Auxotrophy in rhizobia revisited

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Abstract Among the various types of mutations studied in rhizobia, the auxotrophic mutations (which confer on the mutants the inability to synthesize certain essential substances such as amino acids, vitamins and nucleic acids), are the most favoured ones as these can be used as suitable markers for genetic analysis. An important property of rhizobia is their effectiveness i.e. their ability to fix atmospheric nitrogen into ammonia within the nodule. Special interest in this category of mutations by rhizobial geneticists is due to the fact that there is a strong correlation between the metabolic defects and the ineffectiveness (Nod⁻ and/or Fix⁻) of the rhizobial strains. Auxotrophic mutants of various species of rhizobia with defects in the synthesis of nucleic bases, vitamins and amino acids have been obtained by mutagenising with physical, chemical and Tn5 mutagens. These mutants have been used in mapping studies as well as in establishing a correlation between its metabolic requirement and symbiotic relationship with the host plant. The present review deals with the isolation of auxotrophs, and their genetic, biochemical and symbiotic characterization. The review also encompasses the studies on the elucidation of biosynthetic pathways of nutritional substances in rhizobia.

Key words Rhizobia · Auxotrophs · Enrichment · Effectiveness · Mapping · Biosynthetic pathways

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Introduction

Bacteria of six genera of rhizobia namely *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium* (recently changed to *Ensifer*), *Mesorhizobium* and *Allorhizobium*¹ are of major agricultural importance because of their ability to form N₂-fixing nodules on the roots of leguminous plants. Rhizobia-legume symbiosis has attracted worldwide attention as it implies lesser dependence on expensive petroleum-based nitrogenous fertilizers for legumes, thus reducing the chance of causing environmental pollution. The importance of this group of bacteria is further enhanced by the fact that the legumes nodulated by rhizobia include some of the most important crop plants of the world. Symbiotic nitrogen fixation is a complicated process involving reciprocal exchange of low-molecular weight signals between rhizobia and the legumes leading to the coordinated gene expression².

For identification and mapping of genes as well as for studying their functions, it is imperative to have mutations at various chromosomal loci. Among the various types of mutations, auxotrophic mutations are most suitable and readily selectable markers for genetic analysis. The interest in these mutations is further enhanced by the fact that the expression of symbiotic genes is closely associated with the metabolism of amino acids, vitamins and nucleic acids in bacteria^{3,4}. Auxotrophic mutants of rhizobia that are symbiotically defective (as a consequence of their nutritional requirement) can be useful tools for studying the complex series of events in the formation of symbiotic nitrogen-fixing nodules^{5,6}.

The auxotrophic mutants requiring purines, pyrimidines, amino acids and vitamins have been isolated in various species of rhizobia using physical (UV rays), chemical [N-methyl-N-nitro-N-nitrosoguanidine (NTG), ethyl methane sulphonate (EMS), nitrous acid (HNO₂), mustard gas]

and transposon (Tn5) mutagens. Among a large number of auxotrophic mutants tested, those requiring purines or pyrimidines^{3,7–9} were ineffective (Nod[−]), whereas, mutants auxotrophic for amino acids i.e. arginine, cysteine, glycine, methionine, phenylalanine, tryptophan, etc. were effective^{3,7–12}. The glycine-requiring mutants were found to be even more effective than the parent strain³. The present review sums up the work till date on the isolation and identification of auxotrophic mutants in rhizobia, their biochemical and enzymatic properties, mapping of auxotrophic mutations and the effect of metabolic alterations on symbiotic properties.

Isolation and identification of auxotrophic mutants

The isolation of auxotrophic mutants in *Escherichia coli* is relatively easy and well documented. But the same is not true in case of *Rhizobium* sp. because of the production of extra-cellular polysaccharides by the organism during growth, which in turn impedes the isolation of auxotrophs. The first step in isolation of auxotrophs in *Rhizobium* is to treat the exponential or stationary phase cells of a wild type strain with a potent mutagen to induce mutations. Normally that dose of mutagen is used which gives less than 10 % survival of the treated cells. The various mutagens used for inducing auxotrophic mutations include U.V. rays^{9,13}, EMS^{14–17}, NTG^{3,9–12,18–23}, HNO₂^{19,24–27}, Tn5 mutagen^{28–39}, nitrogen mustard⁴⁰ and structural analogues of L-amino acids⁴¹.

Not all the mutagens are effective in causing auxotrophic mutations in different species of rhizobia. There appears to be strain variation in the mutagenicity of a particular agent in different strains⁴². For example, EMS was found to be effective in inducing auxotrophs in *Sinorhizobium meliloti* strains Rm2011 and L5-30^{15,16}, *Rhizobium leguminosarum* bv. *trifolii*^{17,64}, *Bradyrhizobium japonicum*¹⁴ and *Bradyrhizobium* sp. (*Vigna*)⁶³ but it was very inefficient in *Rhizobium leguminosarum* bv. *viciae*⁴³ and *R. leguminosarum* bv. *trifolii*²¹. Likewise, NTG, EMS, HNO₂ and UV were mutagenic with the JRC23 strain of cowpea rhizobia, but did not mutate strain IRC256. On the contrary, Tn5 yielded auxotrophs in strain IRC256, but not in strain JRC23⁴⁴. The reasons for mutagen specificity in rhizobia are not known⁴⁵. Likewise, it was impossible to obtain auxotrophs of strain L-1 of *S. meliloti* by UV rays or HNO₂ treatment⁴⁶ but the latter induced auxotrophic mutations in *R. leguminosarum* bv. *viciae*²⁶, *S. meliloti*⁴, *B. japonicum*²⁷, *Bradyrhizobium* sp. (*Lupinus*)²⁴ and *R. leguminosarum* bv. *viciae*^{19,25}. Out of many mutagens used for the induction of auxotrophic mutations, NTG acts as a powerful mutagen under conditions in which over 50 % of the cells survive in *E. coli*⁴⁷.

After mutagenesis, the treated cells are sub-cultured in the complete or enriched medium viz; yeast extract mannitol (YEM)⁴⁸ or glucose salts yeast extract (GSY)⁴⁹ broth or MM (for specific auxotroph) for 4–5 doublings in order to allow the complete segregation of mutant and non-mutant nuclei from multi-nucleate cells and to permit the phenotypic expression of the induced mutations⁵⁰. This step is very crucial because without phenotypic expression, we are not able to isolate any mutants⁵¹. The failure to get mutants depends on a lag in the adjustment of the enzyme composition of the cell to the new genetic composition. Until the cell has gone through enough generations to dilute out the enzyme molecules, which were formed by the gene prior to its mutation, the cell does not lose its capacity to form a given metabolite, and hence is not resistant to penicillin in minimal medium. Another factor, which limits the survival of mutants, is the syntrophic effect of metabolites secreted by the non-mutated cells growing in minimal medium. The density of the population exposed to penicillin is, best limited to 10⁶ cells ml^{−1}.

The next step after phenotypic expression is the enrichment of auxotrophic mutants among prototrophs. This is accomplished by adding the antibiotic penicillin or ampicillin to the phenotypically expressed cells^{51–56}. Penicillin or ampicillin acts on growing cells by preventing a transpeptidation reaction involved in the formation of cross-linkage in the cell wall^{57–59}. The cells in the presence of ampicillin/penicillin become osmotically fragile and lyse. If the medium lacks an essential nutrient (amino acid, vitamin or a base), the cells will not grow and, hence, will not become osmotically fragile. In a mixed culture of prototrophs and auxotrophs growing in unsupplemented minimal medium with ampicillin/penicillin, the prototrophs will be preferentially lysed, thus augmenting the proportion of auxotrophs among the surviving cells⁵⁹.

The yield of auxotrophs depends upon the efficiency with which growing cells are killed as well as on the degree of resistance of the starved cells to the penicillin or ampicillin treatment. In order to achieve sufficient killing of the parent cells, it is essential to incubate the cells in penicillin for 6–24 h. The primary action of ampicillin/penicillin on sensitive bacteria is to induce protoplast formation by interfering with cell wall synthesis⁶⁰. These protoplasts then burst osmotically, releasing nutrients into the minimal medium. The nutrients promote growth of the auxotrophic mutants, so that they in turn become susceptible to penicillin. The population exposed to penicillin has to be kept below a density of about 10⁶ cells ml^{−1} because the auxotrophs are cross-fed by the cells that lyse during penicillin treatment. The efficiency of this method is limited by the restriction on population density, by cross-feeding that occurs even at low

population densities and by the residual growth of mutants on stored metabolites not eliminated by washing.

Gorini and Kaufman⁶¹ modified the method of Davis⁵¹ in *E. coli* by growing the phenotypically expressed cells in a hypertonic minimal medium (MM) before the addition of penicillin. Growth in the presence of ampicillin or penicillin in the complete or enriched medium ordinarily causes lysis but in a hypertonic medium, the cells are instead converted to protoplasts^{57,60,62}. In the method of Gorini and Kaufman⁶¹, 0.6 M sucrose and 0.01 M Mg SO₄ were added to the penicillin selection medium in order to prevent lysis and thereby to decrease crossfeeding. In addition, it is possible to shorten the duration of exposure to penicillin by allowing the culture to reach exponential growth in minimal medium before the addition of penicillin. This step has the additional advantage of permitting the auxotrophs to exhaust their pools of required growth factors. For preparing the hypertonic MM, either 0.6 M sucrose⁶³ or 0.5 M sorbitol⁶⁴ or a combination of both, with reduced concentrations⁶⁵ have been used. By introducing these modifications, it has been possible to screen populations at densities up to 5x10⁸ cells ml⁻¹ i.e. about 10³ times greater than the densities previously feasible. The selection of auxotrophic mutants is, thereby markedly improved.

The auxotrophic mutants are enriched after starvation. In *Agrobacterium tumefaciens*, the enrichment of auxotrophic and fermentation mutants has been done by the combination of carbenicillin with lysozyme⁶⁶. The method is based on the fact that the killing power of carbenicillin is amplified by the addition of lysozyme. In rhizobia, a combination of ampicillin or penicillin with lysozyme^{55,56,63–65} has been useful for the enrichment. For the isolation of auxotrophs, the wild type strain has to be either penicillin or ampicillin sensitive^{63–65}. The aim of choosing an ampicillin or penicillin-sensitive strain for the isolation of auxotrophs is to ensure killing of prototrophic survivors after mutagenesis and to curtail the duration of ampicillin/penicillin treatment during enrichment of auxotrophs. The time periods for phenotypic expression in minimal medium supplemented with the requirement, starvation in hypertonic minimal medium and enrichment by ampicillin or penicillin and lysozyme are adjusted in accordance with the mean generation time of wild type strain in respective media. The careful monitoring is done for each and every step of the protocol followed to facilitate the isolation of auxotrophs. The detailed steps are given in the flowsheet as shown in Figure 1.

Effect of auxotrophy on symbiotic process

An important property of nodule forming bacteria is their effectiveness i.e. how efficiently these bacteria fix at-

mospheric N₂ in symbiotic association with host legumes. The genetic analysis of rhizobial mutants can help to establish the causal relationship between certain types of mutations (leading to metabolic defects) in these bacteria and their symbiotic nitrogen fixation. The studies of symbiotic properties of auxotrophic mutants may provide information useful for improvement of inoculants. Most of the auxotrophs show defective symbiotic properties such as loss of effectiveness, restriction of host range and a decrease in competitiveness for nodule formation. The only auxotrophic mutants known to be more effective than the wild type strain are very bad competitors.

Schwinghamer⁶⁷ was the first to discover a causal relationship between an auxotrophic mutation in rhizobia and their symbiotic effectiveness. In comparison to the effective and prototrophic strains 24 and T37 of *R. leguminosarum* bv. *trifolii*, all the auxotrophic mutants isolated from these were altered for their symbiotic activity⁶⁸. The symbiotic properties of the prototrophic revertants and the auxotrophs were found to be similar. Some of the mutants were more effective and some were ineffective than the wild type strains. All the 11 auxotrophs isolated from Sa10 and 2011 strains of *S. meliloti*³ formed nodules on lucerne. Prototrophic revertants isolated from Ade⁻ and Ura⁻ strains were found to be more effective than the wild type strain. The authors found an association between nutritional requirement and nitrogen fixation on this host. Kowalski *et al.*(1972)¹⁰ found that auxotrophy for leucine in *S. meliloti* strain L5-30 led to the loss of ability to fix nitrogen on *Medicago sativa*. Prototrophic recombinants, obtained after transduction, had nitrogen-fixing ability. Pankhurst *et al.*(1972)⁶⁹ showed that riboflavin was required by the rhizobia for differentiation into bacteroids in *R. leguminosarum* bv. *trifolii*. When riboflavin was removed after the formation of bacteroids, nitrogen fixation continued. It was also found that differentiation of cells into bacteroids is dependent on a high level of riboflavin, while growth within the nodules and nitrogen fixation by the bacteroids were supported by lower levels. A correlation between the formation of effective nodules, by riboflavin-requiring mutant on some varieties of sub-terranean clover, with different levels of flavin in the tissues of the plants revealed that N₂-fixing varieties were having about 5 times more flavin than ineffective varieties⁷⁰.

Beringer¹⁹ and Johnston and Beringer²⁵ tested auxotrophic mutants of *R. leguminosarum* strain 300 for their symbiotic properties and found that all were infective and effective except leucine auxotrophs, which were infective but ineffective. The double auxotroph for adenine and uracil was non-infective. Denarie and Bergeron⁸ demonstrated an association between nutritional defects of mutants and

*N: Any one antibiotic

Fig. 1 Standard protocol for isolation of auxotrophs.

their symbiotic capacity in *S. meliloti*. All purine and pyrimidine-requiring mutants were ineffective but all the 11 leucine-requiring (*leu*) mutants gave different responses; some were effective, some were ineffective and others were intermediate. Two thymine-requiring mutants were fully effective for symbiotic properties. The glutamate auxotrophs isolated from *S. meliloti* strain 41 and A1 were

fully effective in symbiosis and produced healthy and pink nodules on lucerne but their nitrogenase activity was not significantly different from that given by wild type strain¹¹. But, the glutamine-requiring mutant nodulating lucerne was ineffective, whereas glutamine-independent revertant of this mutant was fully effective. Effective strain L5-30 of *S. meliloti* used for the isolation of auxotrophic mutants

by Malek and Kowalski⁷¹ showed that all the revertants and transductants of the histidine-requiring strains were effective suggesting that histidine deficiency was the cause of ineffectiveness. Microscopic observation of nodules induced by *his* mutant of above rhizobial strain, showed that the mutant was defective in liberation of cells from infection thread and their multiplication because supplementation of the plant growth medium with L-histidine resulted in the establishment of a fully effective association⁷².

Mutants auxotrophic for adenine and uracil isolated by Pain²⁶ were found to be symbiotically defective and non-infective. Bacteria re-isolated from the nodules were tested for auxotrophic marker and it was found that nodules were formed by prototrophic revertants. Twenty-three pyrimidine auxotrophs of *S. meliloti* Rmd201⁷³, generated by Tn5 mutagenesis, were classified into *car*, *pyrC*, *pyrE/pyrF* categories on the basis of biochemical characters. All auxotrophs induced white nodules, which were ineffective in nitrogen fixation. The results indicate that some of the intermediates and/or enzymes of pyrimidine biosynthetic pathway of *S. meliloti* play a key role in bacteroid transformation and nodule development.

Cen *et al.*²⁹ observed that out of seven auxotrophic mutants obtained from a slow-growing *Bradyrhizobium* strain CP283 Sm^r, only the *cys*⁻ formed nodules of a reduced size and did not fix nitrogen *in planta* or *in vitro* (even when supplied with exogenous cysteine). Auxotrophic mutants for adenine, arginine, histidine, lysine and valine, isolated by Kohli and Vashishat⁶³ from mungbean *Rhizobium* SMR8, revealed that mutants requiring adenine, arginine and histidine did not form nodules, whereas lysine and valine-requiring mutants were ineffective. A study of symbiotic properties of auxotrophic mutants in strain L5-30 of *S. meliloti* by Fedorov *et al.* (1984)¹³ showed that the nodules were distinguishable in shape, size and number from those of parental strain. Nitrogenase activity of mutants and parental strain was found to be similar. Stable auxotrophic mutants of *B. japonicum* strain 110²² formed nodules on soybean plants and bacteria could be recovered from the nodules. Mutants from the fast-growing, broad-host range *Rhizobium* strain ANU280 (NGR234 Sm^r Rf^r) overproduced polysaccharides and induced poorly developed nodules on *Leucaena leucocephala* and *Lablab purpureus*³⁵. Appelbaum *et al.*³⁴ characterized several auxotrophic mutants of *B. japonicum* strain USDA191 and showed that *nod* and *nif* genes are located within one region of 200MD plasmid (pSym191). Fedorov and Simarov⁷⁴ studied the nitrogenase activity of 44 mutants of *S. meliloti* strains SHM1 and L5-30 as well as 22 spontaneous prototrophic revertants of mutants having no nitrogen fixation activity.

Sadowsky *et al.* (1986)³⁶ found that out of four histidine auxotrophs of *B. japonicum* strain USDA122, two were symbiotically defective and did not form nodules on *Glycine max* and on *Glycine soja*, and other two mutants were symbiotically competent while prototrophic revertants formed nodules on all the soybean cultivars examined. Symbiotically defective auxotrophic mutants of *R. fredii* HH303 isolated by Kim *et al.* (1988)³⁷ induced nodulation on soybean, but the symbiotic effectiveness of each mutant was different. Kumar and Kuykendall (1989)⁷⁵ examined the 23 amino acid auxotrophs of *B. japonicum* and found that 3 out of 11 *trp*⁻ auxotrophs formed nodules without fixing nitrogen while the other *trp*⁻, *pro*⁻, and *his*⁻ mutants formed abortive nodules. The leucine-dependent auxotrophs and the isoleucine partial auxotrophs were Nod⁺ and Fix⁺. Histidine auxotrophs of *S. meliloti* strain L5-30 formed ineffective nodules that contained only a few degenerating bacterioids inside large vacuoles but many starch granules. Prototrophs of this mutant formed pink and effective nodules⁷⁶. The symbiotic properties of prototrophic revertants TA11 NOD⁺ of nodulation-defective tryptophan auxotrophs of *B. japonicum* were compared with that of normally nodulating wild-type strain of *B. japonicum*-110ARS. Plants inoculated with TA11 NOD⁺ contained significantly more nitrogen per plant (due to an increase in overall nodule number) than did plants inoculated with wild type bacteria⁷⁷. Sharypova *et al.* (1994)⁷⁸ showed enhanced symbiotic effectiveness (Eff^r) from *S. meliloti* XMI-105 and XMI-188. The frequency of Eff^r mutants in strain XMI-188 was higher than in the XMI-105 (1.1 % versus 0.4 %).

Prasad *et al.* (2000)⁷⁹ isolated ten aromatic amino acid auxotrophs of *S. meliloti* by Tn5 mutagenesis and studied their symbiotic properties. Normal symbiotic activity, as indicated by morphological features, was observed in tryptophan synthase mutants and the lone tyrosine mutant. The *trpE* and *aro* mutants fixed trace amounts of nitrogen, whereas the *phe* mutant was completely ineffective in nitrogen fixation. These studies demonstrated the involvement of anthranilic acid and at least one more intermediate of tryptophan biosynthetic pathway in bacteroid maturation and nitrogen fixation in *S. meliloti*. The alfalfa plant seems to provide tryptophan and tyrosine but not phenylalanine to bacterioids in nodules.

Abbas *et al.* (2002)⁸⁰ studied the symbiotic effectivity of 21 cysteine and 13 methionine auxotrophs of *S. meliloti*. The nodules induced by cysteine auxotrophs were fully effective, whereas the nodules induced by methionine auxotrophs were completely ineffective.

Das *et al.* (2006)⁸¹ isolated and characterized a Tn5-induced mutant strain (TL68) of *Mesorhizobium ciceri* unable to grow with ammonium as sole nitrogen source.

The serA⁻ mutant (TL68) was unable to elicit the development of efficient nodules on the roots of *Cicer arietinum* L. The addition of serine to the plant-growth medium restored the ability of the mutant to nodulate *Cicer arietinum*, and the nodules were able to fix nitrogen.

Use of auxotrophic mutants in mapping studies

Auxotrophic mutants of *Bradyrhizobium* sp. (*Lupinus*) were used as recipients and the wild type as donor of DNA for transformation studies⁴⁰. Raina and Modi⁹ used strain GR3, an adenine-requiring mutant of *Bradyrhizobium* cowpea group as the recipient and its wild type as the donor for transformation studies. Auxotrophic mutants in *S. meliloti* strain GR4 were used for linkage mapping studies¹². Megias *et al.* (1982)¹⁷ used wild type strain RS55 of *R. leguminosarum* bv. *trifolii* for constructing a circular linkage map. Osteras *et al.* (1989)⁸² studied auxotrophic mutants of NGR234 and a chromosome mobilization system based on Tn5-mob and pJB3JI was utilized. The use of four different strains with Tn5-mob located at different nutritional loci in crosses with double auxotrophic recipients enabled the construction of a circular linkage map of NGR234 based on relative combination frequencies to be constructed. Lewis *et al.* (1990)⁸³ suggested that the glutamate synthase expression requires complementation groups present at this locus. By use of Southern hybridization and plasmid transfer, it was observed in *S. meliloti*⁷⁸ that ten Tn5 insertions were located in the chromosome, five in megaplasmid 1 and six in megaplasmid d2.

Biochemical and enzymatic studies with auxotrophic mutants

Chemically induced mutants have an advantage over spontaneous derivatives (which are symbiotically defective) in the sense that one can be more certain that the phenotypic changes observed have resulted from a single mutation. Hence, clear causal relationship between loss of symbiotic ability and other biochemical or physiological defects observed can be established. This argument is strengthened if there are revertants that coincidentally regain both correlated wild-type properties. The most useful symbiotically defective mutants can be obtained only by using chemical mutagenesis followed by direct screening of survivors for symbiotic nitrogen fixation with the host legume. Wells and Kuykendall (1983)²⁷ observed that the mutants isolated from *B. japonicum* I-110 ARS after HNO₂ did not grow on indole and accumulated indole glycerol phosphate, were confirmed as having tryptophan synthetase activity while

exhibiting other activities comparable to those in the parent strains. Ali *et al.* (1984)³² introduced Tn5 into the genome of two *S. meliloti* strains 444 and M28 *str* using a suicide plasmid vector. Among the clones carrying random Tn5 insertion, about 0.3% mutants were isolated from each strain and found that site occupied by Tn5 was not always identical in the *trp* mutants. Mutants of *B. japonicum* requiring glutamine or glutamate as a nitrogen source showed similar differences in enzymes, slightly lower glutamine synthetase (GS), 3–5 times lower glutamate synthase but more than 10 times higher alanine dehydrogenase (ADH) activity⁸¹. According to Noel *et al.* (1988)³⁸, undiminished metabolic flow through *de novo* purine biosynthesis or a particular intermediate in the pathway is essential in early symbiotic interaction. Mutants in ornithine transcarbamylase, argininosuccinate synthetase or serine glycine biosynthesis isolated by Kerppola and Kahn⁸⁵ from *S. meliloti* 104A14 formed nitrogen fixing (Fix⁺) nodules on the roots of alfalfa (*Medicago sativa*).

Analysis of *R. meliloti trp* auxotrophs suggest that anthranilate biosynthesis by the *R. meliloti trpE* (G) gene product is necessary during nodule development for the establishment of an effective symbiosis⁸⁶. *trpE* (G) mutants as well as mutants blocked earlier along this pathway, form nodules on alfalfa that have novel defects. In contrast, mutants blocked later in the Trp biosynthetic pathway form normal, pink, nitrogen-fixing nodules. Anthranilate is postulated to act as an *in planta* siderophore. *trpCD* deletion mutants of *B. japonicum* were isolated by site-directed marker exchange mutagenesis⁸⁷. The new Trp⁻ deletion mutants lacked activities for the *trpC*- and *trpD*-encoded enzymes, indole glycerol phosphate synthase (EC 4.1.1.48) and phosphoribosyl anthranilate transferase (EC 2.4.2.18), respectively. Exogenous application of the purine precursor 5-aminoimidazole-4-carboxamide (AICA) riboside restores infection and enhances the development of bean (*Phaseolus vulgaris*) root nodules elicited by *R. etli* purine auxotrophs⁸⁸. AICA riboside does not act directly upon the plant, but rather must be converted to AICA ribonucleotide (AICAR) by rhizobia in order to be effective. These results indicate that infection of bean by *R. etli* requires rhizobial production of AICAR, whether by purine biosynthesis, histidine biosynthesis, or conversion from AICA riboside.

The transposon Tn5-induced purine auxotrophs of NGR234 (ANU2861 and ANU2866) are defective for nodule formation on a wide variety of legumes and the non-legume *Parasponia*⁸⁹. The examination of ANU2861 and ANU2866 lipopolysaccharide (LPS) showed that only ANU2861 possessed a defective LPS that was corrected by addition of AICA-riboside to the growth medium. These results indicated that both mutants are defective in the

purine biosynthetic pathway and that metabolic flow through this pathway is essential for nodule morphogenesis. Yadav *et al.* (1998)⁶⁵ analyzed *his* mutants for their biochemical and symbiotic defects in *R. leguminosarum* bv. *trifolii* and it was suggested that a perturbation of the histidine biosynthetic pathway can abolish the symbiotic competence of *Rhizobium*. Analysis of biochemical defects in mutants revealed that defects in early steps of pathway abolished nodulating ability, whereas lesions in the last step did not. But, the last step was required for symbiotic nitrogen fixation.

King *et al.* (2000)⁹⁰ isolated *proC* gene of *Bradyrhizobium japonicum* and constructed a *proC* mutant strain that behaved as a strict praline auxotroph in culture. The *proC* strain elicited undeveloped nodules on soybeans that lacked nitrogen fixation activity and plant hemoglobin. The authors concluded that the *proC* gene is essential for symbiosis and suggested that the mutant does not obtain an exogenous supply of proline in association with soybeans sufficient to satisfy its auxotrophy.

A metagenomic cosmid library was constructed by Li *et al.* (2005)⁹¹, in which the insert DNA was derived from bacteria in a waste-water treatment plant and the vector was the wide host-range cosmid pLAFR3. The library was screened for clones that could correct defined tryptophan auxotrophs of *Rhizobium leguminosarum* and *Escherichia coli*. Several cosmids corrected the auxotrophy of one or more *R. leguminosarum trp* mutants, but not the corresponding mutants in *E. coli*. For one metagenomic cosmid, *lac* reporter fusions confirmed that its cloned *trp* genes were transcribed in *R. leguminosarum*, but not in *E. coli*. Thus, rhizobia, with their many σ -factors, may be well-suited hosts for metagenomic libraries, cloned in wide host-range vectors.

Conclusion

The physiology and biochemical aspects of the symbiotic interaction between rhizobia and the host legumes can be probed by using auxotrophic mutants. Normal nodule development in the legume plants infected by an auxotrophic mutant indicates that the bacterium in some way acquires the needed supplements. In several pathways, such as purine, pyrimidine, amino acids biosynthesis etc. the symbiotic deficiency is consistently correlated to a nutritional requirement. Mutants defective in early steps of biosynthetic pathways are found to be symbiotically defective. The symbiotic competence of amino acid auxotrophs may vary depending upon strain-specific differences in the bacterial symbiont as well as the amino acid biosynthesis involved. Thus, symbiotically defective auxotrophic mutants of

rhizobia, can be potential tools for studying the complex series of events in the development of symbiotic nitrogen-fixing nodules. Also, the auxotrophic mutants having perturbations at different steps of biosynthetic pathways may be useful in elucidating these pathways.

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