Symbiotic Phenotypes of Auxotrophic Mutants of *Rhizobium meliloti* 104A14

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Auxotrophic mutants of *Rhizobium meliloti* 104A14 were isolated using nitrous acid mutagenesis followed by penicillin enrichment. Mutants in ornithine transcarbamylase, argininosuccinate synthetase or serine–glycine biosynthesis formed nitrogen-fixing (Fix⁺) nodules on the roots of alfalfa (*Medicago sativa*). Mutants with defects in ornithine, pyrimidine, purine, asparagine, leucine, methionine or tyrosine biosynthesis, in one-carbon metabolism or in carbamoylphosphate synthetase formed nodules but these nodules were unable to fix nitrogen. Prototrophic revertants were always Fix⁺. Plasmids that would complement many of these auxotrophs were isolated by transduction with a P2 cosmid gene bank of *R. meliloti* 104A14. These plasmids were then introduced into mutants of the same and different classes and the growth and symbiotic phenotypes of the new strains were determined. In all cases, complementation of the nutritional defect restored symbiotic nitrogen fixation.

INTRODUCTION

In the symbiotic relationship between *Rhizobium* and its host legume, a complex set of biochemical interactions takes place during the development and maintenance of nitrogen-fixing nodules on the plant roots (Verma & Long, 1983; Sutton, 1983). Some of these interactions probably involve surface macromolecules such as extracellular polysaccharides and lipopolysaccharides. In addition, small molecules are exchanged by the symbionts both during nodule development and in the mature nodule. The bacteria depend on their host for carbon and energy, but it is unknown what other metabolites they require or have available to them. Analysing the symbiotic capabilities of auxotrophic mutants is one way of identifying some of the metabolic requirements for establishing a successful symbiotic relationship.

One of our laboratory's objectives is to understand intermediary nitrogen metabolism of *Rhizobium* in both the symbiotic and free-living states (Somerville & Kahn, 1983; Kahn *et al.*, 1985). In order to investigate possible paths of nitrogen flow, it is important to determine how intermediary nitrogen metabolism is organized genetically and to assess the role of various biosynthetic pathways in establishing an effective symbiosis. In this paper, we describe the isolation and characterization of several classes of auxotrophic mutants of *R. meliloti* 104A14. We have determined the symbiotic phenotypes of these mutants and for most mutant classes we have cloned genes that complement the auxotrophic mutations.

METHODS

Bacterial strains and plasmids. *R. meliloti* 104A14, the P2 transductional cloning vectors and the strains used in their manipulation have been described previously (Somerville & Kahn, 1983).

Media and chemicals. LB, yeast mannitol (YMB) and yeast sucrose (YSB) complex media and minimal mannitol medium have been described (Somerville & Kahn, 1983). When specified, *Escherichia coli* media were

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supplemented with kanamycin sulphate at $75 \mu\text{g ml}^{-1}$ or tetracycline at $25 \mu\text{g ml}^{-1}$ and *R. meliloti* media were supplemented with kanamycin sulphate at $200 \mu\text{g ml}^{-1}$ and tetracycline at $5 \mu\text{g ml}^{-1}$. Amino acids, nucleosides, nucleotide bases and biosynthetic intermediates were added to the minimal media at $100 \mu\text{g ml}^{-1}$. Cyanocobalamin was added at $10 \mu\text{g ml}^{-1}$. Biochemicals were from Sigma. Gelrite was from Kelco, San Diego, CA, USA.

Nitrous acid mutagenesis and penicillin enrichment. The protocols were similar to those described for *E. coli* (Miller, 1972). All media used for mutant isolation were supplemented with an additional $10 \text{ mM}-(\text{NH}_4)_2\text{SO}_4$. A 10 ml culture was grown to approximately $5 \times 10^8 \text{ cells ml}^{-1}$ in YMB. The cells were washed in 0.1 M-sodium acetate buffer (pH 4.6) and resuspended in 1 ml of a 7 mg ml^{-1} solution of NaNO_2 in the same buffer. Samples were withdrawn from the mixture at 30 s intervals between 8 and 12 min after the start of mutagenesis, washed to remove the nitrous acid, and then grown in YMB medium for 8 h to allow recessive mutants to segregate. The cells were washed in 0.85% NaCl and grown for 6 h in minimal mannitol medium to deplete nutrient pools. Penicillin G was added to a final concentration of $300 \mu\text{g ml}^{-1}$ and the culture was shaken for 2 h to kill bacteria that were still growing in the minimal medium. After the cells had been washed twice in 0.85% NaCl, serial dilutions were plated on YMB medium. The survival rate was determined by titrating the culture before and after mutagenesis. Colonies from mutagenized cultures with survival rates between 0.01% and 0.1% were replica-picked onto minimal and YMB medium and those that could grow only on YMB were classified by testing for growth on various nutritional supplements (Davis *et al.*, 1980).

Plant tests for nodulation and nitrogen fixation. Seedlings of alfalfa (*Medicago sativa*, Ladak variety) were grown in $22 \times 150 \text{ mm}$ culture tubes in sterile Jensen's medium (Vincent, 1970) with 0.2% Gelrite added as a solidifier. When indicated, amino acids and nucleotide bases were added to the medium at $20 \mu\text{g ml}^{-1}$, and tetracycline was added at $1 \mu\text{g ml}^{-1}$. One-week-old seedlings were inoculated with approximately 10^2 cells and were grown under Gro-Lux lights with an 18:6 light:dark cycle at 25°C . Nodules were visible in 2 weeks. Growth and acetylene reduction were measured six weeks after inoculation and compared to uninoculated seedlings and to seedlings inoculated with the wild-type strain. To test for reversion, nodules were dissected from the plants, washed in sterile saline and crushed. A sample of the crushed nodules was then plated on minimal medium. Confluent growth was taken as evidence of reversion.

Transduction into R. meliloti. Transducing lysates were prepared by infecting P2vir1 into *E. coli* cells that carried a plasmid with a P4cos sequence. *R. meliloti* cells were prepared for transduction by vigorously washing early-exponential-phase cultures twice in YSB. The cells were resuspended in 0.1 vol. YMB, transducing phage were added at a multiplicity of infection of 1, and the mixture was incubated at room temperature for 30 min. YMB was added to restore the original volume. To allow antibiotic resistance genes to be expressed, the cells were grown at 30°C for 3–4 h and then plated on selective medium.

Purification and analysis of plasmid DNA. Plasmid DNA was isolated from *E. coli* by the Triton lysis method of Kahn *et al.* (1979). Plasmid DNA from *R. meliloti* was isolated using a modification of the boiling technique of Holmes and Quigley (1981). Stationary-phase *R. meliloti* cultures were washed in 0.1% sodium lauryl sarcosinate to remove extracellular polysaccharides and resuspended in 0.35 ml STET buffer (Holmes & Quigley, 1981) to which $25 \mu\text{l}$ lysozyme solution (10 mg ml^{-1}) was added. The mixture was heated to boiling and incubated at 100°C for 30 s, then $100 \mu\text{l}$ 5 M-NaCl solution was added, and the lysate was centrifuged at $23\,300 g$ for 10 min. An equal volume of 2-propanol was added to the supernatant to precipitate the DNA.

Restriction enzymes were purchased from New England Biolabs or purified in this laboratory and used under the conditions recommended by New England Biolabs. Restriction fragments were separated by agarose gel electrophoresis and stained with ethidium bromide (Kahn *et al.*, 1979).

RESULTS

Isolation of R. meliloti auxotrophic mutants

R. meliloti 104A14 was mutagenized with nitrous acid and treated with penicillin to enrich for auxotrophs. Among cultures with 0.02%–0.1% survival rates, a 4% frequency of auxotrophic mutants was observed. Of 4700 colonies tested, 172 grew on YMB medium but did not grow on minimal mannitol medium. These mutants were tested for growth on different amino acids, nucleosides, nucleotide bases and vitamins and classified by their growth-factor requirements (Table 1). After a compound that would restore growth to a particular mutant had been identified, intermediates in the biosynthetic pathway for that nutrient were tested to further define the nature of the auxotrophy. All of the intermediates listed in Table 1 as being unable to support growth of a class of mutants can be used as nitrogen source by the wild-type bacteria and can therefore probably be transported into the cells.

Table 1. *Growth factor requirements, symbiotic phenotypes and complementing plasmids of auxotrophic mutants*

Mutant class	Probable defect	Growth factor requirements		No. isolated	Symbiotic phenotype		Complementing plasmid(s)*
		Growth	No growth		Effective	Ineffective	
10	Carbamoylphosphate synthetase	Citrulline + uracil	Ornithine + uracil	29	0	24	pTK11A pTK12N pTK13C
20	Ornithine biosynthesis	<i>N</i> -Acetylornithine	<i>N</i> -Acetylglutamate	13	0	5	pTK21A, pTK21G
30	Ornithine transcarbamylase	Citrulline	Ornithine	12	4	0	pTK31A, pTK31L
40	Argininosuccinate synthetase	Argininosuccinate	Citrulline	6	4	0	pTK41A, pTK41D
50	Dihydroorotate dehydrogenase	Orotic acid	Dihydroorotate	6	0	1	pTK51D
60	Asparagine synthetase	Asparagine	Aspartic acid	21	0	17	pTK61L, pTK61O
70	One-carbon metabolism	Methionine or choline or histidine or cyanocobalamine	Homocysteine Formate	4	0	4	
80	Cystathionine β -synthase	Cystathionine	<i>O</i> -Succinylhomoserine	6	0	4	
90	Purine biosynthesis	Inosine Adenosine	Guanosine	8	0	1	
A0	Leucine biosynthesis	α -Ketoisocaproic acid	α -Ketoisovaleric acid	23	0	18	pTKA1A
B0	Serine biosynthesis	Glycine Serine	Pyruvate	10	8	0	pTKB1H
C0	Tyrosine biosynthesis	Tyrosine	Shikimic acid Phenylalanine	4	0	2	pTKC1B
00		Undefined requirements		30			

* Plasmid designations were derived by adding pTK in front of the name of the mutant that was originally complemented by them.

Thirty mutants were unable to grow on any of the nutritional supplements tested. These may require more than one supplement or some complex factor found in yeast extract. Because of the nature of the enrichment, some of the mutants are almost certainly siblings. However, not all of the mutants in each class are siblings. In many cases we have been able to differentiate between auxotrophs in the same class based on differences in their ability to grow on metabolic intermediates, colony morphology, growth rate, or frequency of reversion. The reversion frequencies for the mutants varied from 10^{-5} to less than 10^{-11} . A large fraction (55%) of the mutants were arginine auxotrophs. This uneven distribution of mutants may indicate that these mutants have a selective advantage in the mutagenesis and enrichment procedures.

Four different classes of arginine auxotrophs were found (classes 10–40, Fig. 1). Mutants in class 10 were able to grow only when supplemented with a pyrimidine and either arginine or citrulline (Table 1). This phenotype is characteristic of mutants that cannot make carbamoylphosphate, a precursor of citrulline and of orotic acid (Mergeay *et al.*, 1974), and is likely to result from a defect in carbamoylphosphate synthetase. Some Arg⁺ revertants of mutants in this class were sensitive to uracil. Uracil sensitivity has also been observed in revertants of *E. coli* carbamoylphosphate synthetase mutants (Mergeay *et al.*, 1974). Mutants in class 70 were able to grow on histidine, methionine, choline or cyanocobalamine. Based on these requirements, we speculate that the mutations involve one-carbon metabolism, and that each of the compensating compounds feeds into the pool of methylated tetrahydrofolate derivatives. Purine auxotrophs (class 90) were able to utilize adenosine or inosine, but not guanosine. The mutations probably affect the early part of the purine biosynthetic pathway but a salvage pathway does not exist to recover the purine nucleus from guanosine.

Symbiotic phenotypes of R. meliloti auxotrophs

Each mutant strain was inoculated onto four separate sterile alfalfa seedlings. All plants had a normal frequency of nodules, but for several of the mutant classes a large fraction of the

symbioses were ineffective (Fix⁻) as judged by the appearance of the plants and lack of acetylene reduction activity. Bacteria were reisolated from the plant growth medium and from nodules. In cases where prototrophic revertants were found, the plant was discarded and the experiment was repeated with new plants. For some mutant strains, the reversion frequency was too high to allow us to determine the symbiotic phenotype. The tendency of mutants to revert in plant tests is probably due to multiplication of the bacteria in the plant medium to give rise to large populations (Pain, 1979).

The symbiotic phenotypes of the different classes of mutants are shown in Table 1. Mutations in carbamoylphosphate synthetase and in ornithine, pyrimidine, purine, asparagine, leucine, tyrosine and methionine biosynthesis lead to ineffective symbiosis, as do the mutations we assign to one-carbon metabolism. Mutations in ornithine transcarbamylase, argininosuccinate synthetase, and serine or glycine biosynthesis do not interfere with successful symbiosis.

To verify that the mutation that altered the symbiotic phenotype was identical to that which caused auxotrophy, we isolated prototrophic revertants and determined their symbiotic phenotype. In all cases where revertants could be isolated, symbiotic nitrogen fixation was restored. To further demonstrate that the mutant gene is directly responsible for nitrogen fixation deficiency, mutants in classes 10–50 carrying cloned *R. meliloti* genes that complemented each mutation (see below) were inoculated onto seedlings growing in plant medium supplemented with tetracycline to ensure that plasmid-containing strains had a competitive advantage. Again, all complemented mutant strains had normal symbiotic phenotypes. The vector alone or cloned genes that did not complement the mutation did not restore symbiotic function. Based on these results we are confident that for each mutant strain, the mutation that caused auxotrophy was also responsible for the symbiotic defect.

Cloning of R. meliloti genes

Genes complementing the auxotrophic mutations were cloned by transduction from a cosmid gene bank of *R. meliloti* 104A14. To construct the gene bank, total *R. meliloti* 104A14 DNA was partially digested with *Hind*III and ligated into the *Hind*III site of the P4 cosmid cloning vector pMK318 (M. Kahn & S. Stanfield, unpublished). The cosmid gene bank was transformed into *E. coli* C-1a for amplification and packaged by infection with P2vir1. Auxotrophic mutants from each class were infected with the cosmid bank and the rescue frequency was determined by plating the infected cells on minimal mannitol tetracycline medium and on YMB tetracycline medium. The rescue frequency ranged from 1×10^{-3} to 1×10^{-7} for the different mutant classes. All mutant classes except classes 70, 80 and 90 could be rescued. Cosmid clones rescuing mutants from each of the other classes are listed in Table 1.

Plasmid DNA was purified from the rescued clones and transformed into *E. coli* C-1a. Electrophoretic analysis showed that the *R. meliloti* DNA inserts ranged in size from 5.5 kb to 16.4 kb and included from one to five *Hind*III fragments. All clones isolated by complementation of mutants from the same mutant class appeared to have common *Hind*III fragments except clones that complemented mutant class 10, which could be divided into three groups (11, 12 and 13) on this basis. Clones isolated by complementation of different mutant classes did not appear to share any of their fragments. The isolated plasmids were packaged into P2 capsids and reintroduced into the original mutant. Complementation of the mutant was observed in all cases.

Determination of complementation groups

To determine the complementation groups and possible linkage of the genes in the arginine and uracil biosynthetic pathways, the plasmids pTK11A, pTK12N, pTK13C, pTK21A, pTK21G, pTK31A, pTK41A and pTK51D were transduced into other mutants in the same and other mutant classes. The complementation pattern obtained is shown in Table 2. The complementation groups corresponded to the classification based on growth-factor requirements. A single complementation group was observed for each mutant class with the exception of mutants in class 10. A more complete complementation analysis of these carbamoylphosphate synthetase mutants is presented in the accompanying paper (Kerppola & Kahn, 1988). These

Table 2. *Complementation groups in the arginine biosynthesis pathway*

Mutant	Complementation by cloned plasmids*:								
	pTK11A	pTK12N	pTK13C	pTK21A	pTK21G	pTK31A	pTK31L	pTK41A	pTK51D
11A	+	—	—	—	—	—	—	—	
12N	—	+	—	—	—	—	—	—	
13C	—	+	+	—	—	—	—	—	
21A	—		—	+	+	—	—	—	
21G	—		—	+	+	—	—	—	
21M	—			+		—			
21B				+					
21D				+					
21I				+					
21H					+				
21J					+				
31A	—		—	—	—	+	+	—	
31F	—					+			
31J	—					+			
31D						+			
41A	—		—	—	—	—	—	+	
41D	—		—	—	—	—	—	+	
41C							—	+	
41B								+	
51A	—								+
51B									+
51C									+
51D									+

* Plasmid designations are explained in Table 1.

complementation groups thus represent several steps of the arginine biosynthetic pathway, and the complementing plasmids most probably carry structural genes coding for enzymes required in the pathway. We find no evidence that any of the genes involved in arginine biosynthesis are linked.

DISCUSSION

The physiology of the symbiotic interaction between *Rhizobium* and the host legume can be probed by using auxotrophic mutants. Correlation of a nutritional defect with a symbiotic defect indicates that at some step in nodule development the required nutrient can not be obtained from the plant. Likewise, normal nodule development by an auxotrophic mutant indicates that the bacterium is in some way acquiring the needed supplement. The *R. meliloti* 104A14 mutants isolated in this study are defective in amino acid biosynthesis, nucleotide biosynthesis, and one-carbon transfer reactions. Of the 13 classes of mutants we recovered, 10 were ineffective. This fraction is high compared to previous studies of *R. meliloti* auxotrophic mutants (Scherrer & Denarié, 1971; Denarié *et al.*, 1976; Fedorov & Zaretskaya, 1977; Malek & Kowalski, 1977). These differences may be due to variations between alfalfa cultivars or bacterial strains, or to different genetic causes for the same nutritional requirement.

There are, however, several pathways, such as purine, pyrimidine and leucine biosynthesis, where symbiotic deficiency is consistently related to a nutritional requirement. We found that mutants defective in the early steps of either purine or pyrimidine biosynthesis were symbiotically ineffective. Adenine-requiring mutants have been found to be ineffective both in *R. meliloti* (Denarié *et al.*, 1976; Fedorov & Zaretskaya, 1977) and *R. leguminosarum* (Pain, 1979). Pyrimidine-requiring mutants that are symbiotically ineffective have also been isolated previously (Scherrer & Denarié, 1971; Malek & Kowalski, 1977). The leucine auxotrophs formed very small, white nodules, a phenotype also seen by Scherrer & Denarié (1971). Truchet

Fig. 1. Location of mutational defects with biosynthetic pathways. The numbers refer to classes of mutants discussed in the text (see also Table 1). The dashed line indicates an alternative pathway for putrescine synthesis that may not exist in *R. meliloti* 104A14.

et al. (1980) showed that adding leucine to the medium around plants infected with this mutant led to the development of effective nodules. Addition of leucine did not restore effectiveness to any of our mutants.

A large fraction of the nitrogen that leaves the alfalfa nodules is transported as asparagine (Sutton, 1983). However, since asparagine synthetase mutants formed small, white and ineffective nodules, it appears that this amino acid is not available to the bacteria. Our methionine auxotrophs were ineffective. In contrast, Scherrer & Denarié (1971) found that methionine auxotrophs formed effective nodules. The difference may be due to the step affected by the mutations, or it may be a case of strain-specific requirements.

Analysis of the arginine auxotrophs led to a surprising conclusion. Mutants blocked in the latter part of the arginine biosynthetic pathway (Fig. 1), in either ornithine transcarbamylase or argininosuccinate synthetase, were effective. These results are consistent with those obtained by Denarié *et al.* (1976) and Fedorov & Zaretskaya (1977). In contrast, mutants blocked in the early part of the arginine biosynthetic pathway that leads to ornithine were ineffective. Therefore, the symbiotic defect of the ornithine auxotrophs was probably not related to the role of ornithine as an arginine precursor. Ornithine is also a precursor in polyamine synthesis (Tabor & Tabor, 1985) and lack of polyamines may directly lead to the symbiotic defect. Alternatively, *Rhizobium* may require a spermidine-derived siderophore, similar to agrobactin (Ong *et al.*, 1979), in order to acquire iron during its symbiotic development. Both of these interpretations require that *Rhizobium* be unable to make putrescine by degrading arginine via agmatine (Fig. 1). The absence of this pathway is suggested by recent work of P. Tower & A. Ferro (personal communication), who find no arginine decarboxylase activity in *R. meliloti*. The ornithine-requiring mutants isolated by Denarié *et al.* (1976) had effective or partly effective symbiotic phenotypes.

Mutants that require both arginine and uracil probably have defects in carbamoylphosphate synthetase (Mergeay *et al.*, 1974). These mutants were ineffective. Since our arginine auxotrophs produced effective nodules, a possible reason for the symbiotic deficiency of these mutants is their inability to synthesize pyrimidines. A detailed analysis of these mutants is presented in the accompanying paper (Kerppola & Kahn, 1988). Malek & Kowalski (1977) also isolated an auxotrophic mutant that required both arginine and uracil. However, although their mutant was symbiotically defective, the mutation that caused the symbiotic defect was separable from the mutation causing the requirement for arginine and uracil. This is not the case for the mutants we

have isolated since spontaneous revertants and strains complemented by the cloned genes have normal symbiotic phenotypes.

From these results we tentatively conclude that during the development of the nodule *R. meliloti* strain 104A14 must synthesize a number of its own metabolites, including both amino acids and nucleosides, and that these are not being supplied by the plant in quantities sufficient for normal nodule development. That the bacteria do not have access to all of the potential nutrients present in the nodule is suggested by the large proportion of auxotrophs that are ineffective and especially by the ineffective phenotype of the asparagine auxotrophs. Extension of this work to other auxotrophic mutants will give a more complete picture of the nutrients available to the bacteria during the development of the symbiotic association.

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REFERENCES

- DAVIS, R. W., BOTSTEIN, D. & ROTH, J. R. (1980). *Advanced Bacterial Genetics, a Manual for Genetic Engineering*, p. 209. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- DENARIÉ, J., TRUCHET, G. & BERGERON, B. (1976). Effects of some mutations on symbiotic properties of *Rhizobium*. In *Symbiotic Nitrogen Fixation*, pp. 47–61. Edited by P. S. Nutman. Cambridge: Cambridge University Press.
- FEDOROV, S. N. & ZARETSKAYA, A. N. (1977). Ethylmethane sulfonate induction of auxotrophic mutants of *Rhizobium meliloti* and their characteristics. *Mikrobiologiya* **47**, 728–732.
- HOLMES, D. S. & QUIGLEY, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Analytical Biochemistry* **114**, 193–197.
- KAHN, M., KOLTER, R., THOMAS, C., FIGURSKI, D., MEYER, R., REMAUT, E. & HELINSKI, D. R. (1979). Plasmid cloning vehicles derived from plasmids ColE1, F, R6K and RK2. *Methods in Enzymology* **68**, 268–280.
- KAHN, M. L., KRAUS, J. & SOMERVILLE, J. E. (1985). A model of nutrient exchange in the *Rhizobium*–legume symbiosis. In *Nitrogen Fixation Research Progress*, pp. 193–199. Edited by H. J. Evans, P. J. Bottomley & W. E. Newton. Dordrecht, the Netherlands: Martinus Nijhoff.
- KERPPOLA, T. K. & KAHN, M. L. (1988). Genetic analysis of carbamoylphosphate synthesis in *Rhizobium meliloti* 104A14. *Journal of General Microbiology* **134**, 921–929.
- MALEK, W. & KOWALSKI, M. (1977). Auxotrophic mutations related to symbiotic properties of *Rhizobium meliloti* strain L5-30. *Acta microbiologica polonica* **26**, 345–350.
- MERGEAY, M., GIGOT, D., BECKMANN, J., GLANS-DORFF, N. & PIÉRARD, A. (1974). Physiology and genetics of carbamoylphosphate synthesis in *Escherichia coli* K12. *Molecular and General Genetics* **133**, 299–316.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- ONG, S. A., PETERSON, T. & NEILANDS, J. B. (1979). Agrobactin, a siderophore from *Agrobacterium tumefaciens*. *Journal of Biological Chemistry* **254**, 1860–1865.
- PAIN, A. N. (1979). Symbiotic properties of antibiotic-resistant and auxotrophic mutants of *Rhizobium leguminosarum*. *Journal of Applied Bacteriology* **47**, 53–64.
- SCHERRER, A. & DENARIÉ, J. (1971). Symbiotic properties of some auxotrophic mutants of *Rhizobium meliloti* and of their prototrophic revertants. *Plant and Soil* (special volume), pp. 39–45.
- SOMERVILLE, J. E. & KAHN, M. L. (1983). Cloning of the glutamine synthetase I gene from *Rhizobium meliloti*. *Journal of Bacteriology* **156**, 168–176.
- SUTTON, W. D. (1983). Nodule development and senescence. In *Nitrogen Fixation*, vol. 3, pp. 144–212. Edited by W. Broughton. New York: Academic Press.
- TABOR, C. W. & TABOR, H. (1985). Polyamines in microorganisms. *Microbiological Reviews* **49**, 81–99.
- TRUCHET, G., MICHEL, M. & DENARIÉ, J. (1980). Sequential analysis of the organogenesis of lucerne (*Medicago sativa*) root nodules using symbiotically defective mutants of *Rhizobium meliloti*. *Differentiation* **16**, 163–172.
- VERMA, D. P. S. & LONG, S. R. (1983). The molecular biology of the *Rhizobium*–legume symbiosis. *International Review of Cytology* (supplement) **14**, 211–245.
- VINCENT, J. M. (1970). *A Manual for the Practical Study of Root Nodule Bacteria*. Oxford: Blackwell Scientific Publications.