, 47, 49, 60) and nitrogen fixation (*nif* and *fix*) genes (8, 11, 26, 41, 42, 45, 48, 53, 58) have been located in this megaplasmid.

Studies of the *R. trifolii* - clover symbiosis (13) indicate that the specific ("Phase I") attachment process of the bacteria to root hairs is initiated by an interaction between the lectin, trifoliin A, associated with the root hair surface and carbohydrate receptors on the bacterial symbiont. *Roa* genes for root hair attachment of *R. trifolii* to clover root hairs are also encoded on the *sym* plasmid (59) and the genetic information required for trifoliin A-binding, "Phase I" attachment, marked root hair curling (shepherd crooks), root hair penetration and infection thread formation was expressed in pTi-cured *A. tumefaciens* containing the *sym* plasmid of *R. trifolii* 5035 (32, F. B.

Dazzo, G. L. Truchet, and P. J. Hooykaas, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K9, p. 178.). It has been hypothesized that the specific attachment of different Rhizobium species to host root hairs results from the interaction between the lectin on the host roots and Rhizobium CPS (15, 37, 44, 50, 52).

Characterization of symbiotically defective mutant strains would be useful to identify the biochemical events leading to successful nodulation. In the present study, three different classes of mutant strains of R. trifolii 0403 rif were obtained after Tn5 mutagenesis, based on their symbiotic phenotype with the white clover host plant. This wild type strain was selected because of the available information on its surface properties and its documented interaction with clover root hairs (16, 17). We used transposon (Tn5) mutagenesis as has been done for other Rhizobium species (4, 41), since Tn5 produces random insertion events at high frequency tagging the DNA lesion physically and genetically with a selectable resistance marker (3, 36). Once inserted, Tn5 has very low frequencies of transposition to a new site and of precise excision (41). These characteristics have made Tn5 the most widely used transposon in gene manipulations in different bacteria. We describe the isolation, symbiotic properties, and biochemical characterization of symbiotically defective mutant strains of R. trifolii 0403 rif.

A preliminary report of this work was presented at the Second International Symposium on the Molecular Genetics of the Bacteria-Plant Interaction, Cornell University, Ithaca, New York, June 1984.

MATERIALS AND METHODS

Bacterial strains and phages. The following bacterial strains and bacteriophages were kindly provided to us. R. trifolii 0403 and E. coli 1830 nal pro met (pJB4JI) were obtained from J. Beringer , Rothamsted Experimental Station, Harpenden, United Kingdom. R. trifolii 0403 rif was obtained from K. Nadler, Michigan State University, East Lansing. R. trifolii 4S and bacteriophage 4S were from M. Abe and S. Higashi, Kagoshima University, Kagoshima, Japan. Bacteriophage λ::Tn5 used for the Tn5 probe was from A. Christensen, Michigan State University, East Lansing. E. coli HM8305 F' pro^+ lac z 8305 :: Mu cts 62/ (pro - lac) his met tyr str was obtained from M. Howe, University of Wisconsin, Madison.

Media and growth conditions. R. trifolii strains were grown on either minimal Y (4), minimal BIII (12) or rich (38) media. Minimal Y contained succinate (YS) or glucose (YG) as C source. KNO_3 was used as nitrogen source instead of sodium glutamate for growth studies on single carbon sources. Rifampicin (R) (20 μg/ml) or Kanamycin (K) (80 μg/ml) were added to YS or YG when indicated (YSRK, YGRK). R. trifolii strains were kept on BIII slants and R. trifolii Tn5 mutant strains were kept on YGRK slants or in stocks of YGRK-grown cells brought to 15% glycerol and kept

at -80°C in screw capped vials. E. coli 1830 was grown on rich TY medium (4) and E. coli HM8305 was grown on minimal Ozeki (43) or rich SB (33) media.

Transposon (Tn5) mutagenesis. Escherichia coli 1830 nal pro met carrying the suicide plasmid pJB4JI (pPH1JI::Mu::Tn5) constructed by Beringer et al. (5) was used. E. coli 1830 was grown on TY broth to stationary phase and R. trifolii 0403 rif on BIII slants for 5 days. Mating was performed by mixing 1.2 x 10^{10} cells of donor with 5 x 10^{9} cells of recipient. The cell suspensions were pipetted onto 0.45 μm filters (Type HAWP; Millipore Corp., Bedford, Mass.). The filter was washed with water and incubated at 28°C on TY plates for 5h. Cells were resuspended in water, plated on selective YSRK medium, and incubated at 28°C. Transfer frequencies were calculated per number of recipient cells after the cross. Transconjugants were restreaked twice on YSRK to obtain single colonies.

Screening for symbiotically defective mutant strains. Surface-sterilized seeds (12) of Trifolium repens var. Ladino were germinated into humid air for 48h and transferred to the surface of (0.8%) agar slopes containing Fahraeus (N-free) medium (12). 5 day-old cultures of Rhizobium strains on BIII agar slants were suspended in Fahraeus medium and 5 x 10^{6} cells were inoculated per seedling. The tubes were incubated in a plant growth chamber with a 14h photoperiod at 22°C (26,900 lux) and 10h darkness at 20°C. Roots were scored for nodulation every week and

plants were assayed for nitrogen fixation by the acetylene reduction technique (12) after 6 weeks.

Rhizobium DNA isolation. Cells grown in Y broth were harvested and lysed. Cells were suspended and incubated at 0°C for 30 min in 50 mM EDTA-Tris (pH 8.0) containing lysozyme (Sigma Chemical Co.) (1 mg/ml). The solution was brought to 0.1% SDS, 100 mM EDTA, and 1 mg/ml of proteinase K (Sigma Chemical Co.), and incubated at 50°C for 30 min. The lysate was extracted with the same volume of water-saturated phenol adjusted to pH 7.0 with 1 M Tris-HCl (pH 8.0) buffer. Aqueous phase was brought to 0.3 M sodium acetate (pH 7.0) and DNA was precipitated with 2 volumes of ethanol. The precipitate was wound out with a glass micropipette, dissolved in 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, digested with RNAse (Type 1-A; Sigma Chemical Co.) (50 μg/ml) for 30 min at 37°C, extracted once with chloroform, and then ethanol-precipitated. The DNA was dissolved in 50 mM Tris-HCl (pH 7.0) containing 1 mM EDTA and stored at 4°C. The DNA concentration was determined by absorbance at 260 nm.

Restriction endonucleases and DNA hybridization. Restriction endonucleases were purchased from Bethesda Research Laboratories, Rockville, Md. and used as recommended by the manufacturer. DNA was digested for 8h with Eco RI or Hind III and separated by electrophoresis in submerged horizontal 0.7% agarose gel in 40 mM Tris buffer (pH 7.5) containing 20 mM sodium acetate and 1 mM EDTA at

150 mA for 4h. The gel was treated with 0.4 M NaOH, 0.8 M NaCl for 30 min to denature the DNA, and neutralized with 0.5 M Tris-HCl (pH 7.5) containing 1.5 M NaCl. DNA transfer to nitrocellulose filters (Scheicher & Schuell) was performed for 24h with 10 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as described by Southern (51). Filters were rinsed with 5 x SSC, air dried and vacuum baked for 3h at 80°C. The DNA probe was obtained by labeling the DNA with ^{32}P by the nick translation method (46). Hybridization and washing of the filters was performed as described by Engel and Dodgson (24). Autoradiography was performed for 8h at -80°C using XAR-5 X-ray film (Eastman-Kodak, Rochester, N. Y., USA) with a Cronex Lightning-Plus intensifying screen (Du Pont, Co., Wilmington, Del.).

Plasmid profile analysis and location of Tn5. Agarose gel electrophoresis was performed as described by Eckhardt (23) with slight modifications. Cultures were grown on Y broth to exponential phase (50 Klett units in a Klett-Summerson colorimeter with a No. 66 filter). Approximately 2 x 10^8 cells were pelleted for 5 min with an Eppendorf microfuge and the supernatant was discarded. Cells were washed with 1 ml of 0.1% sodium sarkosylate in 80 mM Tris-HCl (pH 8.0) containing 20 mM EDTA (A. Christensen, personal communication) and resuspended in 40 µl of the lysozyme (Sigma Chemical Co.) mixture for Gram negative bacteria (23). After 10 min incubation at room temperature, the lysates were added to the wells. 40 µl of the SDS mixture

were carefully layered on top of the bacteria-lysozyme mixture, and the slots were then filled with the overlay mixture and sealed with 0.7% agarose gel as described (23). 0.7% vertical agarose gels were run in a buffer containing 89 mM each Tris-boric acid (pH 8.2) at 8 mA for 1h and then at 28 mA for 14h. Gels were stained for 30 min with ethidium bromide (1 μg/ml) in the same electrophoresis buffer, rinsed with distilled water for 10 min, and the DNA was visualized with a U.V. transilluminator and photographed. Agrobacterium tumefaciens C58 harboring three plasmids of known molecular weights: RP4 36 Md, Ti 130 Md, and pAtt58 214 Md was used for plasmid standard sizes. Plasmid DNA on Eckhardt agarose gels was partially depurinated by treatment with 0.25 M HCl for 15 min (55), and then was denatured, neutralized, transferred to nitrocellulose filters and hybridized to ^{32}P λ::Tn5 as described for restriction endonuclease digested DNA. Autoradiography was performed for 24h.

Root hair interactions. (A) Attachment. Slide culture assay. Bacterial attachment to root hairs on primary seedling roots was examined by a previously described assay (12) in hydroponic, modified Fahraeus slide cultures without agar (25). 4 replicate seedlings of Louisiana Nolin var. of white clover were used. Rhizobium strains were grown for 5 days on BIII agar plates, centrifuged twice in 10 mM K phosphate buffer (pH 7.0) with 0.15 M NaCl (PBS) at 6,000 x g and resuspended in Fahraeus medium. 2 x 10^7 bacteria were inoculated per seedling. After incubation in a plant growth

chamber for 12h, the slide cultures were disassambled. The roots were rinsed gently with a stream of Fahraeus medium while still on the slides, then covered with a glass cover slip and examined by phase contrast microscopy at 500 x along the optical median planes of the root. 15-20 root hairs (ca. 200 µm in length) were counted per treatment. For orientation of attachment studies, 50 root hairs along the optical median planes were examined per strain 4h after inoculation of 4 x 10^7 cells per seedling in modified Fahraeus slide cultures. 1A attachments represent randomly oriented cells clumped to root hair tips and 1C attachments represent single cells polarly attached to root hairs. Beaker assay. Approximately 10 root hairs (ca. 200 µm in length) were examined per strain after incubation of the seedlings for 2h in a cell suspension containing 10^6 cells/ml with slow shaking at 28°C. Seedlings were rinsed and observed as described before. (B) Deformation and infection studies. 10 replicate seedlings of T. repens var. Louisiana Nolin were inoculated with 5 x 10^6 cells per seedling in Fahraeus slide cultures and incubated for 4 days. Seedlings were examined as described under (A) and the number of roots hairs with marked deformations (shepherd crooks) and of infected root hairs along the entire seedling root were counted.

Protein determination. Protein concentration of lectin preparations was measured by the Bio-Rad protein assay.

Bacterial agglutination assay. Harvesting and washing of cells, removal of nondispersible flocs, and quantitative bacterial agglutination assays were performed as described (14) with modifications by J. Sherwood (personal communication). Cells grown for 5d on BIII agar were removed from the plates with PBS and centrifuged at 12,000 x g for 10 min. The cell pellets were washed twice, resuspended in M buffer (pH 7.0) (0.2 mM K phosphate buffer with 0.15 M NaCl, 0.5 mM $CaCl_2$, 0.15 mM $MnCl_2$, and 0.5 mM $MgSO_4$), passed through glass wool in a Pasteur pipette to remove cell aggregates, and adjusted to 3 x 10^8 cells/ml (30 Klett units in a Klett-Summerson colorimeter with a No. 66 filter). Cell suspensions (25 μl) were added to a two-fold dilution series of the lectin in M buffer (25 μl) in U-shaped polyvinylchloride microtiter plates (Dynatech Laboratories, Alexandria, VA.). Plates were sealed and incubated at room temperature for 14h. The maximum titer achieved without autoagglutination was determined by hourly examination of the plates. Trifoliin A used was kindly provided by J. Sherwood and corresponded to the active fraction after the DEAE-Sephadex purification step of trifoliin A (J. Sherwood, personal communication).

Purification of phage Mu and preparation of Mu DNA probe. Mu lysate preparation and phage purification were performed essentially as described (27). E. coli HM8305 (Mu lysogen) was grown at 32°C in 4 flasks with 250 ml of SBM medium (34) to 4 x 10^8 cells/ml. Phage growth was heat-

induced by addition of an equal volume of 58°C medium and growth was continued at 42°C for 45 min. The culture was quickly centrifuged by allowing the rotor to accelerate to 4000 x g and then braking. The cell pellets were resuspended in one-twentieth vol. of 37°C SBM and slowly shaken at 37°C for 4h. The Mu lysate was centrifuged to remove debris. 22 ml of lysate ($1x10^{11}$ PFU/ml) were layered per ultracentrifuge tube over a cesium chloride step gradient consisting of 7 ml of 1.4 g/ml CsCl, 6 ml of 1.5 g/ml CsCl, and 3 ml of 1.7 g/ml CsCl, and centrifuged at 20,000 rpm (52,200 x g) in an SW27 rotor for 2h at 4°C. The resulting phage bands located at approximately 1.47 g/ml were pooled, approximately adjusted by addition of CsCl to a density of 1.50 g/ml and centrifuged to equilibrium at 22,000 rpm (62,700 x g) in an SW27 rotor at 4°C for 48h. Presence of the phage in each step was confirmed by negative staining with neutralized phosphotungstic acid followed by transmission electron microscopy using a Philips 300 TEM. Purified phage was dialyzed against 0.1 M Tris-HCl (pH 7.9) containing 0.3 M NaCl and 2.5 mM $MgCl_2$. DNA was isolated from phage particles by phenol extraction (56) and labeled with ^{32}P by the nick translation method (46). Hybridization, washing of the filters and autoradiography were performed as described above.

Nodulation and host range studies. Seedlings of Medicago sativa var. Veronal, Trifolium subterraneum var. Clare, Trifolium pratense, Trifolium fragiferum and

Trifolium repens vars. Ladino, Louisiana Nolin and White Dutch were inoculated with 2 x 10^7 cells and incubated under the same conditions described for screening of mutant strains. In addition, surface-sterilized seeds of Phaseolus vulgaris var. Black Turtle Soup and line 21-58 (from F. Bliss, Univ. of Wisconsin) and of Pisum sativum lines 8221 and 9888 F were germinated into humid air for 3 days, planted on the surface of Smucker (A. Smucker, personal communication) or Fahraeus agar medium, respectively, and inoculated with 10^8 cells per seedling. All plants were incubated under the same conditions described for screening of mutant strains and scored for nodulation every week.

Isolation of polysaccharide depolymerase PD-I. The β-lyase enzyme was isolated from the clarified phage lysate of R. trifolii 4S by precipitation with $(NH_4)_2SO_4$ (70% of saturation) and purified by DEAE-cellulose (DE52 Whatman, Inc., Clifton, N. J.) column chromatography (29).

Oligosaccharide isolation. BIII plates were inoculated with a suspension of 10^7 cells previously grown for 5 days on BIII agar medium. Cells grown for 5 days were harvested by centrifugation in PBS (pH 7.2). CPS was extracted from pelleted cells with PBS (pH 7.2) containing 0.5 M NaCl, then precipitated with 2 vol. of cold ethanol, centrifuged, redissolved in water, dialyzed against water, and lyophilized. CPS was depolymerized with polysaccharide depolymerase PD-I and the oligosaccharide products were purified by gel filtration chromatography through Bio-Gel

P10 (Bio-Rad Laboratories, Richmond, Ca.) in 20 mM Tris-HCl (pH 7.2), concentrated by flash evaporation, desalted by gel filtration through Bio-Gel P2 and lyophilized (31).

Kinetic study of CPS depolymerization rates. Depolymerization rates of different CPS samples were measured with constant amount of PD-I enzyme under saturating conditions of CPS substrate. CPS solutions in 25 mM Tris-HCl buffer (pH 7.2) containing 2 mM $CaCl_2$ were adjusted to a final concentration of 0.15 mg/ml after assaying for total carbohydrate using the phenol-sulfuric acid assay (22). One unit of PD-1 depolymerase was defined as the amount of enzyme producing an increase of absorbance at 235 nm of 0.01 per minute. PD-I enzyme preparation (4 μg protein, 0.5 Units) was added to 1-ml samples of each CPS solution. The depolymerization activity was measured by an increase in absorbance at 235 nm (due to the unsaturated sugar formed by PD-I cleavage of CPS at glucuronic acid) (31) over a 4h period using a Gilford Response spectrophotometer interfaced with a computer kinetic program. The computer program calculated a quadratic equation of the data by the least-square fitting method. Initial rates were calculated from the value of the derivative of the quadratic expression at time zero.

^{1}H-NMR analysis of oligosaccharides. Oligosaccharide (OS) samples were deuterium-exchanged and the spectra were obtained on a Bruker WM-250 instrument at room temperature

(31). The chemical shifts were measured relative to an external tetramethyl-silane standard.

Oligosaccharide glycosyl composition. Glycosyl composition of the oligosaccharide repeating unit was analyzed by GLC as described (31).

RESULTS

Symbiotic characterization of mutant strains. Nod and Fix phenotype. Rhizobium trifolii 0403 rif was mutagenized by transposon (Tn5) mutagenesis and the mutagenized pool was screened for mutant strains defective in root nodule symbiosis with Ladino white clover. Eight hundred Kan^r Rif^r isolates were analyzed on the clover host plant. Thirteen of these (1.6 %) were symbiotically defective strains, falling into three different classes based on their symbiotic phenotype with the host plant: unable to nodulate (Nod^-), defective in nitrogen fixation (Fix^-), and partially defective in nitrogen fixation ($Fix^{\pm}$). Nod^- and Fix^- phenotypes were assigned after five independent experiments with 5-10 replicates each. Five mutant strains: R. trifolii 308 Nod^-, R. trifolii 43 Nod^-, R. trifolii 251 $Fix^{\pm}$, R. trifolii 738 Fix^- and R. trifolii 755 Fix^- were further characterized. Strains 308, 43, 251, and 755 grew like wild type in minimal medium containing different single carbon sources, whereas strain 738 had slower growth rate than that of the wild type strain. The average number of nodules per plant (5 replicates) induced by the different strains 40 days after inoculation of Ladino white clover was 6 for 0403 rif, 6.8 for 251, 19 for 755, and 12 for 738. Fix^- phenotype was confirmed by acetylene reduction (13). Nodules with R.

trifolii 738 or R. trifolii 755 failed to reduce acetylene. R. trifolii 251 had 50% (8.1 nmol/h/plant) of the wild type activity (17.4 nmol/h/plant) on Trifolium repens var. Ladino.

Physical analysis of mutant strains for presence of Tn5. Autoradiograms of ^{32}P-labeled λ::Tn5 DNA hybridized to Eco RI-digested or Hind III-digested total DNA showed one and three bands respectively (Figure 1). This result demostrated the presence of a single Tn5 insertion since Tn5 lacks Eco RI restriction sites but has two Hind III restriction sites.

Plasmid patterns of mutant strains. R. trifolii 0403 contains at least three plasmids with sizes of 235, 205, and 190 Mdal. The sym plasmid of this strain was identified as the smallest plasmid (190 Mdal) separated on Eckhardt agarose gels (R. Taylor, M. S. Thesis, University of Florida, Gainesville, 1981). The 205 Mdal plasmid appears as a partially resolved doublet and may contain two co-migrating plasmids. Strains 251, 738 and 755 showed the same plasmid profile as the wild type strain when analyzed by Eckhardt agarose gel electrophoresis, whereas strains 308 and 43 showed a deletion in the sym plasmid (Figure 2).

Attachment, deformation and infection studies. Quantitative attachment studies in Fahraeus slide cultures and in beaker assays showed that strain 251 attached better and strains 308 and 43 attached less to clover root hairs than did the wild type strain (Table 1). The percentage of

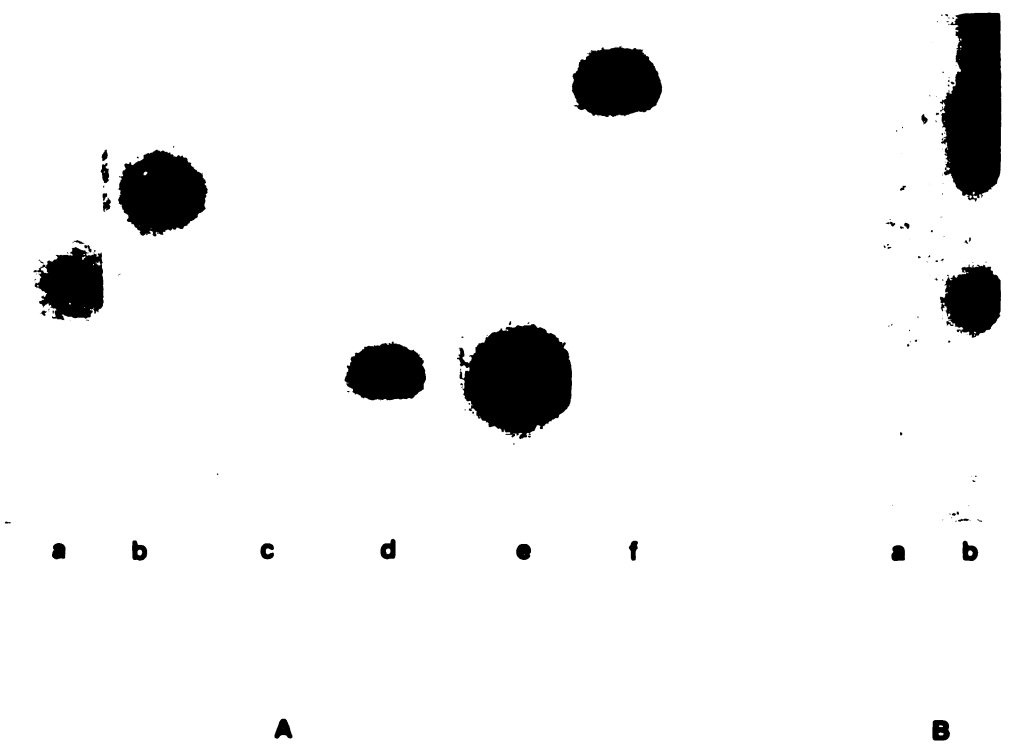

FIGURE 1. Autoradiogram of ^{32}P-labeled λ::Tn5 hybridized to digested total DNA. Eco RI digestion. (A) Lanes: a, R. trifolii 738; b, R. trifolii 43; c, R. trifolii 0403 rif; d, R. trifolii 308; e, R. trifolii 755; f, R. trifolii 251. Hind III digestion. (B) Lanes: a, R. trifolii 0403 rif; b, R. trifolii 251.

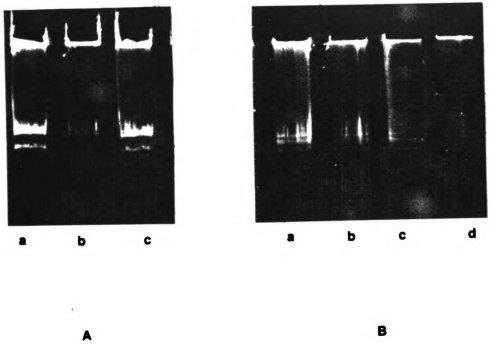

FIGURE 2. Plasmid patterns by Eckhardt agarose gel electrophoresis. (A) Lanes: a, R. trifolii 308; b, R. trifolii 0403 rif; c, R. trifolii 43. (B) a, R. trifolii 738; b, R. trifolii 755; c, R. trifolii 251; d, R. trifolii 0403 rif.

TABLE 1. Attachment of wild type *R. trifolii* 0403 *rif* and mutant strains to white clover root hairs.

Strain	Attached cells per root hair ($\bar{x} \pm$ s.d)	
	Slide culture assay[a]	Beaker assay[b]
0403 *rif* (wild type)	36 ± 3	25 ± 2
251	65 ± 2	41 ± 3
308	15 ± 3	12 ± 2
43	16 ± 3	14 ± 3
755	29 ± 5	31 ± 4
738	ND[c]	24 ± 2

[a] 15-20 root hairs (*ca*. 200 m in length) of Louisiana Nolin var. of white clover seedlings were examined per strain 12h after inoculation of 2×10^7 cells per seedling in Fahraeus slide cultures.

[b] 10 root hairs were examined per strain after incubation of the seedlings for 2h with a cell suspension containing 10^6 cells/ml in beakers.

[c] Not done.

root hairs with the combined 1A+1C pattern of attachment (clump at tip and polar attachments along the sides of the same root hair) was lower for Nod$^-$ strains 308 and 43 than for the wild type strain (Table 2). The wild type R. trifolii 0403 rif and mutant strains 251, 738 and 755 induced similar number of marked root hair deformations (shepherd crooks) (Table 3). The number of infection threads in Fahraeus slide cultures induced by strains 251 and 738 were similar to those induced by the wild type strain, whereas strain 755 induced fewer infection threads (Table 3). Neither shepherd crooks nor infected root hairs were detected on seedlings incubated with R. trifolii 308 or 43 (Table 3).

Quantitative agglutination with trifoliin A. Quantitative agglutination assays of the different mutant strains showed that trifoliin A had a significantly higher specific agglutinating activity using strain 251 and lower specific agglutinating activities with strains 308 and 43 than with wild type strain (Table 4).

Nodulation and host range studies. The nodulation patterns (Table 5) of R. trifolii 251 were similar to those of the wild type 0403 rif strain on T. pratense and Ladino var. of T. repens. However, it induced more nodules on White Dutch and Louisiana Nolin var. of T. repens, var. Clare of T. subterraneum, and T. fragiferum. Neither strain nodulated Veronal alfalfa or pea lines 8221 and 9888F. However, both strains ineffectively nodulated bean varieties

TABLE 2. Orientation of attachment of *R. trifolii* 0403 *rif* and mutant strains to clover root hairs.[a]

Strain	Percentage of root hairs with bacterial attachments			
	No attachment	1A only	1C only	1A + 1C
0403 rif	6	28	22	44
251	13	21	21	45
308	33	18	38	11
43	21	7	54	18
755	7	25	23	45
738	4	30	21	45

[a] Approximately 50 root hairs (*ca.* 200 μm in length) of Louisiana Nolin var. of white clover seedlings were examined per strain 4h after inoculation of 4 x 10^7 cells per seedling in Fahraeus slide cultures. 1A attachments represent randomly oriented cells clumped to root hair tips, and 1C attachments represent single cells polarly attached to root hairs. The 1A+1C pattern has both on the same root hair.

TABLE 3. Shepherd crooks and infection threads induced by *R. trifolii* 0403 *rif* and mutant strains.

Strain	# shepherd crooks per seedling ($\bar{x} \pm$ s.d.)[a]	# infected root hairs per seedling ($\bar{x} \pm$ s.d.)[b]
0403 *rif* (wild type)	17 ± 2	12 ± 1
251	13 ± 1	10 ± 1
308	0	0
43	0	0
755	11 ± 4	6 ± 3
738	11 ± 4	12 ± 3

[a,b] Values are average of four replicate seedlings of *Trifolium repens* var. Louisiana Nolin inoculated with 5 x 10^6 cells per seedling in Fahraeus slide cultures and incubated for 4 days.

TABLE 4. Trifoliin A - agglutinating activity of *R. trifolii* 0403 *rif* and mutant strains.[a]

Strain	Specific agglutinating activity[b] (agglutinating units/mg trifoliin A protein)
0403 *rif*	5,333
251	42,664
308	2,667
43	2,667
755	5,333
738	5,333

[a] Bacterial agglutination was performed in microtiter plates. Trifoliin A (60 µg/ml) isolated from seeds of *Trifolium repens* var. Louisiana Nolin was used. Cells were grown for 5 days on BIII agar medium and prepared as described in the text.

[b] Cell suspensions (25 µl) were added to a two-fold dilution series of the lectin (25 µl). Specific agglutinating activity is the number of agglutinating units per mg of trifoliin A (18).

TABLE 5. Nodulation patterns of *R. trifolii* 0403 *rif* (wt) and *R. trifolii* 251 (ms) on *Trifolium repens*, *Trifolium subterraneum*, *Trifolium fragiferum*, and *Trifolium pratense*.

Clover host	Average number of nodules per plant							
	10d		15d		20d		40d	
	wt	ms	wt	ms	wt	ms	wt	ms
T. repens vars.								
White Dutch	1.0	1.4	2.5	4.0	3.0	5.4	3.0	11.0
Louisiana Nolin	1.0	1.4	1.0	3.0	3.5	4.0	4.5	8.4
Ladino	2.0	1.8	4.0	3.8	6.0	5.6	6.0	6.8
T. subterraneum	0.0	1.3	0.0	1.5	0.5	2.0	1.5	2.3
T. fragiferum	0.0	2.6	1.5	3.6	2.0	5.8	3.0	8.0
T. pratense	2.0	1.0	4.0	3.0	9.0	6.0	9.0	12.0

[a] Average number of nodules per plant (5 replicates) induced by strains 0403 *rif* and 251 were recorded at indicated times after inoculation.

(Black Turtle Soup or line 21-58 at 40 days). Nodulation of P. vulgaris by R. trifolii 0403 has been previously reported (57).

Location of Tn5 insertion in R. trifolii 251. The presence of a Tn5 insertion in the sym plasmid of R. trifolii 251 was demonstrated by hybridization of ^{32}P-labeled λ::Tn5 DNA to plasmid DNA which had been separated by agarose gel electrophoresis (Figure 3).

Physical analysis of R. trifolii 251 for the presence of Mu DNA. DNA was isolated from phage Mu which had been purified by CsCl equilibrium gradient centrifugation (Figure 4) and labeled with ^{32}P by nick translation. Autoradiograms of ^{32}P- labeled Mu DNA hybridized to Eco RI-digested total DNA from strain 251 did not show any hybridization bands, indicating that Mu sequences are not present in R. trifolii 251 total DNA.

CPS depolymerization rates. Kinetic studies showed that the R. trifolii 0403 rif CPS substrate was depolymerized by PD-I at a faster rate than was CPS of R. trifolii 251 (Figure 5, Table 6). A one-way analysis of variance using the F distribution indicated that these values were significantly different at a probability of chance ($P<0.005$) (Table 6). Depyruvylated CPS was not cleaved by the enzyme, and therefore, pyruvate substitutions were essential for PD-I lyase activity.

^{1}H-NMR analysis of CPS for non-carbohydrate components. CPS was converted into structurally analyzable

FIGURE 3. Plasmid profile and location of Tn5 insertion in R. trifolii 251. (A) Plasmid Eckhardt gel. (B) Autoradiogram of ^{32}P-labeled λ::Tn5 hybridized to plasmid Southern blot.

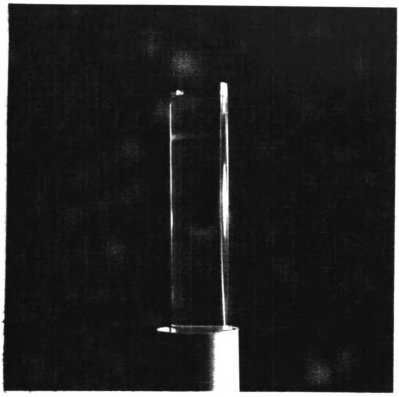

FIGURE 4. Phage Mu purified by CsCl equilibrium gradient centrifugation.

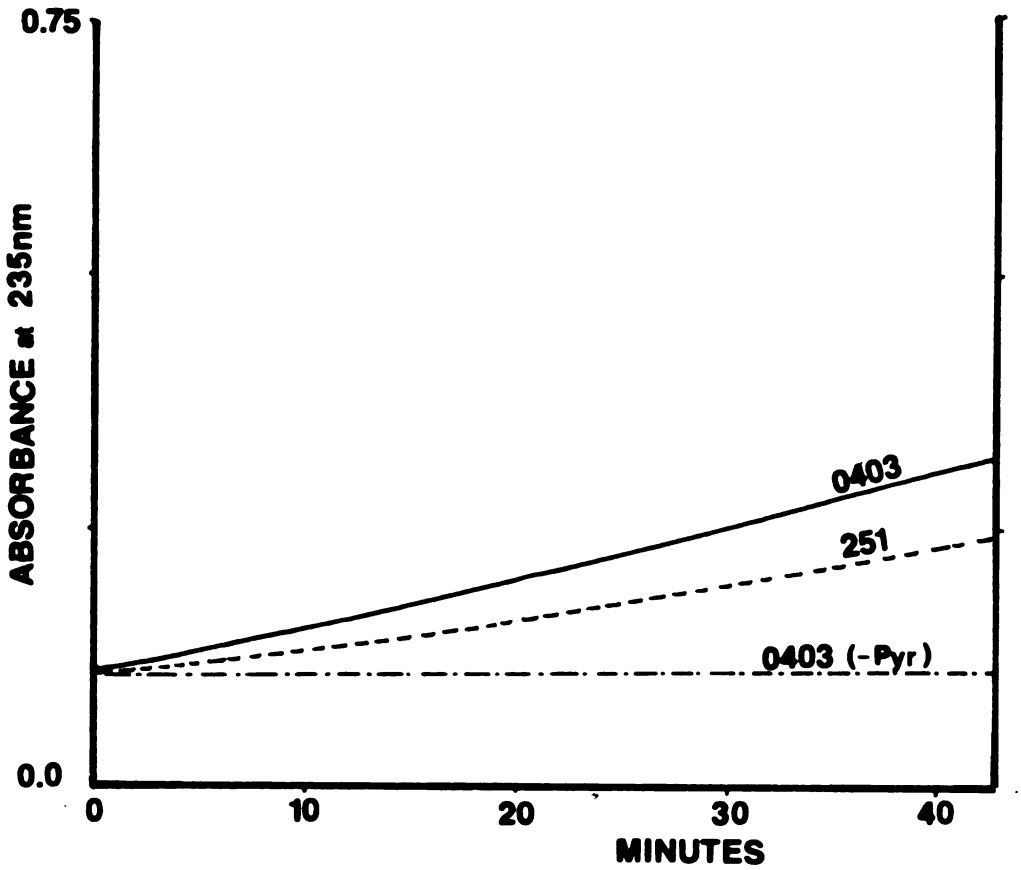

FIGURE 5. Depolymerization rates of CPS from R. trifolii 0403 rif (——), R. trifolii 251 (- - -) and depyruvylated CPS (-·-·-) with enzyme PD-I followed by increase of absorbance at 235 nm under saturating conditions of substrate and identical protein concentration.

TABLE 6. Depolymerization rates of CPS from *R. trifolii* 0403 *rif* and *R. trifolii* 251.

Strain	No. of replicates	Initial rate[a] Mean ± s.d.[b]
0403 rif	3	44 ± 4
251	3	27 ± 2

[a] Expressed as 10^4 x Abs 235 nm/min.

[b] Standard deviation of the mean.

oligosaccharides using a bacteriophage depolymerase system and examined by 250-MH_Z Fourier-transform ^{1}H-NMR. The spectra showed the presence of acetate esters by resonances (relative to external Me_4Si) between δ 1.88 and 2.15. Signals assigned to ketal-linked pyruvate groups appeared between δ 1.23 and 1.43. Two other groups of resonance, one between δ 1.03 and 1.17 and signals between δ 2.36 and 2.58 were assigned to ether-linked 3-hydroxybutanoic acid. These were quantitated by comparing the integrals of the signals for the respective groups to the area of the signal of the C-4 proton of the unsaturated terminal sugar identified as 4-deoxy-L-threo-hex-4-enopyranosyluronic acid (DEPUA) resulting from the enzymatic cleavage of the CPS at glucuronic acid (31). These NMR measurements indicated that the oligosaccharide fragments from CPS of strain 251 had less acetic and more pyruvic acid substitutions per OS repeating unit than wild type OS (Table 7).

Oligosaccharide glycosyl composition. The glycosyl composition of 251 OS was the same as that of wild type OS (Glu+GluUA: Gal: DEPUA) (6: 1: 1) (31).

TABLE 7. Non-carbohydrate composition of oligosaccharides obtained by PD-I depolymerization of CPS isolated from *R. trifolii* 0403 *rif* and *R. trifolii* 251 [a]

Strain	Molar proportion of non-carbohydrate components per oligosaccharide repeating unit (± 0.05).[b]		
	Pyruvic acid	Acetic acid	3-hydroxybutanoic acid
0403 *rif*	1.74	1.70	0.41
251	2.04	1.48	0.31

[a] CPS was isolated from cells grown for 5 days on BIII agar plates, depolymerized into its oligosaccharide repeating unit (OS) and purified through Bio-Gel P10 and P2. OS were exchanged with deuterium oxide and the ^{1}H-NMR spectra were recorded.

[b] Values were obtained by comparing the integrals for the various groups with that of the single C-4 proton of the unsaturated terminal sugar in each oligosaccharide.

DISCUSSION

Three classes of mutant strains were isolated after Tn5 mutagenesis of R. trifolii 0403 rif based on their symbiotic phenotype on white clover: unable to nodulate (Nod^-), defective in nitrogen fixation (Fix^-) and partially defective in nitrogen fixation ($Fix^{\pm}$). Five strains were further studied: R. trifolii 308 (Nod^-), R. trifolii 43 (Nod^-), R. trifolii 755 (Fix^-), R. trifolii 738 (Fix^-), and R. trifolii 251 ($Fix^{\pm}$).

In comparison with the wild type strain, mutant strains 308 (Nod^-) and 43 (Nod^-) had a deletion in the sym plasmid (Figure 2), attached less to clover root hairs (Table 1) with lower percentage of the combined 1A+1C attachment to the same root hair (Table 2), were unable to deform or infect root hairs (Table 3), and had lower specific agglutinating activities with trifoliin A (Table 4). This similarity of results obtained with two strains having a deletion in the sym plasmid suggested that functions coded in this plasmid may have a positive effect on the ability of Rhizobium trifolii 0403 rif to attach to clover root hairs and to interact with the clover lectin. Complementation of these mutants with a cloned (nod-nif) region should restore wild type phenotype if the deletion of the plasmid is responsible of the phenotype. Analysis of CPS

from strain 308 revealed the presence of a novel N-containing non-carbohydrate substitution which was significantly higher in the mutant than in wild type CPS (R. Hollingsworth and F. Dazzo, personal communication). Strains 755 (Fix^-) and 738 (Fix^-) with normal plasmid patterns (Figure 2), had the same attachment (Table 1) and lectin binding (Table 4) ability as the wild type strain. Both strains induced more nodules than the wild type strain although 755 induced less and 738 the same number of root hair infections as wild type (Table 3). It is well known that many (but not all) Rhizobium ineffective strains induce numerous nodules on the host plants. Thus, the Tn5 insertion of strain 755 in the sym plasmid (data not shown) affected root hair infection, nodulation, and nitrogen fixation.

Strain 251 with a single Tn5 insertion (Figure 1) and a normal plasmid pattern when compared to the wild type (Figure 2) attached in higher numbers to clover root hairs (Table 1), had similar orientation of attachment (Table 2), induced similar number of shepherd crooks and root hair infections (Table 3), and was agglutinated better by trifoliin A than was the wild type strain (Table 4). The degree of distribution of lectin receptors on R. trifolii cells has been correlated with the degree and orientation of attachment (50). The fact that this mutant strain which binds the lectin better is also able to attach to clover root hairs better is consistent with the proposed importance of lectin binding in attachment of Rhizobium to clover root

hairs (50). "Phase I" loose attachment is followed by "Phase II" firm anchoring of the bacterial cell to the root hair surface (17). During Phase II, extracellular fibrillar materials are characteristically found associated with the adherent bacteria. It is not known whether these fibrils consist of bundles of cellulose, fimbriae, or some other fibrillar polymers made by the attached bacteria (17). Strain 251 also showed more Phase II (firm) attachments and fibrillar polymers on the root hair surface than did the wild type strain (K. Smith, H. Yang and F. Dazzo, personal communication). Although strain 251 induced more nodules on several clover hosts (Table 5), this property was not correlated with the induction of more root hair infections in Louisiana Nolin var. of T. repens (Table 3) and may be due to the fact that the strain is partially defective in nitrogen fixation. Interestingly, the white clover variety's response to nodulation (Table 5) by strain 251 matches the number of root hair infections induced by the wild type strain (White Dutch>Louisiana Nolin>Ladino) (D. Gerhold and F. Dazzo, personal communication). This strain would be ideal for studying the effect of improved attachment on the degree of the strain's success in interstrain competition (with a Fix^+ strain) for nodule sites on the root.

Strain 251 was selected for further genetic and biochemical characterization because it has a single Tn5 insertion and showed a significant increase in attachment and lectin binding ability as compared to the wild type

strain. Tn5 was located in the sym plasmid (Figure 3), and presence of Mu DNA was not detected in strain 251 DNA. The latter was investigated since Mu phage was originally present in the suicide plasmid (pPH1JI::Mu::Tn5) used as a vector for Tn5 mutagenesis. CPS isolated from strain 251 was analyzed since the specific attachment of different Rhizobium species to host root hairs has been hypothesized to result from the interaction between the lectin on the host root and Rhizobium CPS (15, 37, 44, 50, 52). In addition, lectin-binding ability of R. trifolii 0403 CPS has been reported to be age-dependent (50) and changes of CPS (1, 50) and EPS non-carbohydrate substitutions with culture age have been reported (9). CPS from strain 251 was different from wild type CPS as shown by kinetic study of depolymerization rates using PD-I β-lyase (Table 6, Figure 5) and quantitative ^{1}H-NMR determination of non-carbohydrate substitutions (Table 7). The rate of depolymerization of 251 CPS by depolymerase PD-I was significantly less than that of wild type CPS and depyruvylated CPS of wild type was not depolymerized by the enzyme, indicating that pyruvate substitutions are essential for PD-I depolymerase activity. Previous reports showed that deacetylated CPS was depolymerized faster than wild type CPS by β-lyase PD-II, a different polysaccharide depolymerase enzyme (31). This indicates that changes in the non-carbohydrate substitutions of the CPS may change the conformation of the substrate thereby altering the kinetics of the depolymerization

reaction. CPS from strain 251 had more pyruvate and less acetate non-carbohydrate substitutions than wild type CPS. Tn5 mutants in the nodulation region I (Hac) of R. trifolii 843 also had alterations in the levels of acetate and/or pyruvate in their CPS (Chapter II). However, strain 251 is Hac^+, and therefore the Tn5 insertion in this strain is not in the Hac region of the sym plasmid. Structural analysis of R. trifolii 0403 oligosaccharides obtained by depolymerization of CPS with our polysaccharide depolymerase system showed that acetate is ester-linked and pyruvate is ketal-linked to the same nonreducing terminal galactose residue (R. I. Hollingsworth, manuscript in preparation). The decrease in acetate levels and corresponding increase in pyruvate levels may indicate that the transferase enzymes adding these substitutions to the glycosyl residues are competing for sites for substitution. Therefore, the alteration in the level of one of these substitutions may affect the level of the other. Interestingly, CPS pyruvyl transferase activity was higher for R. trifolii 251 than for the wild type strain (Chapter III). These results also indicate that functions related to CPS synthesis may be encoded in the sym plasmid. It would be important to further define the location of the mutated gene relative to the nodulation and nitrogen fixation regions already identified in the sym plasmid of other strains of R. trifolii (49, M. A. Djordjevic, Mol. Gen. Genet., in press). This would

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provide important information as to the organization and function of the bacterial symbiotic genes.

Results obtained indicate that CPS non-carbohydrate substitutions are important in *R. trifolii* 0403 *rif* attachment to clover root hairs and lectin binding ability.

LIST OF REFERENCES

1. Abe, M., J. E. Sherwood, R. I. Hollingsworth, and F. B. Dazzo. 1984. Stimulation of clover root hair infection by lectin binding oligosaccharides from the capsular and extracellular polysaccharides of Rhizobium trifolii. J. Bacteriol. 160:517-520.

2. Banfalvi, Z., Sakanyan, C. Koncz, A. Kiss, I. Dusha, and A. Kondorosi. 1981. Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of Rhizobium meliloti. Mol. Gen. Genet. 184:318-325.

3. Berg, D. E., R. Jorgensen, and J. Davies. 1978. Transposable kanamycin-neomycin resistance determinants, p. 13-15. In D. Schlessinger. (ed.), Microbiology.1978. American Society for Microbiology, Washington, D.C.

4. Beringer, J. E. 1974. R factor transfer in Rhizobium leguminosarum. J. Gen. Microbiol. 84:188-198.

5. Beringer, J. E., J. L. Beynon, A. V. Buchanan - Wollaston, and A. W. B. Johnston. 1978. Transfer of the drug - resistance transposon Tn5 to Rhizobium. Nature. London. 276:633-634.

6. Beynon, J. L., Beringer, J. E., and Johnston, A. W. B. 1980. Plasmids and host range in Rhizobium leguminosarum and Rhizobium phaseoli. J. Gen. Microbiol. 120:421-429.

7. Brewin, N. J., Beringer, J. E., Buchanan - Wollaston, A. V., Johnston, A. W. B., and Hirsch, P. R. 1980. Transfer of symbiotic genes with bacteriocinogenic plasmids in Rhizobium leguminosarum. J. Gen. Microbial. 116:261-270.

8. Buikema, W. J., S. R. Long, S. E. Brown, R. C. van den Bos, C. Earl, and F. M. Ausubel. 1983. Physical and genetic characterization of Rhizobium meliloti symbiotic mutants J. Mol. Appl. Genet. 2:249-260.

9. Cadmus, M. C., K. A. Burton, and M. E. Slodki. 1982. Growth - related substituent changes in the exopolysaccharides of fast-growing rhizobia. Appl. Environ. Microbiol. 44:242-245.

10. Christensen, A. H., and K. R. Schubert. 1983. Identification of a Rhizobium trifolii plasmid coding for nitrogen fixation and nodulation genes and its

interaction with pJB5JI, a Rhizobium leguminosarum plasmid. 156:592-599.

11. Corbin, D., L. Barran, and G. Ditta. 1983. Organization and expression of Rhizobium meliloti nitrogen fixation genes. Proc. Natl. Acad. Sci. U.S.A. 80:3005-3007.

12. Dazzo, F. B. 1982. Leguminous root nodules, p. 431-446. In R. G. Burns and J. H. Slater (ed.). Experimental Microbial Ecology. Blackwell Sientific Publications, Oxford.

13. Dazzo, F. B. 1984. Bacterial adhesion to plant root surfaces, p. 85-93. In K. C. Marshall (ed.), Microbial Adhesion and Aggregation. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.

14. Dazzo, F. B., and W. J. Brill. 1979. Bacterial polysaccharide which binds Rhizobium trifolii to clover root hairs. J. Bacteriol. 137:1362-1373.

15. Dazzo, F. B., and D. H. Hubbell. 1975. Cross-reactive antigens and lectin as determinants of symbiotic specificity in the Rhizobium-Clover association. Appl. Microbiol. 30:1017-1033.

16. Dazzo, F. B., and G. L. Truchet. 1983. Interactions of lectins and their saccharide receptors in the Rhizobium - legume symbiosis. J. Membrane Biol. 73:1-16.

17. Dazzo, F. B., G. L. Truchet, J. E. Sherwood, E. M. Hrabak, M. Abe, and S. H. Pankratz. 1984. Specific phases of root hair attachment in the Rhizobium trifolii - clover symbiosis. Appl. Environ. Microbiol. 48:1140-1150.

18. Dazzo, F. B., W. E. Yanke, and W. J. Brill. 1978. Trifoliin: a Rhizobium recognition protein from white clover. Biochim. Biophys. Acta 539:276-286.

19. Downie, J. A., G. Hombrecher, Q-S. Ma, C. D. Knight, B. Wells, and A. W. B. Johnston. 1983. Cloned nodulation genes of Rhizobium leguminosarum determine host-range specificity. Mol Gen. Genet. 190:359-365.

20. Downie, J. A., C. D. Knight, A. W. B. Johnston, and L. Rossen. 1985. Identification of genes and gene products involved in the nodulation of peas by Rhizobium leguminosarum. Mol. Gen. Genet. 198: 255-262.

21. Downie, J. A., Q.- S. Ma, C. D. Knight, G. Hombrecher, and A. W. B. Johnston. 1983. Cloning of the symbiotic region of Rhizobium leguminosarum: the nodulation genes

are between the nitrogenase genes and nifA-like gene. EMBO J. 2:947-952.

22. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350-356.

23. Eckhardt, T. 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid 1:584-588.

24. Engel, J. D., and Dodgson, J. B . 1981. Histone genes are clustered but not tandemly repeated in the chicken genome. Proc. Nat. Acad. Sci. U.S.A. 78:2856-2860.

25. Fahraeus, G. 1957. The infection of clover root hairs by nodule bacteria studied by a single glass slide technique. J. Gen. Microbiol. 16:374-381.

26. Forrai, T., E. Vincze, Z. B anfalvi, G. B . Kiss, G. S. Randhawa,and A. Kondorosi. 1983. Localization of symbiotic mutations in Rhizobium meliloti. J. Bacteriol. 153:635-643.

27. Grundy, F. J., and M. H. Howe. 1984. Involvement of the invertible G Segment in bacteriophage Mu tail fiber biosynthesis. Virology. 134:296-317.

28. Higashi, S. 1967. Transfer of clover infectivity of Rhizobium trifolii to Rhizobium phaseoli as mediated by an episomic factor. J. Gen. Appl. Microbiol. 13:391-403.

29. Higashi, S., and M. Abe. 1978. Phage- induced depolymerase for exopolysaccharide of Rhizobiaceae. J. Gen. Appl. Microbiol. 24:143-153.

30. Hirsch, P. R., van Montagu, M., Johnston, A. W. B., Brewin, N. J. and Schell, J. 1980. Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of Rhizobium leguminosarum. J. Gen. Microbiol. 120:403-412.

31. Hollingsworth, R. I., M Abe, J. E. Sherwood, and F. B . Dazzo. 1984. B acteriophage-induced acid heteropoly saccharide lyases that convert the acidic heteropolysaccharides of Rhizobium trifolii into oligosaccharide units. J. Bacteriol. 160:510-516.

32. Hooykaas, P. J., A. A. N. van Brussel, H. den Hulk-Ras, G. M. S. van Slogteren, and R. A. Schilperoort. 1981. Sym plasmid of Rhizobium trifolii expressed in different rhizobial species and Agrobacterium tumefaciens. Nature.(London) 291:357-353.

33. Howe, M. M. (1973) Prophage deletion mapping of bacteriophage Mu-1. Virology 54:93-101.

34. Howe, M. M., J. W. Shumm, and A. L. Taylor. 1979. The S and U genes of bacteriophage Mu are located in the invertible G Segment of Mu DNA. virology 92:108-124.

35. Johnston, A. W. B ., Beynon, J. L., Buchanan - Wollaston, A. V., Setchell, S. M., Hirsch, P. R. and Beringer J. E. 1978. High frequency transfer of nodulating ability between strains and species of Rhizobium. Nature 276:634-636.

36. Jorgensen, R., S. J. Rothstein, and W. S. Reznikoff. 1979. A restriction enzyme cleavage map of Tn5 encoding neomycin-resistance. Mol. Gen Genet. 177:65-72.

37. Kato, G., Y. Maruyama, and M. Nakamura. 1981. Involvement of lectin in Rhizobium - pea recognition. Plant Cell Physiol. 66:609-614.

38. Keele, B. B . Jr., P. B . Hamilton, and G. H. Elkan. 1969. Glucose catabolism in Rhizobium japonicum. J. Bacteriol. 97:1184-1191.

39. Kondorosi, E., Z. Banfalvi, and A. Kondorosi. 1984. Physical and genetic analysis of a symbiotic region of Rhizobium meliloti: identification of nodulation genes. Mol. Gen. Genet. 193:445-452.

40. Long, S. R., W. J. Buikema, and F. M. Ausubel. 1982. Cloning of Rhizobium meliloti nodulation genes by direct complementation of Nod mutants. Nature (London) 298:485-488.

41. Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants in Rhizobium meliloti induced by transposon Tn5 mutagenesis. J. Bacteriol. 149:114-122.

42. Nuti, M. P., A. A. Lepidi,R. K. Prakash, R. A. Schilperoort, and F. C. Cannon, 1979. Evidence for nitrogen fixation genes on indigenous Rhizobium plasmids. Nature. 282:533-535.

43. Ozeki, H. 1959. Chromosome fragments participating in transduction in Salmonella typhimurium. Genetics 44:457-470.

44. Paau, A. S., W. T. Leps, and W. J. Brill. 1981. Agglutinin from alfalfa necessary for binding and nodulation by Rhizobium meliloti. Science 213:1513-1515.

45. Prakash, R. K., R. A. Schilperoort, and M. P. Nuti, 1981. Large plasmids of fast growing Rhizobia: homology studies and location of structural nitrogen fixation genes. J. Bacteriol. 145:1129-1136.

46. Rigby, P. W. J., M Dickman, C. Rhoodes, and P. Berg. 1976. Labeling deoxyribonucleic acid to a high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113:237-251.

47. Rosenberg, C., P. Boistard, J. Denarie, and F. Casse-Delbart. 1981. Genes controlling early and late functions in symbiosis are located on a megaplasmid in Rhizobium meliloti. Mol. Gen. Genet. 184:325-333.

48. Ruvkun, G. B., and Ausubel, F. M. 1980. Interspecies honology of nitrogenase genes. Proc. Nat. Acad. Sci. U.S.A. 77:191-195.

49. Schofield, P. R., M. A. Djordjevic, B. G. Rolfe, and J. Shine. 1983. A molecular linkage map of nitrogenase and nodulation genes in Rhizobium trifolii. Mol. Gen. Genet. 192:459-465.

50. Sherwood, J. E., J. M. Vasse, F. B. Dazzo, and G. L. Truchet. 1984. Development and Trifoliin A- binding ability of the capsule of Rhizobium trifolii. 159:145-152.

51. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

52. Stacey, G. A. S. Paau, and W. J. Brill. 1980. Host recognition in the Rhizobium-soybean symbiosis. Plant Physiol. 66:609-614.

53. Szeto, W. W., J. L. Zimmerman, V. Sundaresan, and F. M. Ausubel. 1984. A Rhizobium meliloti symbiotic regulatory gene. Cell 36:1035-1043.

54. Vincent, J. M. 1980. Factors controlling the legume-Rhizobium symbiosis, p. 103-129. In W. E. Newton and W. H. Orme-Johnson (ed.), Nitrogen fixation, vol.2. Symbiotic associations and cyanobacteria. University Park Press, Baltimore.

55. Wahl, G. M., M. Stern, and G. R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethal-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. 76:3683-3687.

56. Williams, B. G., Blattner, F. R., Jaskunas, S. R., and Nomura, M. 1977. Insertion of DNA carrying ribosomal protein genes of Escherichia coli into charon vector phages. J. Biol. Chem. 252:7344-7354.

57. Wong, P. P., and S. Shantharam. 1984. Binding between pole bean (Phaseolus vulgaris L.) lectin and rhizobia that do or do not nodulate the pole bean. Can. J. Microbiol. 30:1349-1356.

58. Zimmerman, J. L., W. W. Szeto, and F. M. Ausubel. 1983. Molecular characterization of Tn5-induced symbiotic (Fix$^-$) mutants of Rhizobium meliloti. J. Bacteriol. 156:1025-1034.

59. Zurkowski, W. 1980. Specific adsorption of bacteria to clover root hairs, related to the presence of the plasmid pWZ2 in cells of Rhizobium trifolii. Microbios 27:27-32.

60. Zurkowski, W. and Lorkiewicz. 1979. Plasmid mediated control of nodulation in Rhizobium trifolii. Arch. Microbiol. 123:195-201.

CHAPTER II

ALTERATIONS IN CAPSULAR POLYSACCHARIDE OF TN5 - INDUCED MUTANTS IN THE *RHIZOBIUM TRIFOLII* NODULATION REGION.

ABSTRACT

Capsular polysaccharide (CPS) was isolated from wild type *R. trifolii* 843 and mutant strains with Tn5 insertions in three nodulation regions of the *R. trifolii* symbiotic plasmid. A polysaccharide depolymerase isolated from a phage lysate of *R. trifolii* 4S was used to hydrolyze the CPS into its oligosaccharide repeating unit (OS). CPS from mutant strains in nodulation region I(Hac) depolymerized at different rates than wild type CPS. Non-carbohydrate components (pyruvic, acetic, and 3-hydroxybutanoic) of OS were measured by ^{1}H-NMR. OS from mutants in nodulation region I(Hac) had different levels of pyruvic and acetic acid substitutions. In contrast, CPS depolymerization rates as well as the level of non-carbohydrate substitutions were unchanged by mutations in nodulation regions II and III. Mutant strains examined (ANU 851 (I) *nod*::Tn5 and ANU 845 pSym^{-}) had a significantly lower ability to bind the clover

lectin in *ex planta* and *in situ* assays. We conclude that Tn5 mutations in certain essential *nod* genes on the *sym* plasmid affect the CPS of *R. trifolii* 843 and the lectin-binding ability of mutant strains. Functions related to CPS synthesis may be encoded in the *sym* plasmid.

INTRODUCTION

Rhizobium common nodulation (*nod*) and host specific nodulation (*hsn*) genes are closely linked on the *sym* plasmid in *R. trifolii* (4,11), *R. leguminosarum* (5) and *R. meliloti* (9). However, the functions coded by these genes are not known.

In this study we examined Tn5-induced mutants of *R. trifolii* ANU 843 obtained by extensive mutagenesis of a 14 kb DNA fragment from the *sym* plasmid of this strain which carries *nod* and *hsn* genes. A correlation between the site of Tn5 insertion and the symbiotic phenotype of the mutant strains had been established (M. A. Djordjevic et al., Mol. Gen. Genet., in press) and led to the identification of three regions (I, II, III corresponding to Hac^{-}, Hac^{++}, and Hsp respectively, see Figure 1 and Table 1) which affected nodulation ability. The objective of this work was to determine if these mutant strains in regions I, II, and III were altered in their CPS and lectin-binding ability. To analyze the CPS we used a glucuronic acid - specific lyase isolated from a phage lysate of *R. trifolii* 4S (7) to hydrolyze the CPS into its oligosaccharide repeating unit (OS). These OS products contain all of the structural information of the CPS except that one glucuronic acid residue is converted into an unsaturated terminal sugar

(DEPUA, 4-deoxy-L-threo-4-enopyranosyluronic acid) as a result of the enzymatic cleavage of the CPS at one of the glucuronic acid residues (8).

Results obtained showed that CPS of some mutants in nodulation region I (Hac region) had altered levels of pyruvate and acetate and depolymerized at different rates than wild type CPS. In contrast, the level of non-carbohydrate substitutions as well as the CPS depolymerization rates were unchanged by mutations in nodulation regions II and III. Mutant strains examined (ANU 851 (I) nod::Tn5 and ANU 845 pSym$^-$) had a significantly lower ability to bind the clover lectin.

This work was presented in part at the ASM Meeting, Abstr. K102, p. 188, Las Vegas, March 1985.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains used in this study (Figure 1, Table 1) were provided by Dr. Barry Rolfe, Australian National University, Canberra, Australia (M. A. Djordjevic et al., Mol. Gen. Genet., in press).

Media and growth conditions. Cells for CPS isolation were grown on BIII (1) agar plates.

Deformation studies were performed in Fahraeus slide cultures as described in Chapter I.

Protein determination. Protein concentration was measured by the Bio-Rad protein assay.

Lectin binding studies. The ability of wild-type R. trifolii 843 and symbiotically-defective mutant derivatives to bind to trifoliin A, a white clover lectin, was examined by indirect immunofluorescence after growth of the bacteria in a chemically-defined medium (ex planta) and in the clover root environment (in situ)(2). For both studies, the inoculum consisted of a 5 day-old culture grown on plates of BIII agar. For ex planta binding studies, cells were inoculated on BIII plates and grown for 3-10 days at 30°C. Cells were removed from plates daily, suspended in phosphate-buffered saline (PBS, 10 mM potassium phosphate, 140 mM NaCl, pH 7.2), heat-fixed on microscope slides, rinsed with distilled water, and air-dried. Cells were then

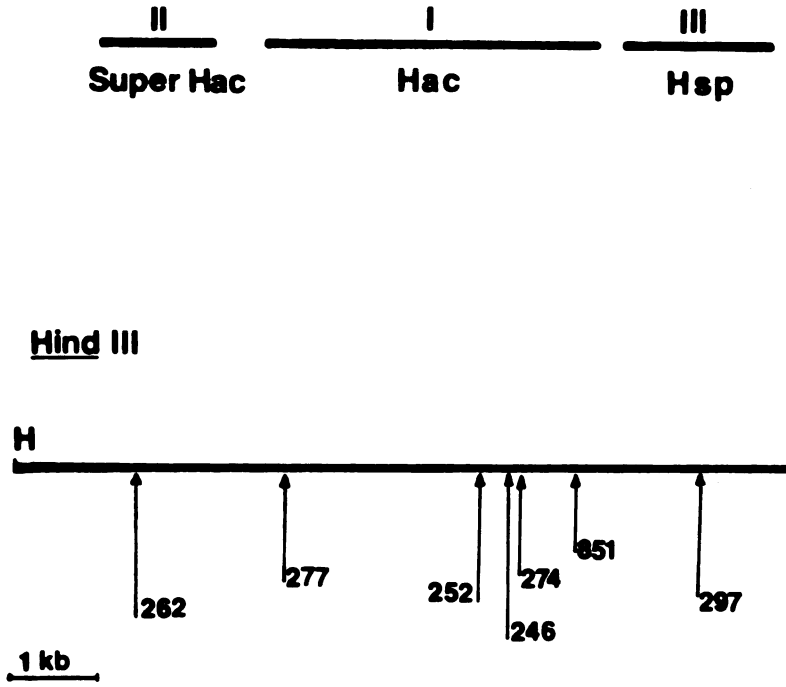


FIGURE 1. Nodulation regions in the sym plasmid of R. trifolii 843. 14 Kb Hind III restriction fragment showing location of Tn5 insertions. The regions were established based on the symbiotic phenotype of the mutant strains (M. A. Djordjevic et al.,Mol. Gen. Genet., in press).

TABLE 1. List of bacterial strains used in this study.

R. trifolii strain [a]	Characteristics	
	Genotype	Phenotype
ANU 843	Wild type	[b] Hac^+Nod^+
ANU 277	[c] (I) *nod*C::Tn5	Hac^-Nod^-
ANU 252	(I) *nod*A::Tn5	Hac^-Nod^-
ANU 246	[d] (I) *nod*A::Tn5	Hac^+Nod^+
ANU 274	(I) *nod*D::Tn5	Hac^-Nod^-
ANU 851	(I) *nod*D::Tn5	Hac^-Nod^-
ANU 262	(II) *nod*::Tn5	[e] $Hac^{++}Nod^{(+)}$
ANU 297	(III) *nod*::Tn5	[f] $Hac^{++}Nod^{(+)}$
ANU 845	[g] $pSym^-$	Hac^-Nod^-

[a] All the strains were obtained from Dr. Barry Rolfe, Australian National University (M. A. Djordjevic et al., Mol. Gen. Genet., submitted).

[b] Hac^+ is ability to induce marked curling (shepherd crooks) of white clover root hairs and Nod^+ is ability to form nodules on white clover roots.

[c] Location of Tn5 insertions are shown in Figure 1. I, II, III are nodulation regions I, II, III respectively (Figure 1). Insertions into region I eliminated root hair curling (Hac^-), infection thread formation, and the mutants were unable to nodulate all tested plant species (Nod^-).

[d] Tn5 is inserted before *nod* A gene coding region.

[e] Hac^{++} means super Hac^+. Mutants in region II induced delayed and fewer nodules and aborted infection threads.

[f] Region III mutants were altered and extended in host-range properties (Hsp). This strain was able to induce marked root hair distortions, infection threads and nodules on *Pisum sativum* but showed a poor nodulation ability on *Trifolium repens* while the ability to induce infection threads on this host was retained.

[g] Lost the *sym* plasmid (heat-cured).

incubated for 1h each with trifoliin A (10 µl at 20 g protein/ml M buffer) isolated from white clover seed (Trifolium repens var. Louisiana Nolin), rabbit anti-trifoliin A IgG (4 mg protein/ml PBS), and FITC-conjugated goat anti-rabbit IgG (Miles Laboratories, Israel). The in situ binding of trifoliin A to cells in the clover root environment was examined after growth in axenic slide cultures. Seedlings of Louisiana Nolin var. of white clover were germinated from surface-sterilized seeds and inoculated with 2×10^7 bacteria per seedling in modified slide cultures without agar supports. Slide cultures were incubated for 7 days in a growth chamber programmed for a 14h photoperiod of 26,700 lux (mixed fluorescent and incandescent lighting), 22°C/20°C day/night cycle. During this time, physiological levels of trifoliin A in the root exudate would interact with the bacteria (2). Afterwards, the cover slip was gently removed from the slide culture and the volume of suspended cells collected (ca 0.2 ml). This volume was diluted to 1 ml with filter-sterilized, N-free Fahraeus medium and centrifuged for 3 min at 1000 x g. The pelleted cells were gently resuspended and heat-fixed on microscope slides. They were then incubated with rabbit anti-trifoliin A IgG, followed by FITC goat anti-rabbit IgG. For epifluorescence microscopy, stained cells were mounted with FA glycerol mounting fluid (pH 9, Difco Labs, Detroit, MI) and examined at a magnification of 1250 x with a Zeiss Photomicroscope I equipped with quartz-halogen FITC-

epifluorescence optics. Five to ten fields were examined per treatment (>50 cells per field). The percentage of cells binding the lectin was calculated by comparing the number of immunofluorescent cells to the total number of cells in the same field recognized by phase-contrast microscopy.

Methods for isolation of polysaccharide depolymerase PD-I from a phage lysate of R. trifolii 4S, and oligosaccharide isolation from CPS of plate-grown cultures have been described in Chapter I.

Kinetic study of CPS depolymerization rates. Depolymerization rates of CPS samples were measured under saturating conditions of substrate and identical protein concentration. CPS solutions in 25 mM Tris-HCl buffer (pH 7.2), 2 mM $CaCl_2$ were adjusted to a final concentration of 0.15 mg/ml after assaying for total carbohydrate by the phenol-sulfuric acid method (3). Five samples with decreasing substrate concentrations were used to establish enzyme saturation with each CPS. PD-I (4 μg protein, 0.5 Units) was added to 1-ml samples of each CPS solution and the depolymerization activity (measured by an increase in absorbance at 235 nm) was followed for 4-5h in a Gilford Response spectrophotometer interfaced with a computer kinetic program. Three to seven independent experiments were run with each substrate. The computer program calculated a quadratic equation of the data by the least-square fitting method. Initial rates were calculated from the value of the derivative of the quadratic expression at time zero. One

unit of PD-I depolymerase was defined as the amount of enzyme producing an increase of absorbance at 235 nm of 0.01 per minute.

^{1}H-NMR analysis of oligosaccharides. Oligosaccharide (OS) samples were deuterium-exchanged and the spectra were recorded on a Bruker WM-250 instrument at room temperature (8). The chemical shifts were measured relative to an external tetramethyl-silane standard.

Oligosaccharide composition. Glycosyl composition of the oligosaccharide repeating unit was analyzed by gas-liquid chromatography-mass spectrometry as described in detail elsewhere (8). Uronic acid residues of OS were methylated and then reduced with sodium borodeuteride. Deuterium - labeled, carboxyl reduced OS were hydrolyzed with trifluoroacetic acid. Monosaccharides were then reduced with sodium borohydride, and the resulting alditols were peracetylated. The ratio of glucose to glucuronic acid was determined by selective ion monitoring of the ions m/z 217 and 219 derived from the unlabeled and deuterium - labeled glucitol hexa-acetates, respectively.

RESULTS

Symbiotic phenotype. Typical root hair deformation responses on Trifolium repens var. Louisiana Nolin induced by wild type, pSym$^-$, and mutant strains in regions I, II, and III in slide cultures are shown in Figure 2.

Trifoliin A - binding activity. R. trifolii 851 (nod (I)::Tn5) and R. trifolii 845 (pSym$^-$) had significantly lower abilities to bind the clover lectin trifoliin A than the wild type strain in both in situ and ex-planta assays (Table 2 and Figure 3). When the cells in the in situ assay with seedlings were compared by FITC-epifluorescence and phase contrast microscopy, three subpopulations could be distinguished, based on the distribution of trifoliin A bound to the bacterial surface. Wild-type cells either bound trifoliin A uniformly, at one cell pole, or were unreactive. The relative proportions of these subpopulations for the wild-type strain, compared to R. trifolii 851 and to R. trifolii 845 in the root environment are presented in Table 2. Interestingly, neither mutant bound trifoliin A at one cell pole, unlike the wild-type strain. The low level of lectin binding ability of R. trifolii 851 in the ex planta assay was transient with culture age as it was for the wild type strain (Figure 3). Binding of trifoliin A to the pSym$^-$ mutant strain 845 was negative (Figure 3).

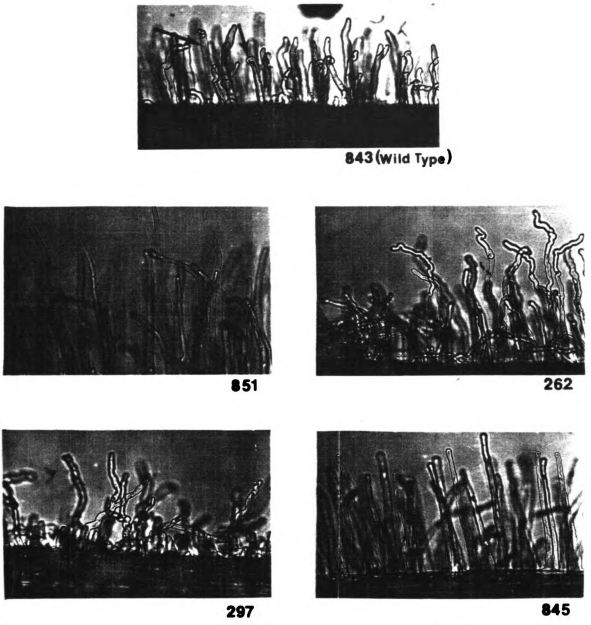


FIGURE 2. Typical root hair response induced by wild type and mutant strains. Strains are R. trifolii 843 (wild type), R. trifolii 851 (nod I::Tn5), R. trifolii 262 (nod II::Tn5), R. trifolii 297 (nod III::Tn5), and R. trifolii 845 ($pSym^-$).

TABLE 2. *In situ* binding of trifoliin A to *R. trifolii* in clover slide cultures.

Strain	% of cells with bound trifoliin A[a] ($\bar{x} \pm$ s. d.)		
	Uniform	Polar	Unreactive
ANU 843 Hac^+Nod^+	11.1±2.3	24.7±2.6	64.2±3.8
ANU 851 Hac^-Nod^-	1.8±1.5	0.0±0.0	97.2±2.6
ANU 845 Hac^-Nod^-	0.0±0.0	0.3±0.7	99.7±0.8

[a] $2x10^7$ bacteria were inoculated per seedling of *T. repens* var. Louisiana Nolin. Cells were removed from the root environment after incubation for 7 days and heat-fixed to slides. Trifoliin A bound to cells was detected by indirect immunofluorescence.

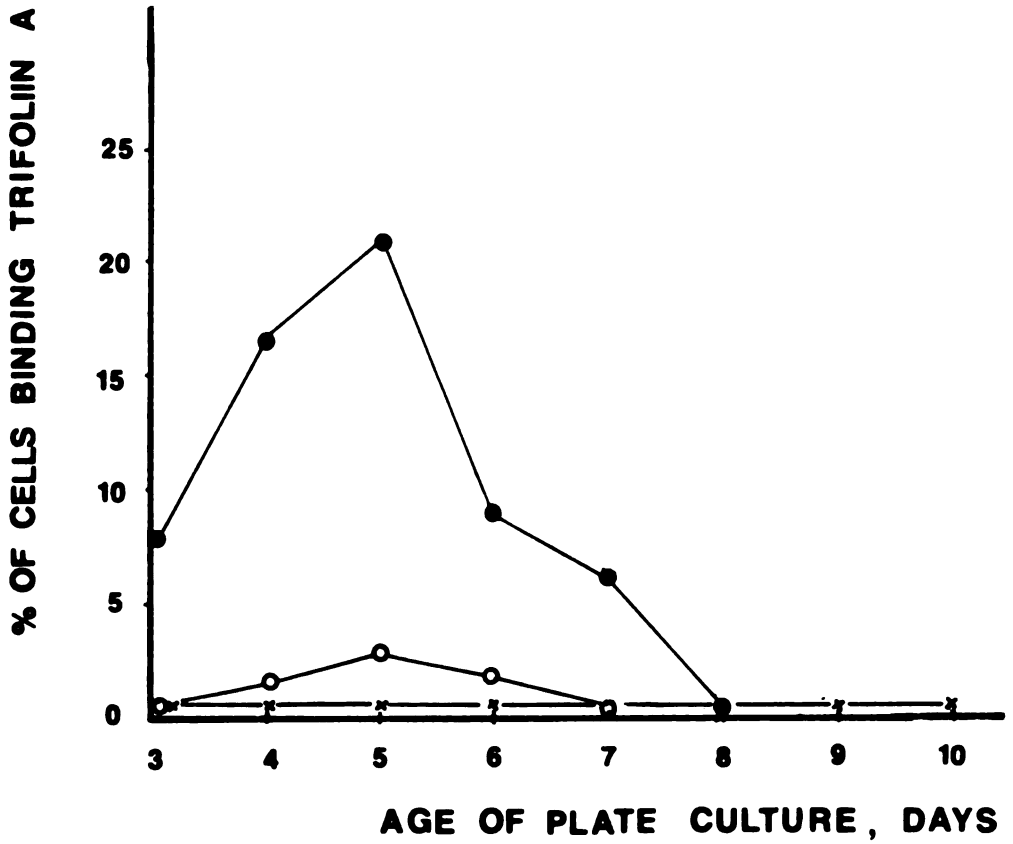


FIGURE 3. Ex-planta binding of trifoliin A to wild type and symbiotically defective mutants of R. trifolii 843. Cells were grown on BIII agar plates for 3 to 10 days and heat fixed to slides. Binding to trifoliin A was examined by indirect immunofluorescence and recorded as the percentage of cells showing fluorescence. (●) R. trifolii 843 (wild type), (o) R. trifolii 851 (nod (I)::Tn5), and (✖) R. trifolii 845 (pSym$^-$).

Kinetic study of CPS depolymerization. PD-I depolymerase enzyme was saturated up to one fourth (0.04mg/ml) of the CPS concentration used in the assays and the increase in absorbance of oligosaccharide products at 235 nm was proportional to the amount of enzyme employed. Completion of depolymerization occurred in approximately 100 minutes for all the CPS substrates. Results of a typical kinetic experiment are shown in Figure 4, illustrating that PD-I depolymerized CPS of some mutant strains faster (e.g. 851) and other mutant strains slower (e.g. 252) than CPS of the wild type strain. Initial depolymerization rates of CPS from different mutant strains are shown in Table 3. To compare rates quantitatively, data were subjected to a one-way analysis of variance using the F distribution (Table 3). The depolymerization rate of CPS from strain 252 was significantly lower ($P<0.05$), whereas that for CPS of strains 851 ($P<0.025$), 274 ($P<0.10$) and 845 ($P<0.10$) were significantly higher than those of wild type CPS (Table 3).

^{1}H-NMR analysis of oligosaccharides. The ^{1}H-NMR spectrum of isolated OS from plate-grown R. trifolii 843 (Figure 5) indicated the presence of acetate esters (δ1.88-2.15), ketal-linked pyruvate (δ1.23-1.43) and ether-linked 3-hydroxybutanoic acid (δ1.03-1.17 and 2.36-2.58). The levels of pyruvic, acetic, and 3-hydroxybutanoic acid substitutions per CPS oligosaccharide repeating unit are presented in Table 4. Strain 252 had more pyruvic and acetic, strain 246 had more pyruvic, strain 851 had less pyruvic, and strain

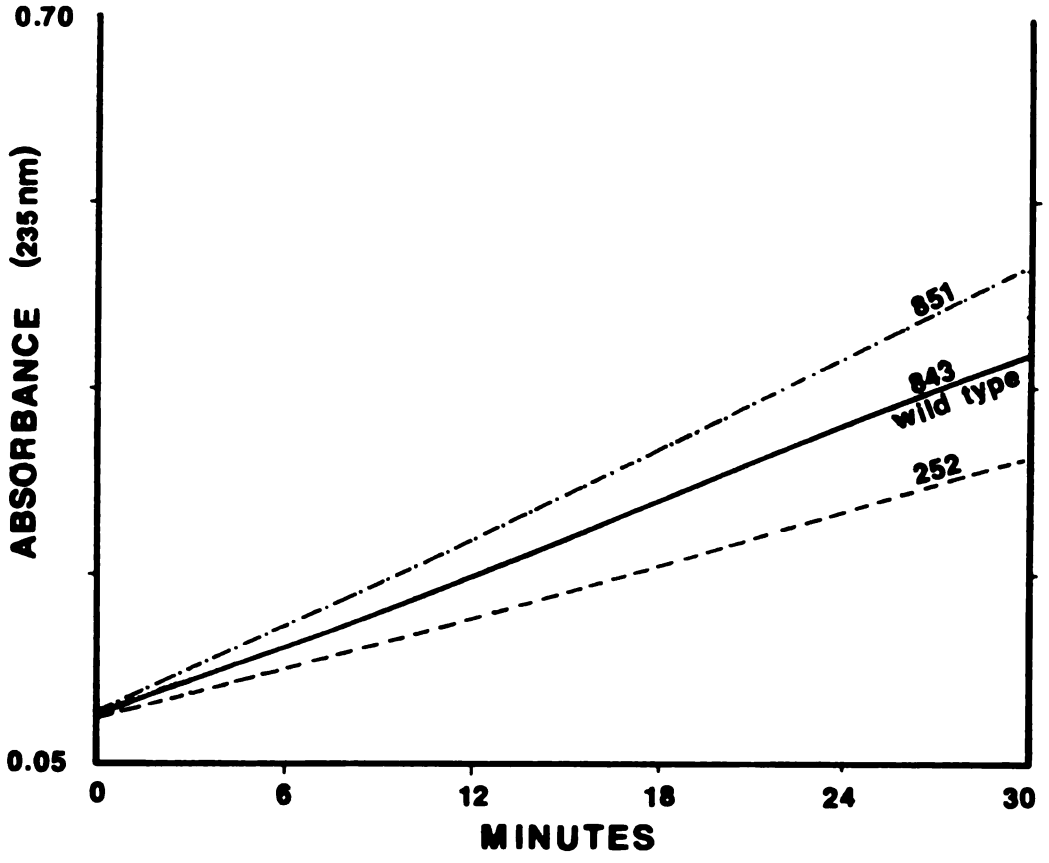


FIGURE 4. Kinetic study of the relative rates of depolymerization of CPS from R. trifolii 843 and mutant strains in region I, by PD-I enzyme under identical conditions. Strains are R. trifolii 843, R. trifolii 252 (nod A::Tn5) and R. trifolii 851 (nod D::Tn5).

TABLE 3. Depolymerization rates of CPS from R. trifolii 843 (wild type) and Tn5 mutant strains in three nodulation regions using enzyme PD-I.

Strain[a]	No. of replicates	Initial rate[b] mean ± s.d.[c]	Significant difference[d]
843 (wild type)	7	48 ± 5	-
277 (I)	3	52 ± 3	No
252 (I)	2	34 ± 3	Yes ($P<0.05$)
246 (I)	2	55 ± 5	No
274 (I)	3	56 ± 5	Yes ($P<0.10$)
851 (I)	5	59 ± 6	Yes ($P<0.025$)
262 (II)	4	50 ± 1	No
297 (III)	4	52 ± 2	No
845 ($pSym^-$)	3	56 ± 2	Yes ($P<0.10$)

[a] Nodulation region with Tn5 insertion is shown in parentheses.

[b] Expressed as 10^4x Abs 235 nm/min.

[c] Standard deviation of the mean.

[d] All data were subjected to a one-way analysis of variance using the F distribution. The indicated probabilities of chance were considered indicative of a significant difference with R. trifolii 843 (wild type).

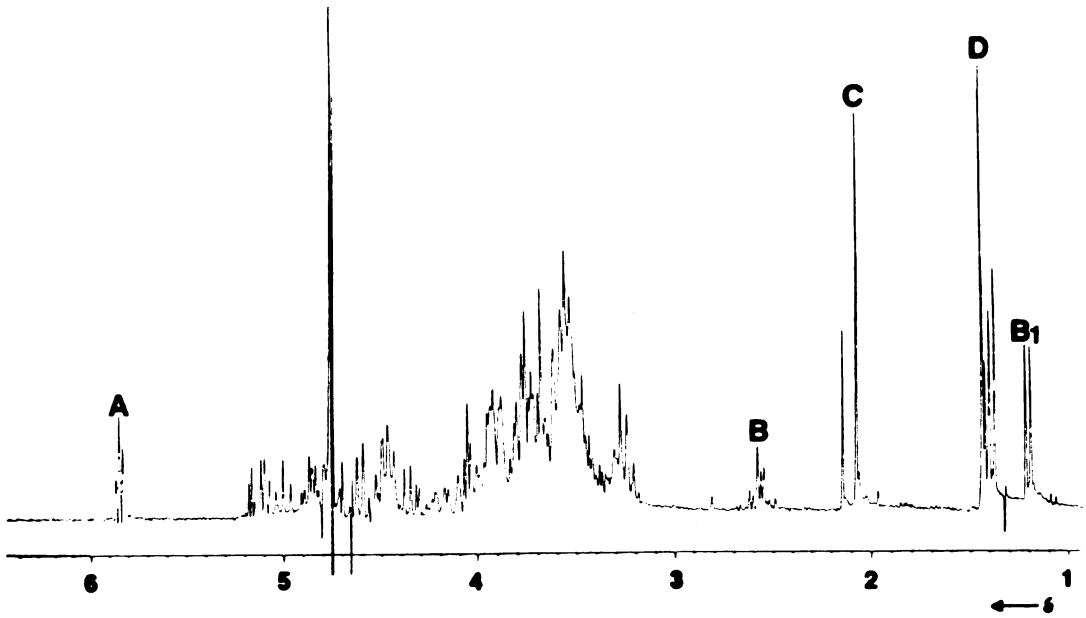


FIGURE 5. A ^{1}H-NMR spectrum of the oligosaccharides produced by depolymerization of CPS from a 5-day-old culture of R. trifolii 843, using PD-I enzyme. Peaks represent (A) H-4 of 4-deoxy-L-threo hex-4-enopyranosyluronic acid, which results from β-elimination of glucuronic acid catalyzed by PD-I, (B) methylene and (B_1) methyl of 3-hydroxy butanoic acid, (C) acetate, and (D) pyruvate protons.

TABLE 4. Non-carbohydrate composition of oligosaccharides obtained by PD-I depolymerization of CPS from *R. trifolii* 843 and mutant strains in three nodulation regions.

R. trifolii strain	Molar proportion of non-carbohydrate components per oligosaccharide repeating unit[a] (± 0.05)		
	Pyruvic acid	Acetic acid	3-hydroxybutanoic acid
ANU 843 (wild type)	1.58	0.93	0.54
ANU 277 (I)	1.55	0.81	0.57
ANU 252 (I)	2.79	1.41	0.62
ANU 246 (I)	1.99	1.09	0.57
ANU 274 (I)	1.54	0.77	0.54
ANU 851 (I)	1.12	0.89	0.49
ANU 262 (II)	1.57	0.92	0.54
ANU 297 (III)	1.57	0.96	0.54
ANU 845 ($pSym^-$)	1.38	0.74	0.60

[a] Values were obtained from ^{1}H-NMR spectroscopy by comparing the integrals for the various groups with that of the single C-4 proton of the unsaturated terminal sugar in each oligosaccharide.

845 had less pyruvic and acetic substitutions, respectively than CPS of the wild type strain. The level of substitutions for OS of other strains was similar to that of the wild type strain.

Glycosyl composition analysis. Gas chromatographic and combined GLC-MS analysis of the wild type oligosaccharide repeating unit indicated that it contained glucose, glucuronic acid, and galactose. The ^{1}H-NMR spectra of the oligomers displayed a downfield multiplet with the same chemical shift at δ 5.8-5.9 as the signal identified as 4-deoxy-L-threo-hex-4-enopyranosyluronic acid in R. trifolii 0403 oligosaccharides produced during β-lyase cleavage of the CPS at glucuronic acid (8). The molar ratio of glycosyl components is (Glu:GluUA:Gal). (5:2:1).

DISCUSSION

Our results showed that CPS of some Tn5 mutant strains of R. trifolii 843 in nodulation region I are altered in levels of pyruvate and acetate non-carbohydrate substitutions relative to wild type CPS. However, several strains had the same levels of these substitutions as the wild type strain, indicating that the entire procedure of CPS isolation, depolymerization into OS and ^{1}H-NMR spectroscopy to quantitate the non-carbohydrate substitutions is very reliable and that these levels are unaffected by the presence of Tn5 genes themselves. 851 CPS had less pyruvate, 246 CPS had more pyruvate and 252 CPS had more pyruvate and more acetate, respectively, than wild type CPS. This indicated that different locations of Tn5 insertions within region I have a different effect on the levels of pyruvate and acetate substitutions. This is consistent with the finding of four nodulation genes within this region for R. trifolii, R. meliloti and R. leguminosarum (4, 6, 9, 10, 12, and T. T. Egelhoff et al., in press). In addition to having different levels of non-carbohydrate substitutions, 851 CPS, 252 CPS, and 845 CPS also displayed different depolymerization rates with PD-I from that of wild type CPS. This is consistent with previous work showing that the activities of CPS depolymerase enzymes

are sensitive to pyruvate (PD-I enzyme) (Chapter I) and acetate (PD-II enzyme) (8) non-carbohydrate substitutions. Therefore, differences between CPS from wild type and mutants can be established based on alterations in depolymerization rates with enzyme PD-I and/or the levels of non-carbohydrate substitutions measured by ^{1}H-NMR. However, the fact that these quantities are the same for CPS of some mutants does not automatically mean that the polysaccharides are the same. ^{1}H-NMR data are only a quantitative measurement of CPS non-carbohydrate substitutions. ^{13}C-NMR would provide further information about CPS structure. CPS with the same depolymerization rates by enzyme PD-I may have differences to which the enzyme is not sensitive. Lectin binding assays showed that mutant strains 851 and 845 with altered CPS were also significantly less reactive with trifoliin A as compared with the wild type strain.

The levels of CPS non-carbohydrate substitutions in some of the mutants may be affected because of different levels of the corresponding transferase enzymes responsible of their incorporation into the CPS, and this study will be addressed in Chapter III.

Since the levels of pyruvate and acetate in CPS from mutant strains with Tn5 insertions in nodulation region I are different from wild type levels we conclude that there must be some expression of essential nod genes in R. trifolii 843 grown in defined BIII medium in absence of the host plant. However, our studies with root exudate (Chapter

III) suggest that some nod genes expression is affected by clover root exudate.

We conclude that Tn5 insertions in certain nod genes within region I cause changes in the CPS of R. trifolii 843 as demonstrated by quantitative determination of pyruvate, acetate and 3-hydroxybutanoate substitutions by ^{1}H-NMR spectroscopy and kinetic study of CPS depolymerization rates and that functions related to polysaccharide synthesis may be encoded in the sym plasmid.

LIST OF REFERENCES

1. Dazzo, F. B. 1982. Leguminous root nodules, p. 431-446. In R. G. Burns and J. H. Slater (ed.). Experimental Microbial Ecology. Blackwell Scientific Publications, Oxford.

2. Dazzo, F. B., G. L. Truchet, J. E. Sherwood, E. M. Hrabak, and A. E. Gardiol. 1982. Alteration of the trifoliin A- binding capsule of Rhizobium trifolii 0403 by enzymes released from clover roots. Appl. Environ. Microbiol. 44:478-490.

3. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350-356.

4. Djordjevic, M.A., P.R. Schofield, R. W. Ridge, N. A. Morrison, B. J. Bassam, J. Plazinski, J. M. Watson, and B. G. Rolfe. 1985. Rhizobium nodulation genes involved in root hair curling (Hac) are functionally conserved. Plant Molec. Biol. 4:147-160.

5. Downie, J. A., G. Hombrecher, Q. S. Ma, C. D. Knight, B. Wells, and A. W. B. Johnston. 1983. Cloned nodulation genes of Rhizobium leguminosarum determine host-range specificity. Mol. Gen. Genet. 190:359-365.

6. Downie, J. A., C. D. Knight, A. W. B. Johnston, and L. Rossen. 1985. Identification of genes and gene products involved in the nodulation of peas by Rhizobium leguminosarum. Mol. Gen. Genet. 198:255-262.

7. Higashi, S., and M. Abe. 1978. Phage-induced depolymerase for exopolysaccharide of Rhizobiaceae. J. Gen. Appl. Microbiol. 24:143-153.

8. Hollingsworth, R. I., M. Abe, J. E. Sherwood, and F. B. Dazzo. 1984. Bacteriophage-induced acid heteropolysaccharide lyases that convert the acidic heteropolysaccharides of Rhizobium trifolii into oligosaccharide units. J. Bacteriol. 160:510-516.

9. Kondorosi, E., Z. Banfalvi, and A. Kondorosi. 1984. Physical and genetic analysis of a symbiotic region of Rhizolium meliloti: identification of nodulation genes. Mol. Gen. Genet. 193:445-452.

10. Rossen, L., A. W. B. Johnston, J. A. Downie. 1984 DNA sequence of the Rhizobium leguminosarum nodulation genes nod A, B and C required for root hair curling. Nucl. Acids Res. 12:9497-9508.

11. Schofield, P. R., R. W. Ridge, B. G. Rolfe, J. Shine, and J. M. Watson. 1984. Host-specific nodulation is encoded on a 14kb fragment in Rhizobium trifolii. Plant Mol. Biol. 3:3-11.

12. Torok, I., E. Kondorosi, T. Stepkowski, J. Posfai, and A. Kondorosi. 1984. Nucleotide sequence of Rhizobium meliloti nodulation genes. Nucl. Acids Res. 12:9509-9524.

CHAPTER III

RHIZOBIUM TRIFOLII CAPSULAR POLYSACCHARIDE BIOSYNTHESIS: PYRUVYLATION OF LIPID-BOUND SACCHARIDES AS AFFECTED BY TN5 MUTATIONS IN THE SYMBIOTIC PLASMID.

ABSTRACT

An *in vitro* assay to measure the enzymatic incorporation of pyruvate into *R. trifolii* CPS was developed using EDTA-treated cells, UDP-sugar donors, and [1-^{14}C]PEP as pyruvate donor. Pyruvylation occurred at the lipid-bound saccharide intermediate stage. CPS pyruvyl transferase activity (CPT) was measured by incorporation of radioactivity into glycoconjugates soluble in organic solvent using two wild type *R. trifolii* strains (843 and 0403 *rif*), and corresponding symbiotically defective mutant strains having alterations in levels of pyruvate in their CPS. CPS pyruvyl transferase activity was affected by Tn5 mutations in the *sym* plasmid, and clover root exudate increased the level of CPT in *R. trifolii* strains having Tn5 insertions in certain nodulation (Hac) genes.

INTRODUCTION

The biosynthesis of exopolysaccharide or capsular polysaccharide of different genera of bacteria including Klebsiella aerogenes (17), Xanthomonas campestris (7), Acetobacter xylinum (3), Aerobacter aerogenes (14), and Neisseria meningitidis (10), have been studied and shown to involve lipid-bound oligosaccharide intermediates as precursors of the polysaccharide. The key lipid carrier is a C55 polyisoprenol (undecaprenol) called bactoprenol which is linked to the growing carbohydrate chain through an acid-labile phosphodiester bond (8).

Synthesis of (1->2) β-glucan from UDP-[^{14}C] glucose by enzyme preparations of Rhizobium japonicum (5) and Rhizobium phaseoli (2) have been reported. Lipid-bound saccharides formed by incubation of UDP-glucose with a particulate enzyme of Rhizobium meliloti were also studied (11,12).

Pyruvic acid ketal residues are present in many bacterial polysaccharides (16). However, there are very few reports (7) on the in vitro pyruvylation of the polysaccharides, and none for Rhizobium polysaccharides.

Rhizobium trifolii Tn5 mutant strains in different locations of nodulation region I had different levels of pyruvate substitutions in their CPS as compared to the wild type strain R. trifolii 843 when grown on defined culture

medium (Chapter II). R. trifolii 251, a mutant strain of R. trifolii 0403 rif containing a single Tn5 insertion in the sym plasmid, also had higher levels of pyruvate in its CPS than did the wild type strain (Chapter I).

Non-carbohydrate substitutions of R. trifolii 0403 CPS have also been related to its transient lectin-binding ability (1,15, Chapter I, and Chapter II).

In view of the importance of these non-carbohydrate substitutions, we decided to study the enzymatic incorporation of pyruvate into CPS of R. trifolii. A second important objective of this study was to determine if the enzymatic lesion of the mutant strains with different levels of pyruvate in the CPS is at the level of this CPS pyruvyl transferase (CPT) activity. A third objective was to examine the effect of clover root exudate on CPT activity in the wild type and mutant strains.

Results of this study showed that pyruvylation of R. trifolii CPS occurs by a phosphoenol pyruvate donor at the saccharide-lipid bound intermediate stage. CPS pyruvyl transferase activity is affected by Tn5 mutations in the sym plasmid and clover root exudate. Portions of this work were presented at the 6th International Symposium on Nitrogen Fixation, Oregon State University, Corvallis, OR, Aug. 4-9, 1985.

MATERIALS AND METHODS

Bacterial strains. R. trifolii ANU 843 (wild type) and corresponding mutant strains (ANU 252, 246, and 851) were obtained from Dr. B. Rolfe, Australian National University, Canberra, Australia, (Chapter II). R. trifolii 252 has a Tn5 insertion in the coding region of nod A gene of nodulation region I and has a $Hac^{-}Nod^{-}$ phenotype. R. trifolii 246 with a Tn5 insertion before the coding region of nod A gene has a $Hac^{+}Nod^{+}$ phenotype. R. trifolii 851 has a Tn5 insertion in the coding region of nod D gene and has a $Hac^{-}Nod^{-}$ phenotype. R. trifolii 0403 rif (wild type) was obtained from K. Nadler, Michigan State University, East Lansing. R. trifolii 251, a mutant strain of R. trifolii 0403 rif, has a Tn5 insertion in the sym plasmid and has a $Hac^{+}Fix^{\pm}$ phenotype.

CPS pyruvylation assay (A) Enzyme preparation. Cells were grown in BIII broth for 36h to stationary phase (120 Klett units in a Klett-Summerson colorimeter with a No. 66 filter), centrifuged at 12,000 x g for 30 min, and washed with BIII medium and with 10 mM EDTA-Tris buffer, (pH 8.0). The pellet was resuspended with one volume of buffer and this cell suspension was frozen at -80°C and thawed at room temperature eight times (hereafter called EDTA-treated cells) (11). For root exudate treatments, cells grown to

stationary phase were then centrifuged aseptically and resuspended in BIII broth without C source (neither mannitol nor glutamate) containing 70 ml of 14d white clover root exudate (0.1 mg/ml protein) and shaken at 30°C for 24 hours. Cell pellets were then washed and EDTA-treated as described. Proteins were measured by the method of Lowry (9) with a standard of bovine serum albumin.

(B) Enzyme assay. Enzymatic incorporation of pyruvate into CPS was measured with an in vitro assay modified from the method of Tolmasky et al. (11). The standard mixture (total volume of 0.05 ml) contained: 70 mM Tris-HCl buffer, (pH 8.2), 8 mM $MgCl_2$, 40 mM 2-mercaptoethanol, EDTA-treated cells (0.4-1 mg protein), 0.3 mM UDP-Glucose, 0.15 mM UDP-Galactose, 0.15 mM UDP-Glucuronic acid, and 0.57 mM [1-^{14}C] phosphoenol pyruvic acid (15 mCi/mmol) as cyclohexylammonium salt (PEP) (Amersham). The reaction was performed in screw-capped plastic tubes kept at 15°C in a water bath for 20 min and stopped by adding 0.5 ml of 70 mM Tris-HCl buffer (pH 8.2) containing 5mM EDTA. The mixtures were centrifuged at 12,000 x g at 4°C (Eppendorf microfuge) and the pellets washed twice with buffer without EDTA. The supernatants were combined and the pellets were extracted twice with 0.2 ml each of chloroform: methanol: water (1: 2: 0.3) (7). Samples of this extract containing lipid-bound oligosaccharides (LBO) were mixed in liquid scintillation mixture (Scinti Verse II; Fisher Scientific Co., Pittsburgh, Pa.) and counted in an LS 7000 Liquid Scintillation Counter (Beckman

Instruments, Irvine, Cal.). Enzymatic activity was expressed as radioactivity (cpm) incorporated into LBO extract in 20 min per mg of protein.

Incorporation of radioactivity into lipid-bound saccharides. The standard incubation mixture without PEP was used to measure incorporation of different labeled UDP-sugar donors into LBO. 0.017 mM UDP-[U-^{14}C]Glucose (293 mCi/mmol), 0.017 mM UDP-[U-^{14}C]Galactose (297 mCi/mmol), and 0.019 mM UDP-[U-^{14}C]Glucuronic acid (269 mCi/mmol) were included separately in the incubation mixtures in which the corresponding cold UDP-sugar was omitted.

Analysis of labeled product obtained from [1-^{14}C]PEP - Mild acid hydrolysis. The incubation mixture using [1-^{14}C]PEP and R. trifolii 843 EDTA-treated cells grown in defined culture medium was scaled-up 30 fold. LBO extract was dried under a stream of nitrogen at room temperature. Mild acid hydrolysis of the pyrophosphate bridge between the lipid and the saccharide was performed in 0.01 N HCl for 10 min at 100°C, and the solution was partitioned in chloroform: methanol: water (3: 2: 1) (11). The aqueous (top) and organic (bottom) phases were separated for further studies. Radioactivity of aliquots was measured.

Analysis of aqueous phase soluble moiety. (A) Column chromatography. Gel filtration chromatography of radioactive moiety soluble in aqueous phase was performed at room temperature in a column (100 x 2 cm) of Bio-Gel P6 (100-200 mesh). The solvent was 0.1 M pyridine acetate (pH 5.0), and

the flow rate was 8 ml/h. Fractions of 3 ml were collected and aliquots of 0.5 ml were mixed in liquid scintillation mixture (Scinti Verse II) and monitored for radioactivity in an LS 7000 Liquid Scintillation Counter. Blue dextran was used to determine the void volume. Elution of unlabeled saccharide standards (isolated octasaccharide from R. trifolii 843 (Chapter II) and lactose) were detected in fractions by the phenol-sulfuric acid method (6).(B) Depyruvylation. Solvent in Fractions I, III, and IV was evaporated under a nitrogen current and the saccharide residues were depyruvylated at pH 2 (HCl) for 90 min at 100°C. Samples were then neutralized, evaporated under a nitrogen current, and redissolved in water. Components were separated by paper chromotography on Whatman No. 1 paper using the solvent n-butanol: propionic acid: water (21: 11: 14). Standards consisting of [1-^{14}C]pyruvate and sodium pyruvate were treated under identical conditions. Radioactivity on the paper was detected with a radiochromatogram scanner, and unlabeled compounds by the alkaline silver nitrate method (13).

Preparation of root exudate. Seedling roots of Louisiana Nolin var. of white clover were grown axenically through agar blocks supported above stainless steel wire mesh into nitrogen-free Fahraeus medium (4). This design protects the rooting medium from contaminating seed exudates. Additional medium was added when necessary to keep its volume constant at 50 ml during the incubation period.

After 14 days of plant growth, the root exudates were collected and used immediately.

RESULTS

Conditions for maximal pyruvylation of lipid-bound saccharides. The conditions for maximal CPT enzymatic activity (incorporation of pyruvate into lipid-bound saccharides) measured as the incorporation of radioactivity from [1-^{14}C] PEP into glycoconjugates soluble in chloroform: methanol: water (1: 2: 0.3) were investigated for R. trifolii 843 EDTA-treated cells grown in defined culture medium. Incorporation of radioactivity was proportional to the amount of enzyme preparation in the range of protein concentration used. The extent of reaction was linear with incubation time up to 30 minutes. The optimal temperature was 15°C and the optimal pH was 8.2. The concentration of UDP-sugar donors used was saturating and the addition of cold PEP to the incubation mixture decreased the incorporation of radioactivity to background levels. Controls consisting of enzyme preparations inactivated by boiling gave negative results.

Incorporation of radioactivity into LBO from labeled UDP-sugars. There was incorporation of radioactivity into lipid-bound saccharides from each of the three labeled UDP-sugar donors used separately in the incubation mixture, UDP-[^{14}C]Glucose, UDP-[^{14}C]Galactose, or UDP-[^{14}C]Glucuronic acid (Table 1).

TAB

sac

suc

UD

UD

UD

UD

a

tr

ir

b

g

w

V

TABLE 1 . Incorporation of radioactivity into lipid-bound saccharides in cells of R. trifolii 843 from labeled UDP-sugars.[a]

UDP-[^{14}C] sugar	Activity[b]
UDP-[^{14}C] Glucose	1499
UDP-[^{14}C] Galactose	2953
UDP-[^{14}C] Glucuronic acid	12746

[a] Assay was performed as described in the text with EDTA-treated cells grown in defined culture medium and the indicated UDP-[^{14}C] sugar donor.

[b] Expressed as the radioactivity (cpm) incorporated into glycoconjugate product soluble in chloroform: methanol: water (1: 2: 0.3) in 20 min per 0.6 mg of cellular protein. Values are average of three replicates.

Mild acid hydrolysis. Labeled product obtained in an incubation mixture containing [1-^{14}C]PEP scaled-up 30-fold was treated by mild acid to cleave the acid-labile pyrophosphate bond linking the lipid carrier to the carbohydrate. This resulted in transfer of all the radioactivity to the aqueous phase when the reaction system was partitioned with chloroform: methanol: water (3: 2: 1) (Table 2).

Analysis of the radioactive moiety soluble in the aqueous phase after mild acid hydrolysis. Gel permeation chromatography of the cleaved radioactive products soluble in aqueous phase (24,000 cpm) through Bio-Gel P6 gave an elution profile shown in Figure 1. Four radioactive peaks were detected. Two peaks (I and II) eluted between the octasaccharide and lactose standards; two other peaks with significantly more radioactivity (III and IV) eluted after lactose. Acid hydrolysates of fractions I, III, and IV were examined by paper chromatography and shown to contain labeled pyruvate.

Pyruvylation of lipid-bound saccharides. CPT activities in EDTA-treated cells of R. trifolii 843 and mutant strains are shown in Table 3. All the data were subjected to a one-way analysis of variance using the F distribution. For cells grown in defined culture medium only, strain 246 showed higher and strain 851 lower CPT activity than wild type strain. The difference between 246 and 851 was statistically significant at a probability of chance $P<0.10$. For cells

TABLE 2. Mild acid hydrolysis of labeled product.

	Radioactivity (cpm)	% recovery
Labeled product[a]	36,000	100
Aqueous phase[b]	37,000	102

[a] LBO extract.

[b] Radioactivity in aqueous phase after mild acid hydrolysis and partition in chloroform: methanol: water (3: 2: 1).

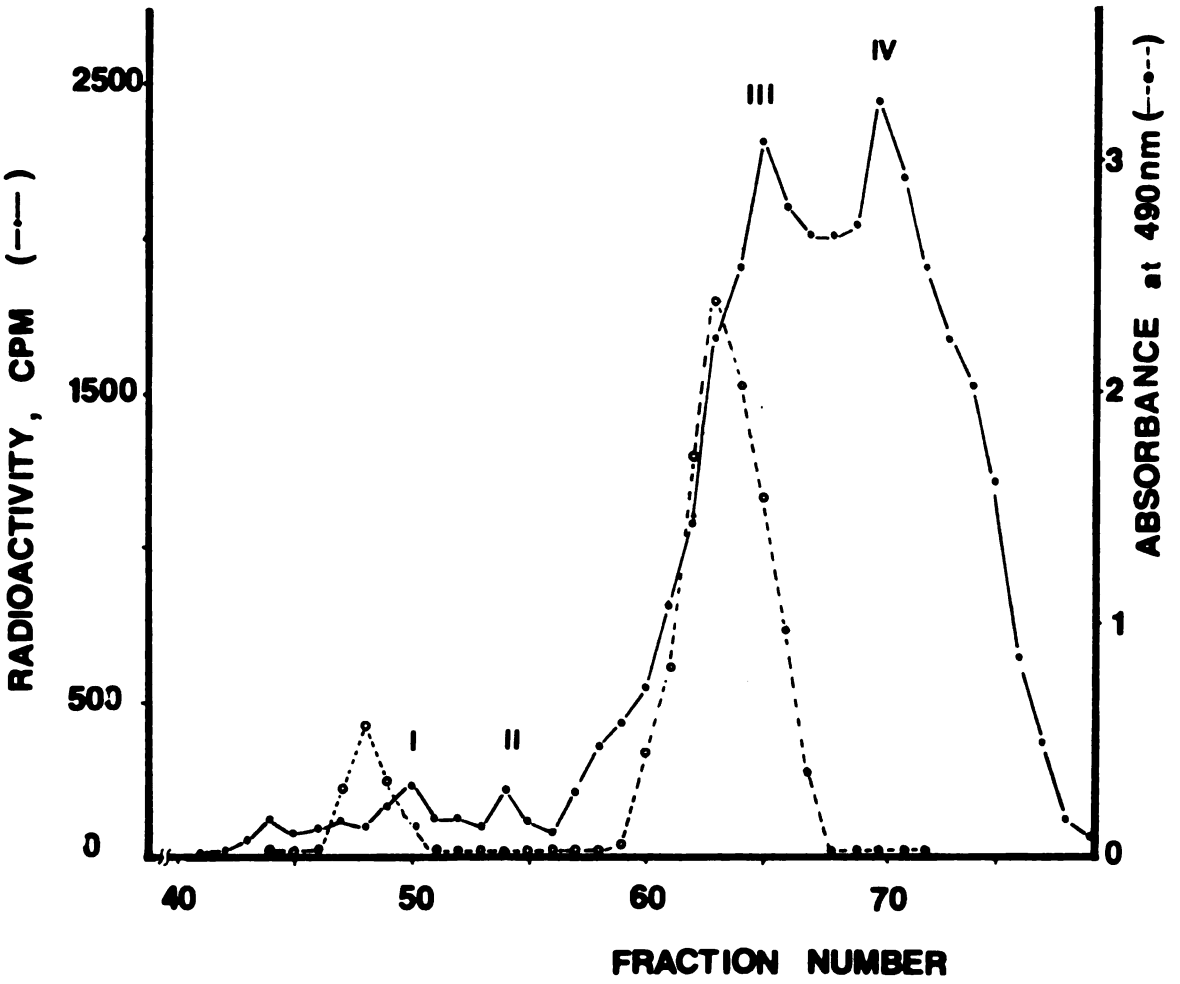


Figure 1. Gel filtration of the radioactive moiety soluble in aqueous phase using Bio-Gel P6. Fractions were collected and aliquots monitored for radioactivity. Unlabeled saccharides were determined by the phenol-sulfuric acid method.

TABLE 3. Pyruvylation of lipid-bound saccharides in cells of *R. trifolii* 843 (wild type) and Tn5 mutant strains[a].

Strain[b]	Pyruvic acid per oligosaccharide repeating unit[c]	Activity[d] ($\bar{x} \pm$ s.d.) -RE[e]	+RE[f]
843 (wild type)	1.58	303±30	295±15
246 (I)	1.99	326±55	447±50
252 (I)	2.79	305±16	374±39
851 (I)	1.12	250±10	286±10

[a] Enzyme assay was performed with EDTA-treated cells, UDP-sugar donors and [1 - ^{14}C]PEP.

[b] Nodulation region with Tn5 insertion is shown in parentheses.

[c] Values were obtained from ^{1}H-NMR spectroscopy by comparing the integrals for the various groups with that of the single C-4 proton of the unsaturated terminal sugar in each oligosaccharide (Chapter II).

[d] Pyruvylation enzyme activity is expressed as the radioactivity (cpm) incorporated into glycoconjugates soluble in chloroform: methanol: water (1: 2: 0.3) in 20 min per 0.4 mg of protein. Values are average of three replicates.

[e] Cells grown to stationary phase in defined culture medium.

[f] Cells grown to stationary phase were then treated for an additional 24h with clover root exudate (RE).

treated with root exudate, strains 246 and 252 had higher CPT activities than did the wild type strain. Treatment of cells of strains 246 and 252 with root exudate significantly increased their CPT activities at $P<0.05$ whereas CPT activities of strains 843 and 851 were unaffected by root exudate. Strain 251 had higher levels of CPT activity than did the corresponding wild type strain *R. trifolii* 0403 *rif* (Table 4).

TABLE 4. Pyruvylation of lipid-bound saccharides in cells of *R. trifolii* 0403 *rif* (wild type) and *R. trifolii* 251.[a]

Strain[b]	Pyruvic acid per oligosaccharide repeating unit[c]	Activity[d]
0403 *rif* (wild type)	1.74	518
251	2.04	685

[a] Enzyme assay was performed with EDTA-treated cells grown to early stationary phase in defined culture medium, UDP-sugar donors and [1-^{14}C]PEP.

[b] Strain 251 has a single Tn5 insertion in the *sym* plasmid of strain 0403 *rif*.

[c] Values were obtained from ^{1}H-NMR spectroscopy by comparing the integrals for the various groups with that of the single C-4 proton of the unsaturated terminal sugar in each oligosaccharide (Chapter I).

[d] Pyruvylation enzyme activity is expressed as the radioactivity (cpm) incorporated into glycoconjugate product soluble in chloroform: methanol: water (1: 2: 0.3) in 20 min per mg of protein. Values are average of three replicates.

DISCUSSION

We have evidence of consistent differences in the levels of pyruvate substitutions in CPS of certain Tn5 mutant strains of R. trifolii 843 with Tn5 insertions in the nodulation region I (Hac) (Chapter II). R. trifolii 251, a mutant strain of R. trifolii 0403 rif containing a single Tn5 insertion in the sym plasmid, also had higher levels of pyruvate in its CPS than the CPS of the corresponding wild type strain. In R. trifolii 0403 the level of non-carbohydrate substitutions in the CPS have an important effect on lectin binding ability (1,15).

In this work we have developed an in vitro assay to measure the enzymatic incorporation of pyruvate into CPS. EDTA-treated cells, UDP-sugar donors and [1-^{14}C] phosphoenol pyruvate as the source of pyruvate, were used in the in vitro assay. There was no incorporation of label into glycoconjugate product soluble in chloroform: methanol: water (1: 2: 0.3) when 4-fold concentration of cold PEP was added to the incubation mixture containing labeled PEP. This indicates that PEP radioactivity is incorporated into this product. By using PEP labeled at C1, we eliminated possible incorporation of label from acetate metabolism. Radioactivity was incorporated into the glycoconjugate product when either UDP-[^{14}C]Glucose, UDP-[^{14}C]Galactose, or

UDP-[^{14}C]Glucuronic acid were used in the incubation mixtures without PEP (Table 1) indicating that the saccharide moiety of the CPS is synthesized from these three UDP-sugar donors. The difference in level of incorporation using each different labeled UDP-sugar precursor probably reflects a different specific activity of the internal pool sizes with UDP-glucuronic acid being the highest since this precursor is not present in the LPS, cyclic β1-2 glucan or cellulose polymers made by this bacterium. In addition, the label incorporated from [1-^{14}C]PEP into product was 3-fold lower when cold UDP-sugar donors were not included in the incubation mixture (data not shown). Incorporation of ^{14}C from PEP into an organic solvent soluble phase indicates that pyruvylation occurs at the lipid carrier intermediate stage of CPS biosynthesis. The labeled glycoconjugate product soluble in organic solvent was hydrolyzed by mild acid to liberate the carbohydrate moiety from the lipid bound carrier. This resulted in the transfer of all the radioactivity to the aqueous phase when partitioned in chloroform: methanol: water (3: 2: 1) (Table 2), indicating that the label was incorporated into the aqueous-soluble carbohydrate moiety of the glycoconjugate product and not the lipid carrier itself. Gel filtration chromatography (Figure 1) of the radioactive products soluble in aqueous phase indicated that pyruvate was incorporated into several products eluting after the oligosaccharide repeating unit of R. trifolii 843 CPS. This indicated that pyruvylation occurs

at several different steps in the polysaccharide biosynthetic pathway before the lipid-bound oligosaccharide is completed. Radioactive pyruvate was detected by paper chromatography after depyruvylation of fractions I, III, and IV proving that pyruvate is the labeled moiety in these various sized fractions.

We examined two mutant strains (246 and 252) having more pyruvate substitutions and one strain (851) having less pyruvate substitutions in their CPS than the wild type strain, R. trifolii 843. As anticipated, all of the strains had CPT activity (Table 3). In the absence of root exudate, there were small differences in CPT activity between the wild type and the three mutant strains, but these were not statistically significant. However, the difference in CPT between strains 246 and 851 was statistically significant. Clover root exudate treatment did not change the levels of pyruvyl transferase activity of either wild type 843 and mutant strain 851. However, treatment of mutants 246 with Tn5 insertion before the coding region of nod A gene and 252 with Tn5 insertion within nod A gene did result in statistically significant increase of pyruvyl transferase activity as compared to cells grown in defined medium. Interestingly, R. trifolii 251, a mutant strain from a different wild type strain, R. trifolii 0403 rif which has more pyruvate in its CPS than wild type CPS had a correspondingly higher level of CPS pyruvyl transferase activity than the wild type strain (Table 4). This suggests

that other loci in the megaplasmid are also affecting CPS pyruvylation.

We conclude that pyruvylation of CPS in R. trifolii occurs from phosphoenol pyruvate at lipid-bound saccharide intermediate stages. CPS pyruvyl transferase level is affected by Tn5 insertions in the sym plasmid of R. trifolii 843 and 0403 rif and mutations in the essential nod A gene on this plasmid affect the response of CPS pyruvyl transferase activity to root exudate of its clover host.

LIST OF REFERENCES

1. Abe, M., J. E. Sherwood, R. I. Hollingsworth, and F. B. Dazzo. 1984. Stimulation of clover root hair infection by lectin-binding oligosaccharides from the capsular and extracellular polysaccharides of Rhizobium trifolii. J. Bacteriol. 160: 517-520.

2. Amemura, A. 1984. Synthesis of (1->2)-β-D-Glucan by cell-free extracts of Agrobacterium radiobacter IFO 12665b1 and Rhizobium phaseoli AHU 1133 Agric. Biol. Chem. 48(7): 1809-1817.

3. Couso, R. O., L. Ielpi, R. C. Garcia, and M. A. Dankert. 1982. Synthesis of polysaccharides in Acetobacter xylinum. 123:617-627.

4. Dazzo, F. B . 1982. Leguminous root nodules, p. 431-446. In R. G. Burns and J. H. Slater (ed.). Experimental Microbial Ecology. Blackwell Scientific Publications, Oxford.

5. Dedonder,R. A. and W. Z. Hassid, 1964. The enzymatic synthesis of a (β-1,2-)-linked glucan by an extract of Rhizobium japonicum. Biochim. Biophys. Acta 90:239-248.

6. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350-356.

7. Ielpi, L., R. O. Couso, and M. A. Dankert. 1981. Xanthan gum biosynthesis. Biochem. Biophys. Res. Commun. 102:1400-1408.

8. Lennarz, W. J. and H. G. Scher. 1972. Metabolism and function of polyisoprenol sugar intermediates in membrane-associated reactions. Biochim. Biophys. Acta 265:417-441.

9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

10. Masson, L., and B. E. Holbein. 1985. Role of lipid intermediate(s) in the synthesis of serogroup B Neisseria meningitidis capsular polysaccharide. J. Bacteriol. 161:861-867.

11. Tolmasky, M. E., R. J. Staneloni, R. A. Ugalde, and L. F. Leloir. 1980. Lipid - bound sugars in Rhizobium meliloti. Arch. Biochem. Biophys. 203:358-364.

12. Tolmasky, M. E., Staneloni, R. J. and Leloir, L. F. 1982. Lipid - bound saccharides in Rhizobium meliloti. J. Biol. Chem. 257:6751-6757.

13. Trevelyan, W. E., D. P. Procter, and J. S. Harrison, (1950). Detection of Sugars on Paper Chromatograms. Nature (Lond.) 166:444-445.

14. Troy, F. A., F. E. Frerman, and E. C. Heath. 1971. The biosynthesis of capsular polysaccharide in Aerobacter aerogenes. J. Biol. Chem. 246:118-133.

15. Sherwood, J. E., J. M. Vasse, F. B. Dazzo, and G. L. Truchet. 1984. Development and trifoliin A-binding ability of the capsule of Rhizobium trifolii. J. Bacteriol. 159: 145-152.

16. Sutherland, I. W. 1979. Microbial exopolysaccharides. Trends Biochem. Sci. 4:55-59.

17. Sutherland, I. W., and Norval, M. (1970). Synthesis of exopolysaccharide by Klebsiella aerogenes membrane preparations and the involvement of lipid intermediates. Biochemical Journal 120:567-576.

CHAPTER IV

SUCCINATE METABOLISM AS RELATED TO GROWTH, BACTEROID DIFFERENTIATION AND FUNCTION IN *RHIZOBIUM MELILOTI*

ABSTRACT

The effect of succinate metabolism on growth and the *"in vitro"* and *in vivo* bacteroid differentiation and function of *Rhizobium meliloti* was investigated with a wild type strain (L5-30), a succinate-dehydrogenase mutant strain (UR6), and a spontaneous revertant strain (UR7).

Meristematic nodules induced by strains L5-30 and UR7 on alfalfa plants were effective (Fix^+) in acetylene reduction, and had typical histologies and ultrastructures. Nodules induced by UR6 were ineffective (Fix^-). In these Fix^- root nodules, three weeks after inoculation, very few transformed bacteroids were observed and premature degeneration and lysis occurred in the central zone. Six weeks after inoculation, the Fix^- nodules were fully senescent and the bacteria were lysed in all zones. In contrast, L5-30 and UR7 bacteroids were typically degenerated only in the normal senescent zone of the Fix^+

nodules. This difference in nodule ultrastructures indicated that UR6 had a Bad$^-$ symbiotic phenotype (defective in bacteroid differentiation).

In vitro effects of succinate on growth and cell morphology in defined culture medium were concentration dependent. At a low concentration (5-10 mM), succinate was utilized preferentially before (5-10 mM) glucose or mannitol and diauxic growth was observed for the wild type strain L5-30 but not for UR6. No changes in cell morphology were detected for any of the strains under these growth conditions. However, at a higher concentration (20 mM), both succinate or malate affected growth in mannitol (27 mM) minimal medium. Under these conditions, growth of L5-30 was inhibited, accompanied by cell elongation and pleomorphism in 15% of the population, and [^{14}C] mannitol uptake and mannitol dehydrogenase activities were lowered. At 20 mM, succinate did not inhibit growth, induce cell pleomorphism, or repress mannitol uptake and mannitol dehydrogenase activities in strain UR6. These results suggest that metabolism of succinate through a complete TCA cycle is necessary for normal differentiation and maintenance of alfalfa bacteroids, as well as for the in vitro effects of succinate on growth and the induction of bacteroid - like cell morphologies in Rhizobium meliloti.

INTRODUCTION

Succinate and malate are abundant organic acids within legume root nodules (9, 18, 33) and succinate is transported and metabolized by free-living bacteria (12, 16, 22, 27) and bacteroids (6, 16, 28, 32) of different Rhizobium species. Organic acids support the highest rate of oxygen respiration by bacteroid suspensions (37) and succinate is a very effective substrate for supporting nitrogen fixation by both free-living Rhizobium species (7, 42) and bacteroids (26, 34). Utilization of tricarboxylic acid intermediates are related to symbiotic effectiveness (1), and Rhizobium mutant strains defective in their uptake and metabolism are ineffective in nitrogen-fixing symbiosis with the host plant (13, 15, 27).

"In vitro" induction of bacteroid - like cell morphology by succinate in R. trifolii (39, 40, 41) and induction of sphere to rod morphogenesis in Arthrobacter crystallopoietes (19) have been described.

The objective of this work was to investigate the effect of succinate metabolism on growth and the in vivo and "in vitro" bacteroid differentiation in Rhizobium meliloti L5-30 (wild type effective strain). We used for this study a succinate dehydrogenase mutant strain (UR6) unable to grow on succinate and ineffective in symbiosis and a spontaneous

revertant strain (UR7) which regained succinate dehydrogenase activity, normal growth phenotype, and was effective in symbiotic nitrogen fixation (15).

The present study of the histology and ultrastructure of alfalfa nodules by combined light and transmission electron microscopy showed that UR6 was released from infection threads but was defective in bacteroid differentiation "in vivo". The effect of succinate metabolism on the "in vitro" induction of bacteroid-like cell morphology was found to be concentration dependent. At low concentration (5-10 mM), succinate was used preferentially to other carbon sources and did not induce bacteroid-like cell morphology. At high concentrations (20 mM) succinate inhibited growth and induced "in vitro" bacteroid formation in the wild type strain but not in the mutant strain unable to grow on succinate.

This work was presented in part at the 5th International Symposium on Nitrogen Fixation, Noordwijkerhout, The Netherlands, August 28 - September 3, 1983 (p. 253).

MATERIALS AND METHODS

Bacterial strains. R. meliloti L5-30 (wild type strain) was kindly provided by J. Denarie. UR6 is a succinate-dehydrogenase mutant strain of R. meliloti L5-30 and UR7 a spontaneous revertant strain. UR6 was unable to grow on succinate, lacked succinate dehydrogenase enzyme activity and induced ineffective nodules on alfalfa plants (15). UR7 regained succinate dehydrogenase enzyme activity, had normal growth phenotype and induced effective nodules in symbiosis with alfalfa plants.

Symbiotic phenotype. Surface-sterilized seeds (8) of Medicago sativa var. Vernal were germinated, transferred to Jensen agar slopes (18) and were inoculated with $5x10^6$ cells per seedling with an inoculum of cells grown for 5 days on minimal medium (MM) (15) mannitol agar slants. The tubes were incubated in a plant growth chamber with a 14-h photoperiod at 22°C (26,900 lux) and 10-h darkness at 20°C. Roots were scored for nodulation every week and plants were assayed for nitrogen fixation by the acetylene reduction technique (8) after 6 weeks. The mutant strain recovered from surface-sterilized nodules had the original $Succ^-$ Str^r phenotype.

Electron and light microscopy of nodules. Nodules were fixed in 4% glutaraldehyde in sodium cacodylate buffer (pH

7.2), post-fixed in 1% OsO_4, dehydrated with ethanol, embedded in Epon 812, and thin sections were stained by the uranyl acetate-lead citrate method (35). Transmission electron microscopy (TEM) was performed with a Philips TEM 300 at 80 kv. Semi-thin sections of Epon embedded material were deposited on glass slides and treated by the basic fuchsin - methylene blue method (17) for direct light microscopy.

Growth conditions. Bacteria were grown in minimal medium containing streptomycin (100 g/ml)(MM) (15) and kept on mannitol MM slants. For growth studies, a standardized inoculum size of 0.5% glycerol-grown cells was inoculated into MM containing the indicated carbon sources. Growth was followed turbidimetrically at 660 nm in side-arm flasks with a Klett-Summerson colorimeter using the No. 66 red filter. Data shown are mean values of three replicate parallel cultures.

Cell-free extract preparation. Cell-free extracts were obtained as described (2) by three 1-min treatments of cell suspensions with a Branson Sonifier instrument.

Protein determination. Protein was measured by the method of Lowry (20) using bovine serum albumin as standard.

Glucose utilization. Glucose was measured (25) in the supernatants of culture samples centrifuged at 12,000 x g.

Succinate utilization. The culture supernatants obtained by 12,000 x g centrifugation were filtered through 0.45-μm-porosity membrane filters (Type HAWP; Millipore

Corp., Bedford, Mass.) and succinate in the filtrates was quantitated on a Water's Associates HPLC with a Bio-Rad HPX - 87 H Organic Acid Analysis column at 50°C using 0.005 N H_2SO_4 as the solvent, and was detected at 210 nm with a Gilson Holochrome UV monitor.

Enzyme activities. Mannitol uptake was measured as previously described (14). The incubation mixture consisted of 0.05 ml of cell suspension ($7x10^9$cells/ml); 0.45 ml of MM; 10 nmol of unlabeled mannitol and 2 nmol of [^{14}C] mannitol (30,000 dpm/nmol). 0.1 ml samples were removed each 45 sec and filtered through 0.45-μm filters (Type HAWP; Millipore Corp., Bedford, Mass.). The filters were washed with 2ml of MM, and the radioactivity retained was counted in an LS 7000 Liquid Scintillation-Counter (Beckman Instruments, Irvine, Cal.) using Scinti Verse II (Fisher Scientific Co., Pittsburgh, Pa.) as the scintillation mixture. Mannitol dehydrogenase activities were measured by following the reduction of NAD^+ at 340 nm in a Gilford updated Beckman model DU spectrophotometer as described (21).

Electron microscopy of cells. 2-ml culture samples were removed at various times, centrifuged at 12,000 x g and the cells washed twice in phosphate-buffered saline (PBS, 10 mM potassium phosphate, 140 mM NaCl, pH 7.2). Cell suspensions deposited on Formvar-coated grids were stained by the glutaraldehyde-ruthenium red-uranyl acetate technique (24) and observed by TEM. The percentage of cells displaying

elongation and/or pleomorphism was recorded from a total of 100 cells examined for each sample.

Oxygen consumption. L5-30 and UR6 strains were grown as shaken cultures in MM broth containing 27 mM mannitol plus 20 mM succinate. At indicated times shaking was stopped, an oxygen electrode (New Brunswick Scientific, N. J.) was introduced immediately into the cultures, and the decrease of dissolved oxygen was recorded over a 30 minutes period. The oxygen electrode was calibrated with 5% Na_2SO_3 (0%) and distilled water saturated with air (100%) at 25°C according to manufacturer's instructions.

RESULTS

Ultrastructure of nodules. Meristematic nodules induced by strains L5-30 and UR7 were effective in nitrogen fixation (acetylene reduction), and had typical histologies and ultrastructures throughout a growth period of 6 weeks (Figure 1). Nodules induced by UR6 were ineffective in acetylene reduction. Examination of these Fix^- nodules 3 weeks after inoculation showed that the UR6 cells were released from infection threads into the host cells, but very few of the bacteria had transformed into bacteroids. Also premature degeneration and lysis of the bacteria and host cells occurred in the central zone (Figure 2). 6 weeks after inoculation, the nodules induced by UR6 were fully senescent and the bacteria were lysed in all zones. At this time, L5-30 and UR7 bacteroids were typically degenerated only in the normal senescent zone of the Fix^+ nodules.

Growth studies, cell morphology and enzyme activities. *R. meliloti* L5-30 had a higher growth rate in succinate (10 mM) than in glucose (10 mM) (Figure 3A). L5-30 grown in 10 mM glucose plus 10 mM succinate (Figure 3A) underwent diauxic growth and the glucose concentration in the medium decreased only during the second phase of growth. UR6 grown under the same conditions did not undergo diauxic growth (Figure 3B). L5-30 grown in 10 mM mannitol plus 10 mM

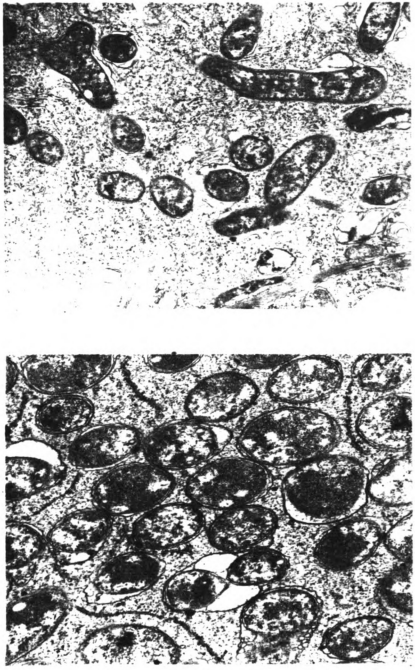


FIGURE 1. Ultrastructure of root nodules on alfalfa 6 weeks after inoculation with UR7 strain. (A)(B) Enlargement of plant cells in the central zone filled with bacteroids. Results with wild type L5-30 were identical. X 12,000.

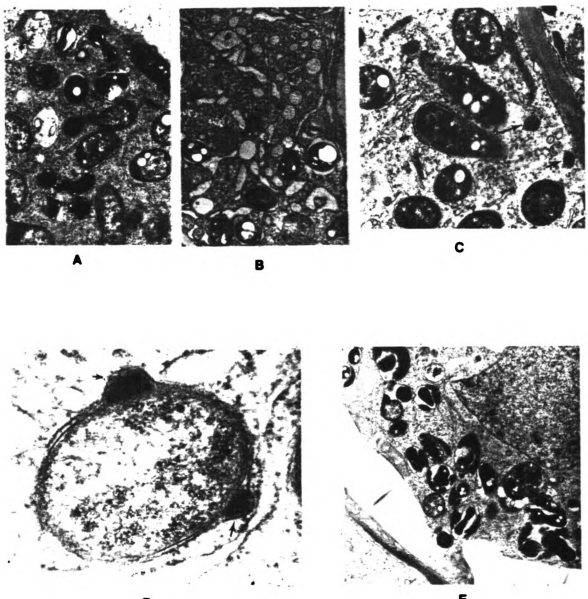


FIGURE 2. Ultrastructure of root nodules on alfalfa induced 3 weeks after inoculation with UR6 strain. (A) Some degenerated bacteria in the proximal infection zone, X 4,100. (B) Enlarged profiles of rough endoplasmic reticulum in the distal infection zone, X5,500. (C) Lysosomes (arrows) in distal infection zone, X9,000. (D) Electron-dense granules (arrow) associated with bacteria in the distal infection zone, X3,700. (E) Bacteria undergoing degeneration in host cells in distal central zone. Note the ultrastructurally well preserved host cytoplasm, X6,800.

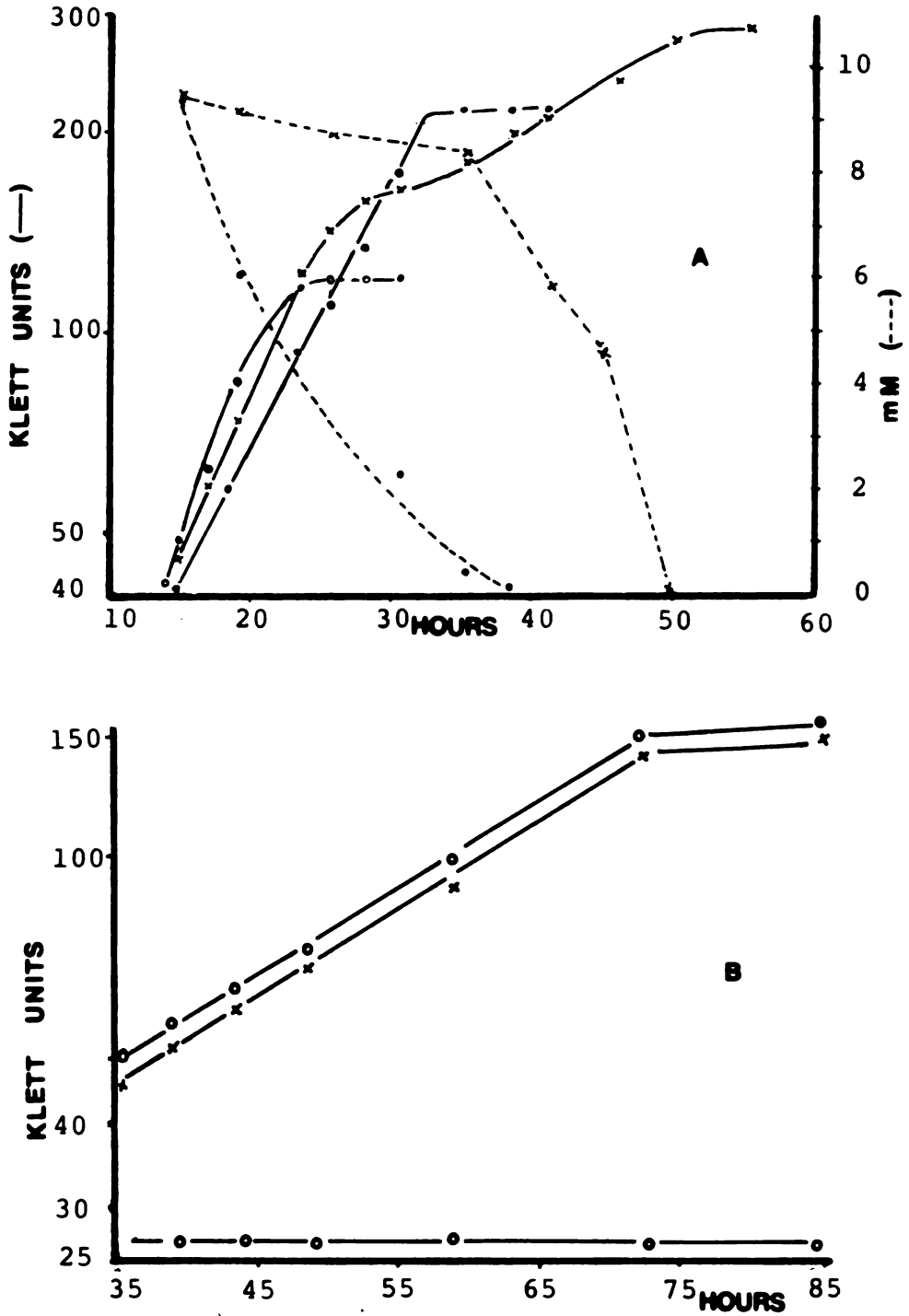


FIGURE 3. Growth of R. meliloti L5-30 (A) and UR6 (B) in minimal medium containing 10 mM glucose plus 10 mM succinate (x); 10 mM glucose (●); or 10 mM succinate (o). (—) Klett units; (---) glucose concentration in the culture medium (mM).

succinate also grew biphasically, although not as markedly as for glucose (Figure 4A). Succinate was consumed in the first phase of growth although at a slower rate than with cells grown in succinate alone (Figure 4A). In contrast, UR6 growth in 10 mM mannitol plus 10 mM succinate was the same as in 10 mM Mannitol (Figure 4B).

Succinate or malate affected cell growth of L5-30 in 27 mM mannitol minimal medium. At 20 mM succinate or malate, L5-30 had lower yields at stationary phase (Figure 5). At times indicated by arrows in Figure 5, 15% of the cells were elongated and pleomorphic (Figure 6). UR6 grew on fumarate or malate and was able to transport but not to grow on succinate. 20 mM succinate or malate neither inhibited growth of UR6 in mannitol (Figure 7), nor induced cell pleomorphism (Figure 8). Under these conditions, mannitol uptake and mannitol dehydrogenase activities were lowered for the wild type strain but not for the UR6 strain (Table 1).

Oxygen consumption. L5-30 consumed oxygen at a higher rate in 27 mM mannitol plus 20 mM succinate than did UR6 (Figure 9).

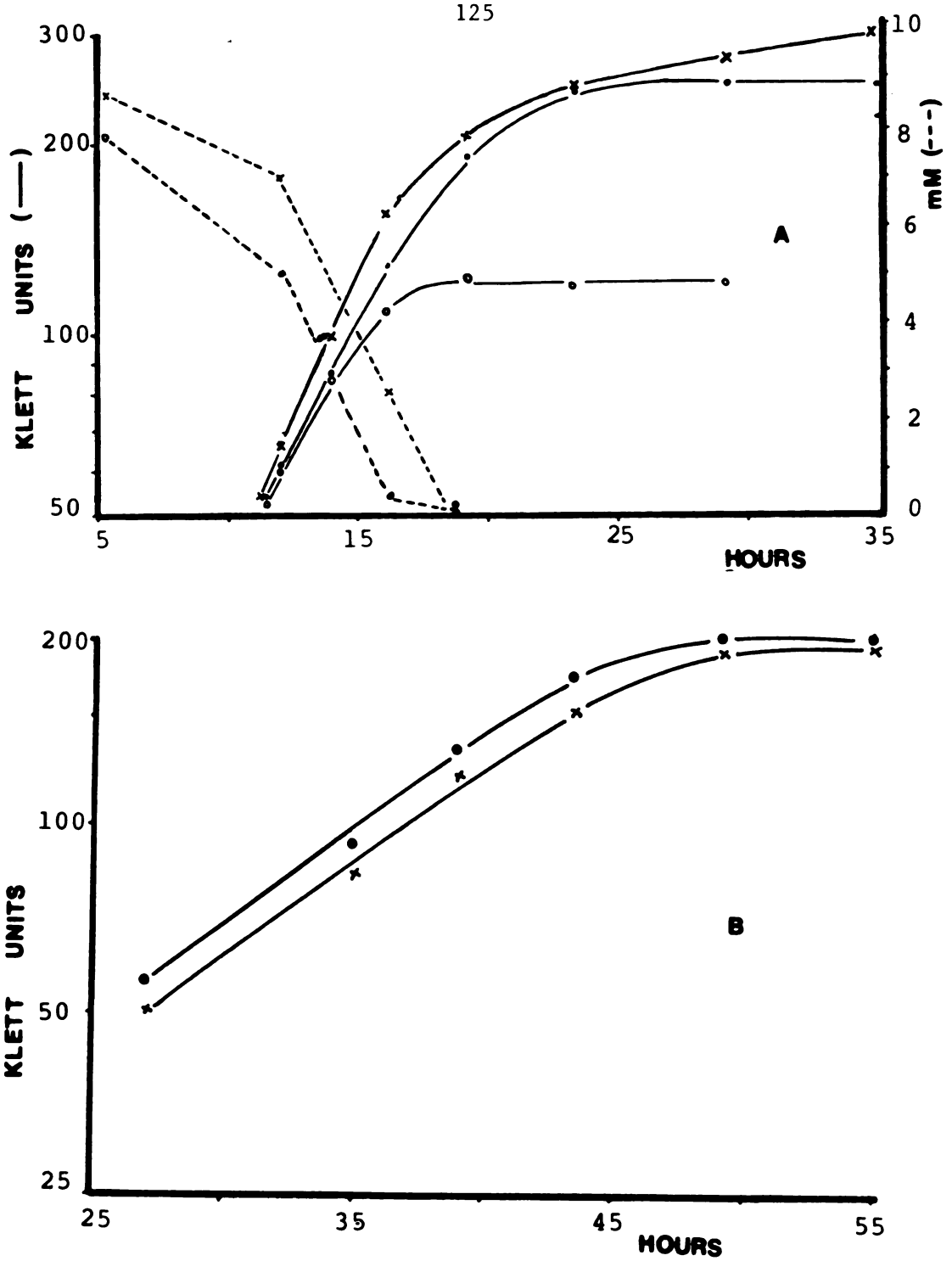


FIGURE 4. Growth of R. meliloti L5-30 (A) and UR6 (B) in minimal medium containing 10 mM mannitol plus 10 mM succinate (✱); 10 mM mannitol (●); or 10 mM succinate (⊙). (——) Klett units; (----) succinate concentration in the culture medium (mM).

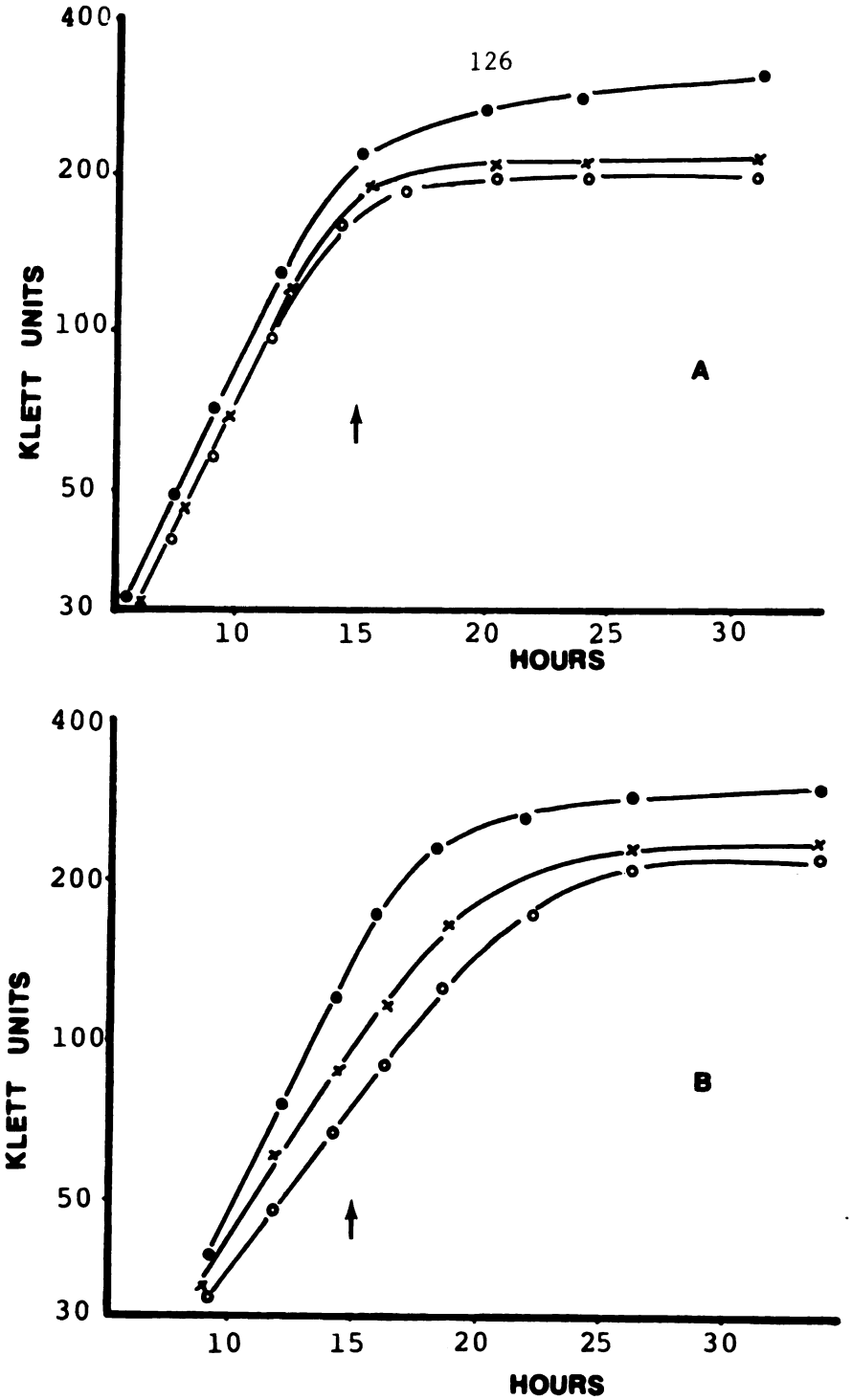


FIGURE 5. Growth of R. meliloti L5-30 in mannitol minimal medium containing (A) succinate or (B) malate. In (A), labels are 27 mM mannitol only (●); 27 mM mannitol plus 20 mM succinate (✖); 20 mM succinate only (○); in (B), they are 27 mM mannitol only (●); 27 mM mannitol plus 20 mM malate (✖); 20 mM malate only (○).

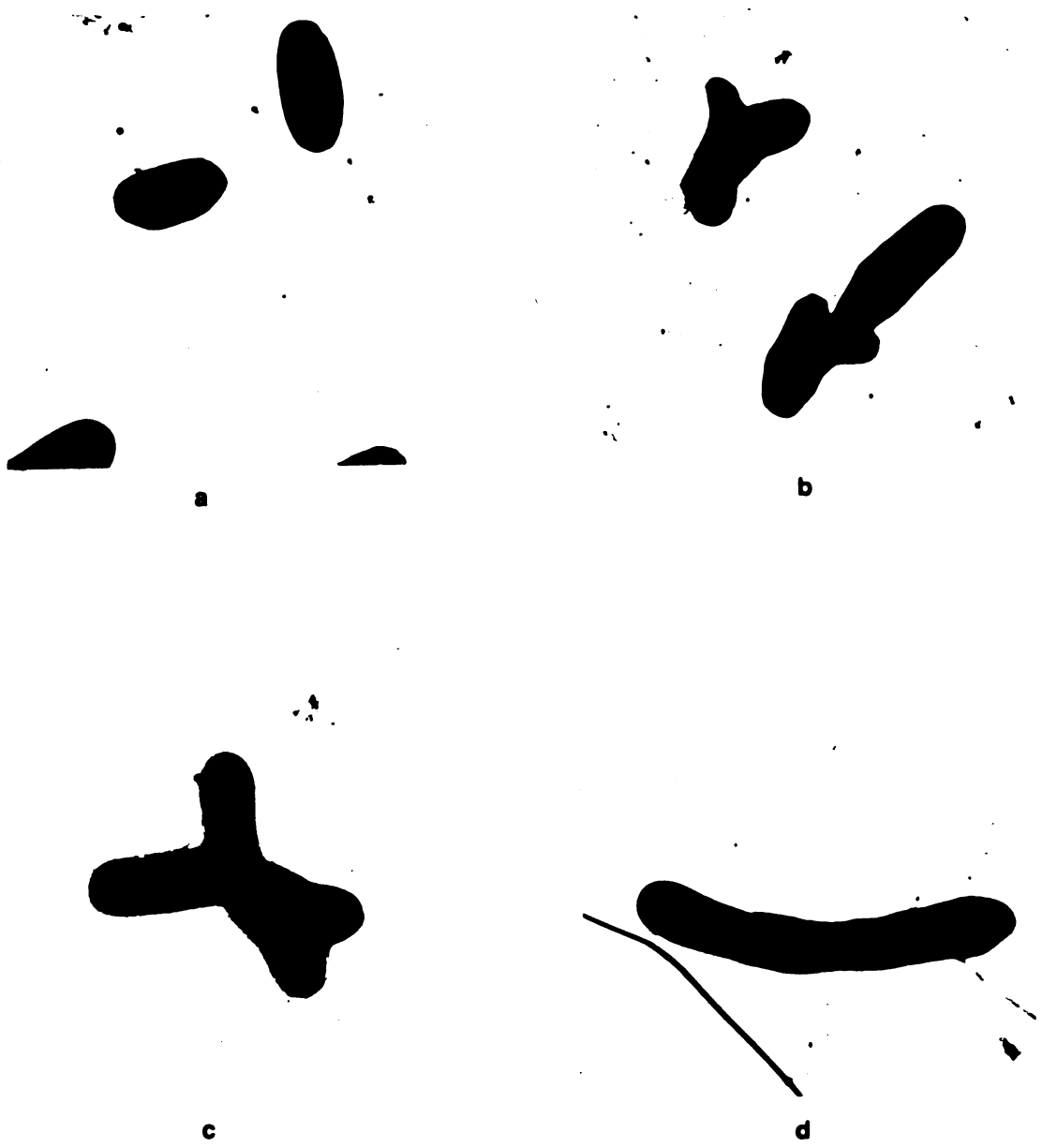


FIGURE 6. Cell morphology of L5-30 strain grown in different media. Culture samples removed at indicated times (arrows) in Figure 5, were observed by TEM. L5-30 grown in mannitol (a); mannitol plus succinate (b) (c); mannitol plus malate (d). X11,000.

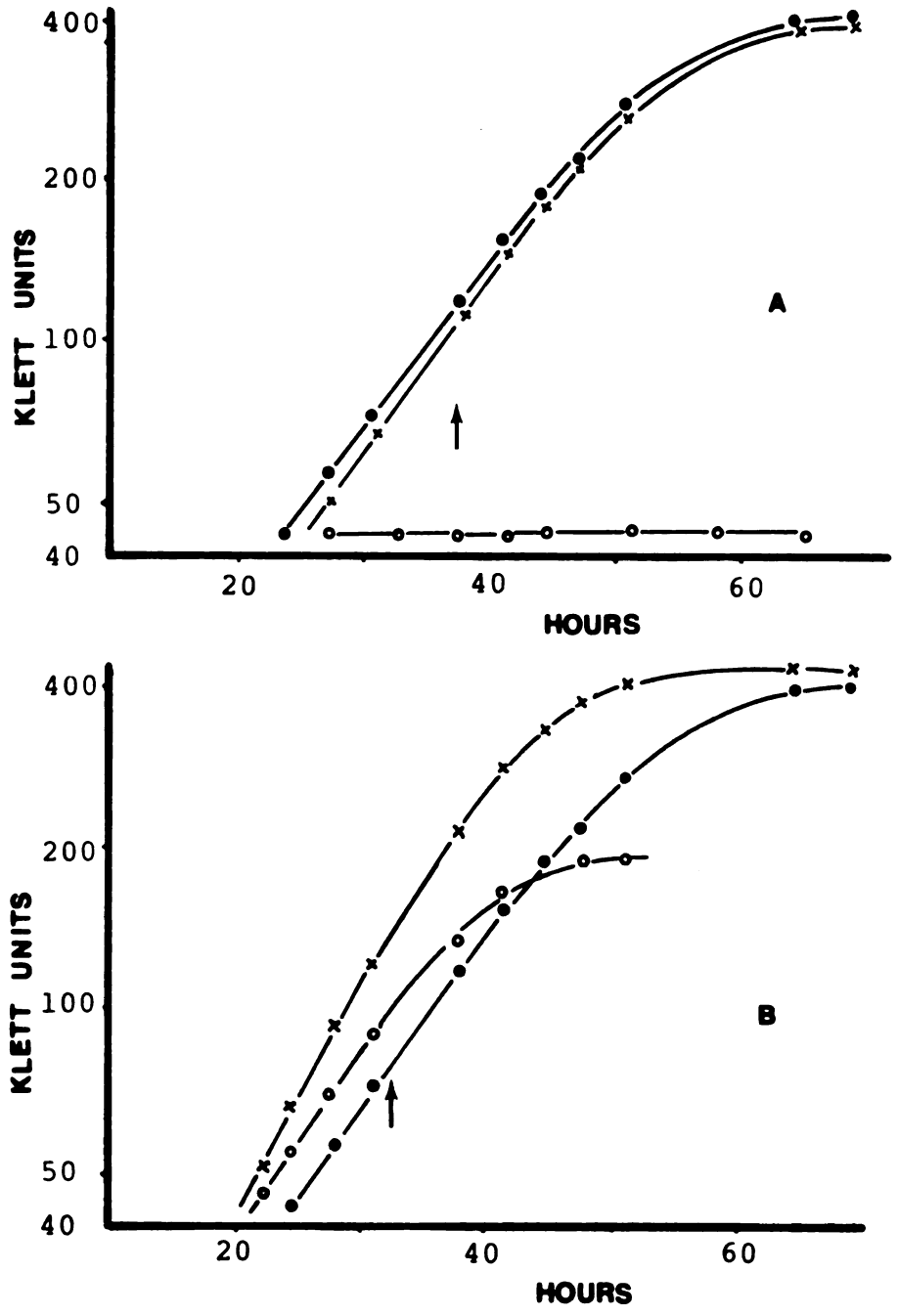


FIGURE 7. Growth of R. meliloti UR6 in mannitol minimal medium containing (A) succinate or (B) malate. (A) 27 mM mannitol only (●); 27 mM mannitol plus 20 mM succinate (✖); 20 mM succinate only (○). (B) 27 mM mannitol (●); 27 mM mannitol plus 20 mM malate (✖); 20 mM malate (○).

FIGURE 8. Cell morphology of UR6 strain grown in different media. Culture samples removed at indicated times (arrows) in Figure 7, were observed by TEM. UR6 grown in mannitol (a); mannitol plus succinate (b); mannitol plus malate (c). x 3,200.

TABLE 1. Mannitol uptake and mannitol dehydrogenase activities in L5-30 and UR6 strains.[a]

Strain	Carbon source	Mannitol uptake nmol/min/mg protein	%[b]	Mannitol dehydrogenase nmol/min/mg protein	%[c]
L5-30	Mtl	150	100	76.0	100
"	Mtl+Succ	45	29	50.5	65
"	Mtl+Mal	63	42	52.2	68
UR6	Mtl	92	100	102.9	100
"	Mtl+Succ	86	94	107.9	105
"	Mtl+Mal	72	78	87.3	85

[a] Cells were grown in parallel cultures in MM containing the indicated carbon sources, Mannitol (Mtl) was used at 27 mM and succinate (Succ) or malate (Mal) at 20 mM. Cells were harvested when the cell density of mannitol-grown cells reached a Klett value of 150. [^{14}C] mannitol uptake and mannitol dehydrogenase activities were measured as described in the text.

[b,c] Expressed as percentage of the respective activity of each strain grown in mannitol.

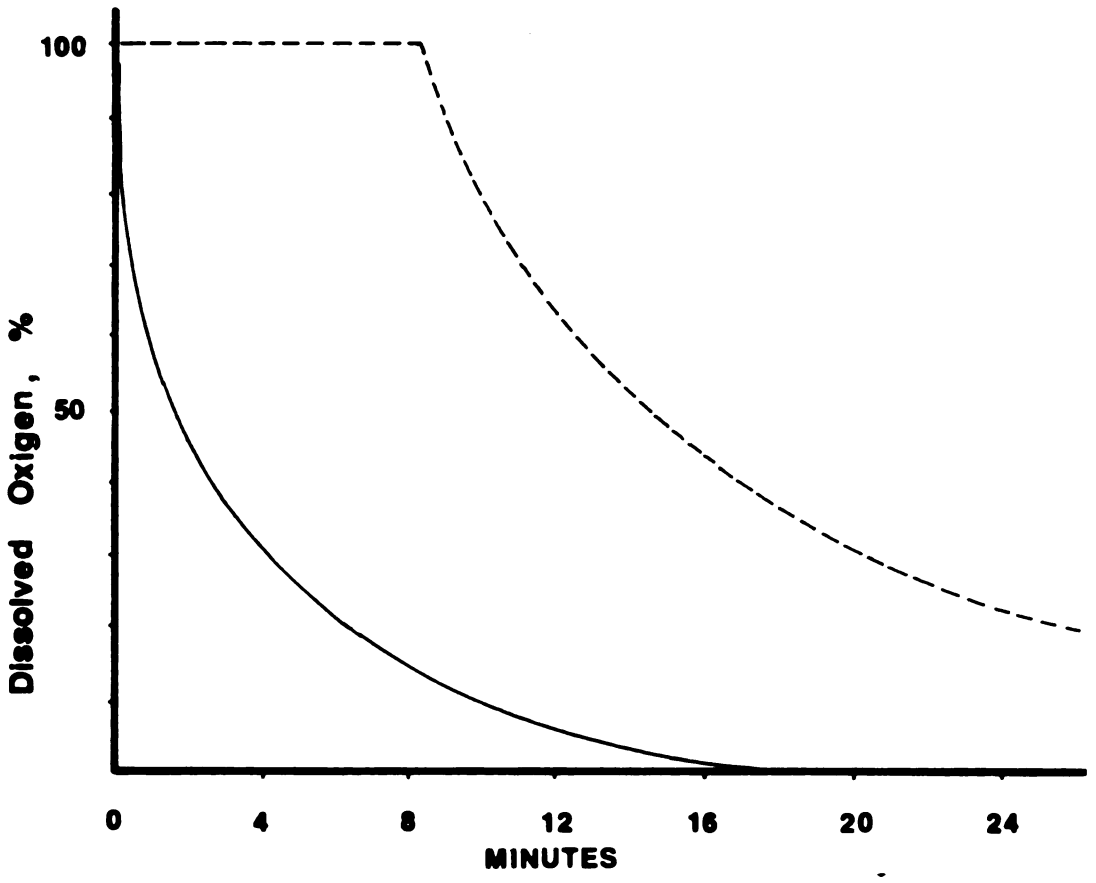


FIGURE 9. Decrease of dissolved oxygen in cell suspensions of L5-30 (——) and UR6 (----) strains. Cells were grown as for Figures 5 and 7. At indicated times (arrows) shaking was stopped, a NBS oxygen electrode was introduced into the cultures and the decrease of dissolved oxygen was followed as a function of time. The oxygen electrode was calibrated with 5% Na_2SO_3 (0%) and distilled water saturated with air (100%) at 25°C.

DISCUSSION

Ultrastructural study of the Fix^- nodules induced by the succinate dehydrogenase mutant strain UR6 indicated that this *R. meliloti* strain was defective in bacteroid differentiation (Bad^-) in symbiosis with alfalfa plants, while a spontaneous revertant strain UR7 possessed the same Bad^+ Fix^+ characteristics as the wild type strain, *R. meliloti* L5-30 (Figures 1 and 2). Specific ultrastructural changes in nodules induced by the mutant strain were different from the general changes that result of nitrogen deficiency in the case of an ineffective symbiosis (36). These results indicated that symbiosis by UR6 was blocked at a step following release of bacteria from the infection threads and that a complete TCA cycle is required for normal bacteroid differentiation of *R. meliloti* in alfalfa nodules.

The use of this combination of strains was ideal for studying the *"in vitro"* effects caused in *R. meliloti* by succinate. The *in vitro* succinate effect was concentration dependent. At low concentration, succinate was utilized preferentially to glucose or mannitol (Figures 3 and 4) and did not induce a bacteroid-like cell morphology. The effect of succinate on glucose growth was more accentuated than on mannitol growth (Figures 3 and 4). In media containing glucose plus succinate, there was no co-utilization of

substrates by the wild type strain and glucose was only consumed in the second phase of diauxic growth (Figure 3). Biphasic growth has been reported for R. meliloti grown in succinate plus lactose, and cells in the second phase of growth had higher levels of β-galactosidase activity than did the cells in the first phase and this effect was not reversed by addition of cAMP (38). Succinate was totally consumed in the first phase of growth in media containing both mannitol plus succinate, but this consumption occured at slower rate than with cultures grown with succinate alone (Figure 4). This indicated that succinate and mannitol were co-utilized. The differences in the effect of succinate on glucose and mannitol growth could be because R. meliloti L5-30 grows slower on glucose than on mannitol. Similar effects have been reported in R. leguminosarum 3841 (11) grown on a mixture of carbon sources, e. g. succinate plus p-hydroxybenzoate. The cells co-utilized both substrates, however consumption of p-hydroxybenzoate was substantially lower than that of succinate. Consistent with results using R. trifolii 0403 (41), succinate at high concentrations (20 mM) decreased growth yields and induced bacteroid-like morphology in R. meliloti L5-30 (Figures 5 and 6). Finan at al. (12) reported that inhibition of R. leguminosarum growth caused by succinate was eliminated in a medium containing 4 mM Mg^{+2} and 0.2 mM Ca^{+2}, and they suggested that the succinate effect on cell morphology was due to chelation of divalent cations and extraction from the cells.

The pleomorphism induced by glycine in rhizobia (29, 29, 30, 31) can be avoided by increasing the calcium concentration in glycine-containing media (29). The medium used in our study contains 1 mM Mg^{+2} and 0.4 mM Ca^{+2} and does not contain aminoacids. Although, chelation of divalent cations may contribute to the succinate effect, this is not the only reason since the mutant strain grown under identical conditions of high succinate concentration as the wild type strain did not show growth inhibition or cell pleomorphisms at all (Figures 7 and 8). Furthermore, studies in R. trifolii (41) showed that succinate induces bacteroid-like swellings in the presence of Ca^{+2} in excess of the amount that could be chelated by succinate. Neither of these effects occuring with wild type strain were obtained for the succinate dehydrogenase mutant strain UR6 under identical conditions, indicating that normal metabolism of succinate is necessary for the effects of succinate on growth (at low concentration) and for the "in vitro" induction (at high concentration) of bacteroid-like cell morphology in R. meliloti.

Succinate also lowered the levels of mannitol uptake and mannitol dehydrogenase activities in cells grown on mannitol plus succinate as compared to cells grown on mannitol alone (Table 1). In R. leguminosarum 3841 (11) glucose or succinate produced about 50% repression of the inducible p-hydroxybenzoate catabolic system when the cells were grown on a mixture of carbon sources, e. g. succinate

plus p-hydroxybenzoate. Repression of mannose uptake by succinate in R. meliloti (3) and of glucose uptake by malate in R. leguminosarum (10) have also been reported.

Bacteroid characteristics were reported to develop in Rhizobium in microaerobic conditions (4, 5). Also, the concentration of dissolved oxygen regulated the pathway of glucose metabolism in Pseudomonas aeruginosa (23). L5-30 decreased the concentration of dissolved oxygen in media containing succinate at a much higher rate than did UR6 (Figure 9). Presumably there are regulatory molecules in R. meliloti that respond to the catabolic situation in the cell.

We conclude that metabolism of succinate through the TCA cycle is necessary in R. meliloti for normal differentiation and function of alfalfa bacteroids and for the "in vitro" effects of succinate on growth and induction of bacteroid-like cell morphology.

LIST OF REFERENCES

1. Antoun, H., L. M. Bordeleau, and R. Sauvageau. 1984. Utilization of the tricarboxylic acid cycle intermediates and symbiotic effectiveness in Rhizobium meliloti. Plant and Soil 77:29-38.

2. Arias, A., C. Cervenansky, A. Gardiol, and G. Martinez-Drets. 1979. Phospho-glucose isomerase mutant of Rhizobium meliloti. J. Bacteriol. 137:409-414.

3. Arias, A., A. Gardiol, and G. Martinez-Drets. 1982. Transport and catabolism of D-mannose in Rhizobium meliloti. J. Bacteriol. 151:1069-1072.

4. Avissar, Y. J., and R. Gollop. 1982. Bacteroid characteristics in microaerobic Rhizobium. Israel J. Botany 31:112-118.

5. Avissar, Y. J., and K. D. Nadler. 1978. Stimulation of tetrapyrrole formation in Rhizobium japonicum by restricted aeration. J. Bacteriol. 135:782-789.

6. Bergersen, F. J. 1977. Physiological chemistry of dinitrogen fixation by legumes p. 519-555. In R. W. F. Hardy and W. S. Silver (ed.), A treatise on dinitrogen fixation, section III. John Wiley and Sons, New York.

7. Bergersen, F. J., G. L. Turner, A. H. Gibson, and W. F. Dudman. 1976. Nitrogenase activity and respiration of cultures of Rhizobium spp. with special reference to concentration of dissolved oxygen. Biochim. Biophys. Acta 444:164-174.

8. Dazzo, F. B. 1982. Leguminous root nodules, p. 431-446. In R. G. Burns and J. H. Slater (ed.). Experimental Microbial Ecology. Blackwell Scientific Publications, Oxford.

9. de Vries, G. E., P. In't Veld, and J. W. Kijne. 1980. Production of organic acids in Pisum sativum root nodules as a result of oxygen stress. Plant Sci. Lett. 20:115-123.

10. de Vries G. E., A. A. N. van Brussel, and A. Quispel. 1982. Mechanism and regulation of glucose transport in Rhizobium leguminosarum. J. Bacteriol. 149:872-879.

11. Dilworth, M. J., I. McKay, M. Franklin, and A. R. Glenn. 1983. Catabolite effects on enzyme induction and substrate utilization in Rhizobium leguminosarum. J. Gen. Microbiol. 129:359-366.

12. Finan, T. M., J. M. Wood, and D. C. Jordan. 1981. Succinate transport in Rhizobium leguminosarum. J. Bacteriol. 148:193-202.

13. Finan, T. M., J. M. Wood, and D. C. Jordan. 1983. Symbiotic properties of C_4-dicarboxylic acid transport mutants of Rhizobium leguminosarum J. Bacteriol. 154:1403-1413.

14. Gardiol, A., A. Arias, C. Cervenansky, C. Gaggero, and G. Martinez-Drets. 1980. Biochemical characterization of a fructokinase mutant of Rhizobium meliloti. J. Bacteriol. 144:12-16.

15. Gardiol, A., A. Arias, C. Cervenansky, and G. Martinez-Drets. 1982. Succinate dehydrogenase mutant of Rhizobium meliloti. J. Bacteriol. 151:1621-1623.

16. Glenn, A. R., P. S. Poole, and J. F. Hudman. 1980. Succinate uptake by free-living and bacteroid forms of Rhizobium leguminosarum. J. Gen. Microbiol. 119:267-272.

17. Huber, J. D., F. Parker, and G. F. Odland. 1968. A basic fuchsin and alkalinized methylene blue rapid stain for epoxy-embedded tissue. Stain Techn. 43:83-87.

18. Kawai, S., and S. Mori. 1984. Detection of organic acids in soybean nodules by high performance liquid chromatography. Soil Sci. Plant Nutr. 30:261-266.

19. Krulwich, T. A., and J. C. Ensign. 1969. Alteration of glucose metabolism of Arthrobacter crystallopoietes by compounds which induce sphere to rod morphogenesis. J. Bacteriol. 97:526-534.

20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

21. Martinez-Drets, G., and A. Arias. 1970. Metabolism of some polyols by Rhizobium meliloti. J. Bacteriol. 103:97-103.

22. McAllister, C. F., and J. E. Lepo. 1983. Succinate transport by free-living forms of Rhizobium japonicum. J. Bacteriol. 153:1155-1162.

23. Mitchell, C. G., and E. A. Dawes. 1982. The role of oxygen in the regulation of glucose metabolism,

transport and the tricarboxylic acid cycle in Pseudomonas aeruginosa. J. Gen. Microbiol. 128:49-59.

24. Mutaftschiev, S., J. Vasse, and G. Truchet. 1982. Exostructures of Rhizobium meliloti. FEBS Microbiol. Lett. 13:171-175.

25. Park, J. T., and M. J. Johnson. 1949. Submicro determination of reducing carbohydrates. J. Biol. Chem. 181:149-152.

26. Patterson, T. G., J. B. Peterson, and T. A. LaRue. 1983. Effect of supra-ambient oxygen on nitrogenase activity (C_2H_2) and root respiration of soybeans and isolated soybean bacteroids. Plant Physiol. 70:695-700.

27. Ronson, C. W., P. Lyttleton, and J. G. Robertson. 1981. C_4-dicarboxylate transport mutants of Rhizobium trifolii form ineffective nodules on Trifolium repens. Proc. Natl. Acad. Sci. USA. 78:4284-4288.

28. Saroso, S., A. R. Glenn, and M. J. Dilworth. 1984. Carbon utilization by free living and bacteroid forms of cowpea Rhizobium strain NGR-234. J. Gen Microbiol. 130:1809-1814.

29. Sherwood, M. T. 1972. Inhibition of Rhizobium trifolii by yeast extracts or glycine is prevented by calcium. J. Gen. Microbiol. 71:351-358.

30. Staphorst, J. L., and B. W. Strijdom. 1971. Infectivity and effectiveness of colony isolates of ineffective glycine resistant Rhizobium meliloti strains. Phytophylactica 3:131-136.

31. Staphorst, J. L., and B. W. Strijdom. 1972. The effect of yeast extract concentration in media on strains of Rhizobium meliloti. Phytophy lactica 4:29-32.

32. Stovall, I., and M. Cole. 1978. Organic acid metabolism by isolated Rhizobium japonicum bacteroids. Plant Physiol. 61:787-790.

33. Stumpf, D. K., and R. H. Burris. 1979. A micromethod for the purification and quantification of organic acids of the tricarboxylic acid cycle in plant tissues. Anal. Biochem. 95:311-315.

34. Trinchant, J. C., and J. Rigaud. 1979. Sur les substrats energetiques utilises, lors de la reduction de C_2H_2, par les bacteroides extraits des nodosites de Phaseolus vulgaris. L. Physiol. Veg. 17:547-556.

35. Truchet, G. L. and F. B. Dazzo. 1982. Morphogenesis of lucerne root nodules incited by Rhizobium meliloti in the presence of combined nitrogen. Planta 154:352-360.

36. Truchet, G., M. Michel, and J. Denarie. 1980. Sequential analysis of the organogenesis of lucerne (Medicago sativa) root nodules using symbiotically-defective mutants of Rhizobium meliloti. Differentiation 16:163-172.

37. Tuzimura, K. and H. Meguro. 1960. Respiration of substrates by Rhizobium in the nodules. J. Biochem. 47: 391-397.

38. Ucker, D. S., and E. R. Signer. 1978. Catabolite-repression- like phenomenon in Rhizobium meliloti. J. Bacteriol. 136: 1197-1200.

39. Urban, J. E. 1979. Nondividing, bacteroid-like Rhizobium trifolii: in vitro induction via nutrient enrichment. Appl. Environ. Microbiol. 38:1173-1178.

40. Urban, J. E., and D. B. Bechtel. 1984. Fine structure of succinate-swollen Rhizobium trifolii 0403. Appl. Environ. Microbiol. 47:1178-1181.

41. Urban, J. E., and F. B. Dazzo. 1982. Succinate-induced morphology of Rhizobium trifolii 0403 resembles that of bacteroids in clover nodules. Appl. Environ. Microbiol. 44:219-226.

42. Wilcockson, J., and D. Werner. 1979. Organic acids and prolonged nitrogenase activity by non-growing free living Rhizobium japonicum. Arch. Microbiol. 122:153-160.

SUMMARY

The Rhizobium - legume symbiosis involves a complex sequence of interactions resulting in the formation of a root nodule that fixes nitrogen. In this dissertation, symbiotically defective mutant strains of Rhizobium were isolated and characterized with the objective of identifying biochemical events leading to a successful symbiosis.

In Chapter I, R. trifolii 0403 rif was subjected to transposon (Tn5) mutagenesis. The most useful mutant strain obtained was R. trifolii 251 with a single Tn5 insertion in the sym plasmid. R. trifolii 251 showed a significant increase in attachment to clover root hairs and in lectin-binding ability when compared to the wild type strain. This strain could be useful for studying the role of improved attachment in interstrain competition in the rhizosphere. To verify that the mutant phenotype is caused by the transposon insertion, the mutation could be transferred (6)(17) to a wild type background. The linkage of the Tn5-conferred kanamycin resistance phenotype with the mutant symbiotic phenotype would be indicative that the Tn5 insertion is the cause of the mutation. Alternatively, the fragment into which Tn5 has inserted could be cloned, the gene inserted into R. trifolii wild type background (24) and the resulting mutant strains tested for the mutant symbiotic phenotype. The 251 capsular polysaccharide (CPS) was different from

wild type CPS in terms of non-carbohydrate substitutions. To analyze the CPS, we used a glucuronic acid-specific lyase (PD-I)(isolated from a phage lysate of R. trifolii 4S) to hydrolyze the CPS into its oligosaccharide repeating unit (OS). This approach was very useful since it allowed the detection of differences between CPS from wild type and mutant strains based on both the kinetic study of CPS depolymerization rates with enzyme PD-I and the quantitative ^{1}H-NMR measurement of the levels of non-carbohydrate substitutions (pyruvate, acetate, and 3-hydroxybutanoate) of OS. CPS from strain 251 depolymerized at a lower rate and contained more pyruvate but less acetate substitutions than wild type CPS. These results are consistent with the proposed role of CPS non-carbohydrate substitutions in R. trifolii attachment to clover root hairs and the finding that trifoliin binding to CPS is sensitive to small changes in non-carbohydrate substitutions (1).

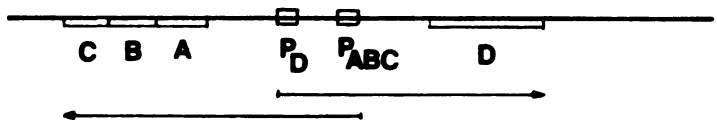
In Chapter II, we examined the CPS isolated from R. trifolii 843 and Tn5-induced mutant strains obtained by extensive mutagenesis of a 14 kb DNA fragment of the sym plasmid carrying nodulation genes. CPS from mutant strains in region I (Hac) had different rates of depolymerization and different levels of pyruvic and acetic acid substitutions when compared with wild type CPS. We will discuss this more below.

An in vitro assay was developed to measure the enzymatic incorporation of pyruvate into R. trifolii CPS in

Chapter III. Pyruvylation was shown to occur at a lipid-bound oligosaccharide intermediate stage. This method was used to measure CPS pyruvyl transferase activity (CPT) in wild type and mutant strains of R. trifolii 843 and 0403 rif having altered levels of pyruvate in the CPS. This procedure, with the necessary modifications, could be useful to study CPS acetylation.

The results obtained in these two Chapters (summarized in Table 3, Chapter III) have to be interpreted in the context of the limited available knowledge about conditions and regulation of Rhizobium nod genes expression. Recent regulation data (19) (B. Rolfe and M. Djordjevic, personal communication) from research groups working with the four common nod (D and ABC) genes (affecting root hair curling (Hac) and nodulation (Nod) phenotypes) of different Rhizobium species suggests the following: a) nod D gene is constitutively expressed at a low level in defined culture medium; and b) nod D gene is required in concert with a plant factor present in root exudate (plant stimulator) for the induction of nod ABC genes which are expressed coordinately in a single transcriptional unit (4)(10)(19). The results of studies of R. trifolii 843 (wild type) and mutant strains 246, 252, and 851 described in Chapters II and III are related to the control of expression of nod D and nod ABC genes. These results will be explained on the basis of data obtained from B. Rolfe and M. Djordjevic (personal communication).

R. trifolii 843. In defined culture medium, P_D (the promoter of nod D) is available for binding of RNA polymerase. The strength of P_D is greater than that of P_{ABC} (promoter of nod ABC), and nod D gene is therefore expressed constitutively. nod ABC genes are expressed transiently at late exponential/early stationary phase in defined liquid culture medium and in cells grown for five days on BIII defined agar medium. This suggests that the nutritional status of the cells may affect expression of nod genes.



This figure shows the relative location of nod D and nod ABC genes and their respective promoters. These genes are transcribed in opposite directions. In presence of root exudate, the nascent nod D gene product together with a plant signal (plant stimulator) improves the quality of P_{ABC} as a promoter. As a result mRNA to nod ABC genes is synthesized and because two opposing promoters can not be read at the same time nod D gene is presumably turned off. It is not known if the transient expression of nod ABC genes in defined culture medium is due to accumulation in the cells at a certain phase of growth of the same factor (plant stimulator) that is present in the root exudate.

Mutant strain 246. In this strain Tn5 is inserted between P_D and the coding region of nod A gene. The Tn5 is

positioned so that it can not affect the expression of nod D gene. However, it does separate effectively the nod ABC genes from their promoter. One would predict then that this mutant would be Nod^-, however it is Nod^+. Tn5 is known to have weak promoters at each end of the transposon. The most likely explanation is that expression of nod ABC genes is taking place from a Tn5 promoter.

Mutant strain 252. Tn5 is inserted in the coding region of nod A gene. This destroys nod A or makes short nonsense products. Either (a) nod BC genes are expressed from a Tn5 promoter ($A^-B^+C^+$) or (b) not expressed and the result will be ($A^-B^-C^-$). A recent report suggested that the expression of nod C was obtained from a Tn5 promoter located in nod A in a mutant of R. meliloti (19).

Mutant strain 851. In this strain, Tn5 has inserted into the nod D gene. Tn5 should destroy nod D or make short nonsense products and nod ABC genes will not be expressed because the nod D gene product is required along with a presumptive plant signal.

Cells for CPS isolation were grown on BIII plates for 5 days because the level of non-carbohydrate substitutions and lectin-binding ability of CPS changes with culture age and, at this time, CPS lectin-binding ability is maximal (1). 246 and 252 CPS had more pyruvate and 851 CPS less pyruvate than wild type CPS. CPS from strain 277 with a Tn5 insertion in the nod C gene had levels equal to wild type CPS. Therefore the nod C gene product does not seem to be involved in

controlling CPS pyruvate levels. Although strain 845 lacks the sym plasmid, its CPS still had pyruvate and acetate substitutions suggesting that the role of nod genes on the levels of pyruvate of CPS may be regulatory. This effect (direct or indirect) may be at the level of the CPS pyruvyl transferase enzyme (affecting synthesis or activity) or at the level of the assembly of the oligosaccharide substrate.

A possible working hypothesis to explain our results (Table 3, Chapter III) would be that the nod A gene product is a negative regulator of the levels of pyruvate in the CPS. In the wild type cells used for CPS isolation, nod ABC genes are expressed transiently from P_{ABC} and therefore this strain has the "right level" of non-carbohydrate substitutions. Strain 246 is Nod^+. Therefore there must be some expression of the nod A gene in this strain. We must assume that this expression takes place from P_{Tn5}. The nod A gene would then be expressed from P_{Tn5} in 246 and not expressed in 252. If the nod A gene product is a negative regulator, the lack of expression of the nod A gene would result in higher levels of pyruvate in these two strains. In the 851 strain, the nod ABC genes are not expressed because a functional nod D gene is not available. This strain should also have higher levels of pyruvate. The fact that it has less pyruvate may be explained if nod D gene product is also affecting the levels of pyruvate. The possibility that nod A gene is responsible for the phenotype could be verified by complementing strain 252 with a cloned wild type nod A gene

and recovering the wild type levels of pyruvate in the CPS.

Cells for measurement of CPT activity were grown in liquid BIII medium (-RE) to stationary phase. This assay measures incorporation of labeled pyruvate into lipid-bound oligosaccharides. The differences in CPT levels between the strains was not as marked as the differences in levels of pyruvate of the CPS (Table 3, Chapter III). This may be explained because the expression of nod ABC genes in liquid culture occurs at early, not late stationary phase, when the cells were harvested. However, the nod ABC genes should have been expressed transiently in the wild type strain. CPT activity was also measured in cells grown to stationary phase and then treated for 24h with root exudate (+RE) isolated after 14 days of incubation of clover seedlings with plant medium. A single batch was used for simultaneous treatment of cells from all the strains. In the presence of an active plant stimulator (+RE) and the nod D gene product, nod ABC genes should be expressed in the wild type strain from P_{ABC}, and subsequently nod D should be turned off. It has been reported that low level expression of nod D from a megaplasmid copy permitted only a 2- to 3- fold increase of nod C expression by plant exudates as opposed to a 30-fold increase when nod D was expressed at a high level from a inc-P vector (19). Since appreciable activation of the nod ABC genes requires high levels of nod D gene product, activation of nod ABC in presence of plant exudate may in

fact be low for the wild type strain. In the 246 and 252 strains the nod D gene should be expressed all the time. The higher values obtained for CPT levels (+RE vs.-RE) may be explained if other nod genes expressed in the presence of the plant stimulator affect the levels of pyruvylation. Consistent with this possibility, other megaplasmid loci have been reported to be involved in the regulation of both nod D and nod C genes expression in R. meliloti (19). Interestingly, R. trifolii 251 a mutant strain from a different wild type strain R. trifolii 0403 rif which has more pyruvate in its CPS than wild type CPS had a correspondingly higher level of CPT activity than the wild type strain. Strain 251 is Hac^+. These results suggest that other loci in the megaplasmid different from the Hac region are affecting CPS pyruvylation.

Alternatively, the nod B gene product may be responsible for the phenotype. The nod B gene should be expressed from P_{Tn5} in 246 and expressed from P_{Tn5} or not expressed in 252 strain. If the nod B gene product is a negative regulator of the level of CPS pyruvylation, the lack of nod B gene expression would also result in higher pyruvate values in CPS and a similar reasoning to the one described for nod A would be valid. If this is the case, a Tn5 insertion in the coding region of the nod B gene should produce higher levels of pyruvate in CPS of the mutant than in wild type CPS and should be complemented with a clone of the wild type nod B gene. If this mutant strain has normal

levels of pyruvate, the participation of nod B gene can be ruled out.

A third alternative would be that the nod B gene product has a positive effect on the levels of pyruvylation and the possible constitutive expression of nod B gene from P_{Tn5} in strains 246 and 252 versus transient expression in the wild type strain is enough to have a positive effect on the levels of pyruvylation. A mutant of strain 252 with a Tn5 mutation in the coding region of nod B should have lower levels of pyruvate than 252 if this alternative is correct. This mutant should be restored to wild type levels with a clone of the nod B gene.

The knowledge about conditions and factors affecting nod genes expression is still developing. In addition, the presence and activity of a plant stimulator in root exudate is highly variable and depends on the experimental conditions for its isolation (e. g. time of incubation of seedlings with plant medium and conditions of storage).

More information is needed about the regulation of nod genes expression, different possible effects of root exudate, and CPS structure of wild type and mutant strains under different conditions to be able to elaborate a definitive model explaining the reproducible and significant differences detected in the levels of pyruvylation in CPS from wild type and mutant strains.

In Chapter IV, the effect of succinate metabolism on growth and bacteroid differentiation of Rhizobium meliloti

was investigated with wild type (L5-30), a succinate dehydrogenase mutant (UR6), and a spontaneous revertant (UR7) strains. UR6 had a Bad^- phenotype (defective in bacteroid differentiation) while L5-30 and UR7 were Bad^+. Ex planta "succinate effects" were concentration dependent. Succinate (at low concentration) was utilized preferentially before glucose. At higher concentration, succinate decreased growth yield and induced bacteroid-like morphology in 15% of the cell population. These effects were observed for the wild type strain but not for the mutant strain, suggesting that metabolism of succinate is necessary for in vivo normal differentiation of alfalfa bacteroids and to produce the ex planta "succinate effects" on growth and morphogenesis in R. meliloti.

The mechanism by which succinate is utilized preferentially before glucose in R. meliloti has not yet been established and deserves special consideration.

In E. coli and other enteric bacteria the induction of several catabolic operons responds to the intracellular concentration of cAMP, which is determined by the carbon source available to the cell (5). The mechanism of catabolite repression of enzyme synthesis (15) is implicit in the model for the role of the CRP(cAMP receptor protein)-cAMP regulatory complex in initiation of transcription (20). There is mounting evidence that additional factors are involved in catabolite repression (30). A factor called catabolite modulator factor (CMF) of low molecular weight,

stable to acid, base and heat, has been proposed as a negative factor by contrast with cAMP, a positive factor in regulation of gene expression (2). Indole acetic acid and imidazole acetic acid can replace cAMP for expression of the arabinose operon (11). The "catabolic potential" of the cell, where cAMP is one factor determining this potential, has been related to catabolite repression (31). Sugars transported by a variety of mechanisms including the PTS mechanism for glucose and mannitol, proton symport mechanisms such as for lactose, and the facilitated diffusion of glycerol, can all inhibit cAMP production (3,25,26). The sugar does not need to be metabolized for this inhibition to occur.

In contrast to the facultative anaerobes, in Pseudomonas species the compounds producing the most severe catabolite repression are succinate and other intermediates of the tricarboxylic acid cycle (21,27). Pseudomonas is a genus of soil bacteria closely related to fast-growing Rhizobium species in its oxidative metabolism. In addition, the presence of a PEP-PTS system has not been demonstrated in these bacteria, nor is this system functional in R. meliloti (8). In Pseudomonas species, the intracellular concentration of cAMP is not markedly affected by the carbon source used by the cells (21,27) and certainly not to the extent seen in E. coli (5). Exogenously applied cAMP does not reverse catabolite repression due to succinate (21,27) and the intracellular cAMP levels are unchanged by the onset

of succinate-related catabolite repression (21). The pathway of glucose metabolism is regulated by the concentration of dissolved oxygen in Pseudomonas aeruginosa (18).

Regulatory processes in carbon metabolism in Rhizobium and Bradyrhizobium are not well understood. Glucose catabolite repression of polyol metabolism has been reported for R. trifolii (23) and R. meliloti (16) but not for B. japonicum (12). Differences have been observed between slow (B. japonicum) and fast (R. meliloti) growing species. Malate represses hydrogenase activity in B. japonicum (14) and exogenously applied cAMP can overcome this repression. Intracellular cAMP pool sizes have also been reported to change with the carbon source used in B. japonicum (14, R. McClung and B. K. Chelm, Abstr. Second International Symposium on the Molecular Genetics of the Bacteria-Plant Interaction. 1984, #59). Intracellular cGMP levels were also found to change in microaerophilic-aerobic shift experiments in B. japonicum (13). In contrast, catabolite repression-like phenomena caused by succinate on galactosidase activity in R. meliloti (29) was not reversed by addition of exogenous cAMP.

It has been reported that during growth in two carbon sources, succinate dehydrogenase activities paralleled intracellular cAMP which varied independently of the carbon source whereas peak succinate-transport rates of succinate-grown cells were 4-fold those of arabinose-grown cells in B. japonicum (S. Ferrenbach, and J. Lepo Abstr. 6th

International Symposium on Nitrogen Fixation 1985,#4-23). C4-dicarboxylates, succinate, fumarate and malate have a symport transport with protons in E. coli (9) and are actively transported by a common inducible system in R. leguminosarum (7), R. trifolii (22) and B. japonicum (28).

In wild type R. meliloti L5-30 we obtained similar ex planta effects with succinate and malate suggesting a common cause. However, the dicarboxylate transport is not likely to be responsible for these effects since the UR6 succinate dehydrogenase mutant strain is able to transport succinate and malate and did not show these effects. In contrast, our results suggest that metabolism of succinate rather than uptake is necessary for the in vivo and ex planta "succinate effects". In addition, we found that the wild type and mutant strains consumed oxygen at different rates in the presence of succinate. These results suggest that there may be regulatory molecules in R. meliloti that respond to the metabolism of succinate.

LIST OF REFERENCES

1. Abe, M., J.E. Sherwood, R.I. Hollingsworth, and F.B. Dazzo. 1984. Stimulation of clover root hair infection by lectin-binding oligosaccharides from the capsular and extracellular polysaccharides of Rhizobium trifolii. J. Bacteriol. 160:517-520.

2. Dessein, A., F. Tillier, and A. Ullmann. 1978. Catabolite modulation factor: physiological properties and in vivo effects. Mol. Gen. Genet. 162:89-94.

3. Dills, S. S., A. Apperson, M. R. Schmidt, and M. H. Saier, Jr. 1980. Carbohydrate transport in bacteria. Microbiol. Rev. 44:385-418.

4. Egelhoff, T. T., Fischer, R. F., Jacobs, T. W., Mulligan, J. T. and S. R. Long. 1985. DNA 4:241-248.

5. Epstein, W., L. B. Rothman-Denes, and J. Hesse. 1975. Adenosine 3',5'-cyclic monophosphaate as mediator of catabolite repression in Escherichia coli. Proc. Natl. Acad. Sci. USA 72:2300-2304.

6. Finan, T. M., A.M. Hirsch, J.A. Leigh, E. Johansen, G. A. Kuldan, S. Deegan, G. C. Walker, and E. R. Signer. 1985. Symbiotic mutants of R. meliloti that uncouple plant from bacterial differentiation. Cell 40:869-877.

7. Finan, T. M., J. M. Wood, and D. C. Jordan. 1981. Succinate transport in Rhizobium leguminosarum. J. Bacteriol. 148:193-202.

8. Gardiol, A., A. Arias, C. Cervenansky, C. Gaggero, and G. Martinez-Drets. 1980. Biochemical characterization of a fructokinase mutant of Rhizobium meliloti. J. Bacteriol. 144:12-16.

9. Gutowski, S. J. and H. Rosenberg. 1975. Biochem. J. 152:647-654.

10. Jacobs, T. W., Egelhoff, T. T. and S. R. Long. 1985. J. Bacteriol. 162:469-476.

11. Kline, E. L., C. S. Brown, V. Bankaitis, D. C. Montefiori, and K. Craig. 1980. Metabolite gene regulation: imidazole and imidazole derivatives which circumvent cyclic adenosine 3',5'-monophosphate induction of the E. coli L-arabinose operon. J. Bacteriol. 141:770-778.

12. Kuykendall, L. D. and G. H. Elkan. 1977. Some features of mannitol metabolism in Rhizobium japonicum. J. Gen. Microbiol. 98:291-295.

13. Lim, S. T., H. Hennecke, and B. Scott. 1979. Effect of cyclic guanosine 3',5'-monophosphate on nitrogen fixation in Rhizobium japonicum. J. Bacteriol. 139:256-263.

14. Lim, S. T., and K. T. Shanmugan. 1979. Regulation of hydrogen utilization in Rhizobium japonicum by cyclic AMP. Biochim. Biophys. Acta 584:479-492.

15. Magasanik, B. 1961. Catabolite repression. Cold Spring Harbor Symp. Quant. Biol. 26:249-262.

16. Martinez-Drets, G. and A. Arias. 1970. Metabolism of some polyols by Rhizobium meliloti. J. Bacteriol. 103:97-103.

17. Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants in Rhizobium meliloti induced by transposon Tn5 mutagenesis. J. Bacteriol. 149:114-122.

18. Mitchell, C. G. and E. A. Dawes. 1982. The role of oxygen in the regulation of glucose metabolism, transport and the tricarboxylic acid cycle in Pseudomonas aeruginosa J. Gen. Microbiol. 128:49-59.

19. Mulligan, J. T. and S. R. Long. 1985. Induction of Rhizobium meliloti nod C expression by plant exudate requires nod D. Proc. Natl. Acad. Sci. USA 82:6609-6613.

20. Pastan, I., and S. Adhya. 1976. Cyclic adenosine 5'-monophosphate in Escherichia coli. Bacteriol. Rev. 40:527-551.

21. Phillips, A. T., and L. M. Mulfinger. 1981. Cyclic Adenosine 3',5'-Monophosphate Levels in Pseudomonas aeruginosa during induction and carbon catabolite repression of histidase synthesis. J. Bacteriol. 145:1286-1292.

22. Ronson, C. W., P. Lyttleton, and J. G. Robertson. 1981. C4-dicarboxylate transport mutants of R. trifolii from ineffective nodules on Trifolium repens. Proc. Natl. Acad. Sci. USA. 78:4284-4288.

23. Ronson, C. W., and S. B. Primrose. 1979. Effect of glucose on polyol metabolism by Rhizobium trifolii. J. Bacteriol. 139:1075-1078.

24. Ruvkun, G. B., and F. M. Ausubel. 1981. A general method for site-directed mutagenesis in prokaryotes. Nature (London) 289:85-88.

25. Saier, M. H., Jr. 1977. Bacterial phosphoenolpyruvate:sugar phosphotransferase systems: structural, functional, and evolutionary relationships. Bacteriol. Rev. 41:856-871.

26. Saier, M. H., Jr. 1979. The role of the cell surface in regulating the internal environment, p. 169-225. In J. R. Sobatch and L. N. Ornston (ed.), The bacteria, vol.3. Academic Press, Inc., New York.

27. Siegel, L. S., P. B. Hylenon, and P. V. Phibbs, Jr. 1977. Cyclic adenosine 3',5'-monophosphate levels and activities of adenylate cyclase and cyclic adenosine 3',5'-monophosphate phosphodiesterase in Pseudomonas and Bacteroids. J. Bacteriol. 129:87-96.

28. Stovall, I., and M. Cole. 1978. Organic acid metabolism by isolated Rhizobium japonicum bacteroids. Plant Physiol. 61:787-790.

29. Ucker, D. S., and E. R. Signer. 1978. Catabolite-repression-like phenomenon in Rhizobium meliloti. J. Bacteriol. 136:1197-1200.

30. Ullmann, A. and A. Danchin. 1979. 1980. Role of cyclic AMP in regulatory mechanisms in bacteria. Trends Biochem. Sci. 5:95-96.

31. Wanner, B. L., R. Kodaira, and F. C. Neidhardt. 1978. Regulation of lac operon expression. Reappraisal of the theory of catabolite repression. J. Bacteriol. 136:947-954.