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Agrobacterium Tumefaciens Nitrogen Fixation System Expresses in *Sinorhizobium meliloti* (=*Rhizobium meliloti)*

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Abstract: Kanvinde and Sastry (Appl. and Environ. Microbiol. 56:2087-2092, 1990) had previously demonstrated that *A. tumefaciens* has all the physiological characters of a diazotroph. Genetic evidence indicates that the genes responsible for the reaction are located on the chromosome between *his* and *trp* but nearer to the former. Using this information two recombinant plasmids were isolated: (a) a stable R68.45-prime, pSRK9, bearing *his*$^+$ Nif$^+$ *trp*$^+$ of *A. tumefaciens* and (b) pHN20, a recombinant cosmid (with a *his*$^+$ Nif$^+$ insert, about 30 kb) selected from a gene bank. Both these plasmids have been shown to be able to express clearly and facilitate nitrogen fixation in *S. meliloti nif* K$^-$ and to some extant in *nif* H$^-$ backgrounds; the plasmids acted synergistically with the wild type itself as was evidenced by increased nitrogen fixation and dry weight.

Key words: Acetylene reduction, dry weight, root nodules, Alfalfa. *Sinorhizobium meliloti, Rhizobium meliloti, Agrobacterium tumefaciens.*

INTRODUCTION

Sometime ago, it was shown that *Agrobacterium tumefaciens* has all the physiological hallmarks of a diazotrophic organism --- it grows on a well-defined nitrogen-free medium, it reduces acetylene under microaerophilic conditions which is repressed by the presence of NH_4^+ and it incorporates $^{15}N_2$, when supplied in a gaseous form [1]. However, lack of DNA sequences which visibly hybridize with *nif* probes from *Klebsiella pneumoniae,* [2] and unpublished results from this Laboratory] *Sinorhizobium meliloti* and the genes responsible for vanadium-nitrogenase (*vnf*) (unpublished from this Laboratory) remained as a major obstacle for further characterization of the system.

During a genetic investigation, we found circumstantial evidence to suggest that the genes responsible for nitrogen fixation in *A. tumefaciens* are located on the main chromosome, and are in between *his*19 and *trp*20 (but nearer to the former) of the map produced by [3]. Using this information as the basis, two recombinant plasmids are obtained in the present investigation, and it will be shown with the help of expression studies in *S. meliloti* that they contain the *A. tumefaciens* genes required to fix nitrogen.

MATERIALS AND METHODS

Bacterial Strains and Plasmids: The chromosomal gene bank of *A. tumefaciens* C58 screened in the present investigation was constructed by [4] using a *Bam* H1 partial digest of a Ti plasmid-free strain, NT1. The vector was a broad host range cosmid, pCP13/B [5]. The bank was in *E. coli* DH1 and was supplied as the bacteria containing the recombinant cosmids. It was divided into eight parts and were used as eight donor parents with *E. coli* UNF514 the recipient in eight filter matings; pRK2013 was used as the helper plasmid. It should also be pointed out that *A. tumefaciens* C58 [LBA2238 (R68.45)] used to obtain pSRK9 is also free from the Ti plasmid.

Media: Recipe for the minimal medium AB is in [6]; M9 (supplemented with biotin at 1.0 mg/ml for *S. meliloti*), complete media LB and TY are described [7] Nitrogen free minimal medium, NFDM, is described in [1].

When necessary, filter sterilized antibiotics were added at the following concentrations: tetracycline 15 mg/ml and 10 mg/ml (5 mg/ml for selecting pSRK9) for *E. coli* and *S. meliloti* respectively; kanamycin 25 mg/ ml for *E. coli* and neomycin for *S. meliloti* at 60 mg/ml was used.

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Formula for FP, used as nitrogen free medium for the plant growth is given in [8].

Plasmid Transfer: Plasmids were transferred between the bacteria either using a filter mating [9]. or transformation [see 10 for the procedure]. DNA was prepared using a commercial kit made by QIAGEN (QIAGEN Ltd., Dorking RH4 1HJ, England).

Plants and Infection: Alfalfa (var. Culver) seed were surface sterilized and germinated on water agar (containing 500 mg/ml ticarcillin) in darkness at 25° C. Uniform seedlings at the first true leaf stage were transferred under aseptic conditions to test tubes (one per tube) containing solidified FP agar slopes. Once established for one or two days in growth rooms maintained at 22° C**,** they were infected with overnight bacterial cultures (one ml per tube) grown in TY medium with tetracycline; cultures were washed with FP medium prior to use, to remove the traces of fixed nitrogen. The root system was covered with a foil to prevent the suppressive effect of light on nodulation.

Since the above procedure allowed only 4 to 5 weeks of growth, for testing under relatively long term conditions, an alternative procedure was used. Surface sterilized alfalfa seed was germinated in three-inch pots containing sterilized vermiculite. Plants (one per pot) were watered regularly with double distilled autoclaved water, and once a week with half-strength, sterilized FP medium. Plants were grown and infected as in the preceding case

RESULTS AND DISCUSSION

Production of the Recombinant Plasmid pSRK9: The first recombinant plasmid, pSRK9, was isolated as a R68.45-prime from the following cross: *A. tumefaciens* C58 *phe*1 *pyr*12 (R68.45) X *E. coli his trp rec* A [LBA 2238 (R68.45) X UNF 514]. The self-transmissible P group plasmid, R68.45, is well known for its ability to transfer chromosomal genes in several bacterial genera such as *Psudomonas, Rhizobium* and *Agrobacterium* [see for example 3]. In designing the above cross, we made certain that the *A. tumefaciens* donor strain C58 is free from the Ti plasmid, *nif*$^+$ and has the auxotrophic markers (*phe* and *pyr*) are far from *his*$^+$ and *trp*$^+$*;* we also knew that the latter complement the respective mutant alleles of UNF514.

Several *his*$^+$, *trp*$^+$, and *his*$^+$ *trp*$^+$ recombinants were selected on properly supplemented AB minimal medium (which contains ammonium chloride as the nitrogen source) plates. As expected from the previous indications, most of the *his*$^+$ (80%) and *his*$^+$ *trp*$^+$ (98%) recombinants were Nif$^+$ (125 colonies from each class were tested; Nif$^+$ / Nif$^-$ phenotype was assessed by their ability to grow on nitrogen free medium, NFDM). Further transfer experiments established that in the recombinants all *A. tumefaciens* genes are located on the plasmid R68.45. One of the stable *his*$^+$ Nif$^+$ *trp*$^+$ primes, which from now onwards will be referred to as pSRK9, was chosen for further analysis; this recombinant plasmid carries all the antibiotic markers (Tc, Ap, Km) and the conjugative ability of R68.45.

Additionally, the *his* and *trp* markers of pSRK9 satisfy the auxotrophic requirements of *E. coli* UNF514 alleviating the need to supplement nitrogen free medium with histidine and tryptophan. This avoided doubts raised in early experiments as to whether the *E. coli* strain containing the *A. tumefaciens* genes, was growing on NFDM with the help of aminoacids (especially tryptophan) as the nitrogen source.

In spite of its advantages, pSRK9 did not turn out to be an ideal candidate for molecular analysis: it is a big (~ 85 kb) low copy number (appears to be even lower than the vector, R68.45) plasmid and produced only good DNA after several attempts.

Isolation of pHN20: Two reasons made us to look for an alternate recombinant plasmid:

(a) A plasmid produced using molecular procedures should be smaller and make its characterization relatively simple.

(b) To obtain an *A. tumefaciens* gene bank constructed in a different laboratory and select recombinant plasmids following the same procedure as the one used for obtaining pSRK9, and confirm that our results were not anomalous.

To this end an *A. tumefaciens* C58 chromosomal gene bank (constructed in a broad host range cosmid, pCP/13) was screened (see Materials and Methods). Using similar selection procedures employed to isolate pSRK9, eleven *his*$^+$, nine *trp*$^+$ and seven *his*$^+$ *trp*$^+$ transconjugants were isolated; all of them showed resistance to tetracycline, the antibiotic marker of the cosmid. Further confirmatory tests showed that all the recombinant markers are located on the cosmid. All eleven *his*$^+$ cosmids facilitated *E. coli* UNF 514 to grow extremely well on nitrogen free medium (Fig. 1) but none of the *trp*$^+$ nor (surprisingly) *his*$^+$*trp*$^+$ colonies grew well.

Insert sizes in a representative numbers of all types of recombinant cosmids were examined: they were around 30 kb, and no more. A stable *his*$^+$ carrying recombinant cosmid, pHN20, was chosen for a detailed investigation.

Expression in S. meliloti: Although the initial observations indicated that the *A. tumefaciens* genes carried by the recombinant plasmids do express (as

monitored by the growth on NFDM under anaerobic conditions) in *Klebsiella pneumoniae nif* mutant strains, the results were often erratic; the expression was much better in *A. rubi* and *Alcaligenes eutrophus* (results not shown) but, since genetic knowledge of these bacteria is virtually non-existent they could not be used to define the *nif* system of *A. tumefaciens.* Results with *S. meliloti* were, however, encouraging and therefore we explored it to confirm the nitrogen fixing ability of recombinant plasmids carrying the *Agrobacterium* genes.

First, pSRK9 was transferred to *S. meliloti nif* H^- and *nif* K^- and the wild type strains (all *nod*$^+$) in filter matings. For this purpose, the plasmid was first transferred to *E. coli* SC1800 which was then used as a donor. *S. meliloti* transconjugant selection was achieved on plates containing M9 with tetracycline. In a second stage, pHN20 was transferred from UNF514 into both *nif* H^- and *nif* K^- strains with the help of pRK2013 in triparental matings. Transconjugant selection was achieved in a similar fashion.

After carrying out the relevant microbiological confirmatory tests, the *S. meliloti* transconjugants and the respective controls were used to infect alfalfa seedlings as described in Materials and Methods.

Statistical Analysis: To assess the nitrogen fixing ability of *S. meliloti* strains with and without the recombinant plasmids, three parameters were used: (1) their ability to facilitate infected plants to reduce acetylene to ethylene, (2) dry weight of the plants and (3) to a lesser extant the appearance of nodules. For statistical analysis data obtained from all plants in respective treatments were pooled together. Although the data obtained from plants grown in the test tubes and pots were analysed separately, Table 2 shows only the combined information gathered from both test tube- and pot- grown individuals. The F - values are shown below the Table. However, differences between different modes of growth will be pointed out at appropriate places.

Acetylene Reduction: Ability to reduce acetylene to ethylene was investigated in test tube grown plants after 4 weeks following the infection. Just before initiating the experiment, cotton plugs were replaced with Suba Seals (Freeman & Co., Barnsley, England); 10% of the air in the test tubes was replaced with freshly prepared acetylene and allowed to incubate for 60 min at room temperature. From each tube, 0.5 ml of the gas mixture was analysed on the GLC. In a representative number of cases, 36 to be precise, samples of gas mixture taken out at longer intervals were analysed to make certain the reduction continues with the time. Rest of the procedural details are described in [1]. Apart from several preliminary experiments (to optimise conditions), three complete sets of investigations were carried out. In these experiments about 25 plants were used for each treatment.

Acetylene reduction by plants grown in pots were also assessed in a similar fashion except that they were uprooted carefully from the pots, washed and then were transferred to test tubes that that contained 10 ml FP medium.

Data presented in Table 2 is the summary obtained from both types of experiment. Although pot grown plants showed lower values (than their counterparts in test tubes), the differences (F value 2.31) is still significant ($0.05>P>0.01$) and the trend was same.

Thus, beyond any reasonable doubts these results prove that the *A. tumefaciens* genes located on both recombinant plasmids express in *S. meliloti* to facilitate nitrogen fixation. In fact, the introduction of pHN20 into *nif* K made the strain two and half times more efficient than the wild type! Continuous monitoring of ethylene production showed that it does increase with time for several hours but in controls it has not showed up.

Nodule Counts and Dry Weights: After the acetylene reduction tests, plants were removed from the tubes, washed and nodule counts were made. An attempt made to distinguish effective and ineffective nodules basing on their appearance was not successful since the differences were not always clear. However, irrespective of their appearance, only nodules from the plants infected with plasmid-bearing strains contained the respective plasmids.

After the nodule count, the dry weight of the plants was assessed (Table 2). On the whole plants grown in test tubes gave lower values than those from pots, since the latter were grown for a longer period and in more space. However, it can be seen that both pSRK9 and pHN20 also gave significantly higher dry weights. As with acetylene reduction data, *nif* K^- (pHN20) and wild type (pSRK9) exceeded well over the wild type.

Bacteria from the nodules were isolated on antibiotic-free medium (from ten plants grown in test tubes from each treatment) and were examined for the presence of vector and insert markers. Recombinant plasmids were present in one hundred percent of nodules infected with *nif* K and wild type but nodules produced with *nif* H (recombinant plasmid) gave variable results.

Thus the occupancy was very high in *nif* K^- strain and wild type (100%) but *nif* H^- was variable.

E. coli UNF514 containing the recombinant plasmids produced excessive exopolysaccharides

especially when grown on NFDM medium. Whether this character is related to nitrogen fixation and whether any specific genes are involved has not been investigated.

Discussion: The main goal of the present investigation was to see whether it is possible to confirm the previous genetic indications that the genes responsible for fixing nitrogen in *A. tumefaciens* are in a cluster and are located on its chromosome near *his*19. To do this, apart from *E. coli,* we chose *S. meliloti* as an experimental host with the hope that if it expresses the genes concerned then the evidence will be strong, additional, support to the tenet that *A. tumefaciens* is a nitrogen fixing organism. In the event, our approach proved to be right.

The recombinant plasmid pSRK9, which was produced using a similar procedure employed to isolate an R-prime with *Klebsiella pneumoniae nif*$^+$ cluster (pRD1, a P group plasmid RP4 based prime [11], should have been adequate for the present investigation. However, the further investigation of the molecular biology of the *A. tumefaciens nif* system would still have been slow and laborious with this plasmid. In this regard, isolation of pHN20 should be considered as a significant step forward since it has a well-defined insert (~ 30 kb), and an easily identifiable marker, *his*$^+$.

Results from both pSRK9 and pHN20 established beyond any reasonable doubt that the *A. tumefaciens* genes carried by their inserts do express in *S. meliloti* strains, (especially in *nif* K$^-$), resulting in efficient nitrogen fixation by the latter. Although even a casual perusal of the Figs. 2, 3 and 4 proves the point beyond doubt, the burden of the proof is based on the information gathered from acetylene reduction (Table 2), increase in the dry matter of alfalfa seedlings (Table 2 and Figs. 2 and 3), and to some degree on the appearance of nodules (Fig. 4). Perhaps, the values for dry matter obtained from the plants grown in pots should be considered as nearer to the real situation, since they were allowed to grow for longer periods and without any restriction for space and non - nitrogen nutrients. On that basis, an extra yield of 49% in plants treated with *nif* K$^-$ (pHN20) (compared to those infected with wild type, Table 2) is really remarkable! In all experiments, both reported here and in pilot runs, pHN20 out performed pSRK9. Whether this is due to a simple difference in copy numbers or more complex molecular interactions needs to be seen.

It is not, however, clear that why the *nif* cluster does not express strongly in *nif* H$^-$ strain, while it does so well in *nif* K$^-$ background? There could be many reasons for this; the instability of the plasmids, and the inserts may be one of them. Some simple experiments conducted to investigate this possibility did not give a clear answer. On the other hand, if one assumes that what is observed in the present study is not a mere expression of the whole *Agrobacterium nif* cluster on a *S. meliloti* n*if*$^-$ background but a complementation, then the difference might be attributed to some difficulty with such an interaction with a double mutant, HD, since the *nif* H is a transposon induced one. This assertion, however, requires the interaction of what appears to be two different sets of nitrogen fixation genes.

Visual observations suggested that within a week after infecting with the strains containing the recombinant plasmids the plants looked much better than the respective controls; but the ones treated with *nif* H$^-$ (Plasmid) did not continue with this improvement. This might suggest that *A. tumefaciens nif* genes might start to function before the native system, and in the case of *nif* H$^-$ for some reason the former might turn off after a little while.

In addition to demonstrating the nitrogen fixation systems, two more significant points came out of the present investigation:

(1) The presence of *A. tumefaciens nif* cluster in *S. meliloti nif* K$^-$ significantly increased the performance of the host beyond even its wild type counterpart. Since it is an unexpected result, this part of the experiment was repeated several times, with the same effect -- the increase was never less than 25%, and often much more than that.

An increase in the number (certainly not reduced!) of nodules when the bacterial strains are provided with the recombinant plasmids and the occupancy (see below) is encouraging, if one hopes to explore the system for agricultural uses (see especially wild type with pSRK9 and *nif* K$^-$ with pHN20 in Table 2).

(2) Even the wild type strain of *S. meliloti* is benefited by the presence of *Agrobacterium nif* genes. Perhaps some kind of deregulation of the introduced genes, or synergistic interactions of both nitrogenases or simply the presence of extra copies of the genes are involved.

Finally, the fact that *A. tumefaciens* can fix nitrogen and the genes concerned express in *S. meliloti* should not come as a surprise, since it is closely related to the genus *Rhizobium* and belongs to the same family, *Rhizobiaceae* [12]. Several workers also previously showed that *A. tumefaciens* can express *nod*$^+$ located on the Sym plasmid of *Rhizobium* [see for instance 13] Kanvinde *et al.* [14] showed that *A. tumefaciens* expresses *K. pneumoniae nif* genes in a constitutive fashion. What is surprising, however, is that the *A. tumefaciens nif* appears to be structurally different from the other well-characterized systems, including that of *S. meliloti* (our unpublished results).

Table 1: List of bacterial strains and plasmids.

Bacteria	Genotype	Source
Escherichia coli		
UNF514	*his trp recA*56 *lacX*74 *Spec*^{r}	R. A. Dixon
SC1800	D (*pro* AB - *arg* F - *lac*) XIII *his met* B *arg* I 90 *mal xyl*	M. Sadowsky
Sinorhizobium meliloti		
S259	Rm1021 *nif* K$^-$ :: Tn5-*gusA*2 *nod*$^+$	S. B. Sharma
S237	Rm1021 *nif* H$^-$ :: Tn5-*gusA*2 *nod*$^+$	S. B. Sharma
S392	Wild type (Rm1021)	S. B. Sharma
Agrobacterium tumefaciens		
LBA2238*	C58 *phe*1 *pyr*12 *rif*^{r}	P. J. J. Hooykaas
Plasmids		
R68.45	Km^r Tc^r Ap^r	P. J. J. Hooykaas
pSRK9	R68.45 :: *his*$^+$ *nif*$^+$ *trp*$^+$	This work
pRK2013	Km^r	D. R. Helinski
pHN20	Tc^r *his*$^+$ *nif* $^+$	This work

* = This strain was supplied with the plasmid R68.45

Table 2: Expression of pSRK9 and pHN20 in *S. meliloti*. Summary of the data from the *S. meliloti* infected plants grown in both test tubes and pots. Information obtained from individual plants was pooled, summerized and statistically analysed (see the Text for details). All figures are given as percentage of the wild type.

Strain	Ethylene	Nodules	Dry wt.
Wild type	100.0	100.0	100.0
Wild type (pSRK9)	102.3	140.3	91.4
nif H-	1.4	84.2	21.8
nif H- (pSRK9)	0.9	142.1	49.0
nif H- (pHN20)	1.9	131.6	35.2
nif K-	14.2	128.6	48.6
nif K- (pSRK9)	89.2	138.6	91.0
nif K- (pHN20)	260.3	140.3	163.5
Blank	0.8	0.0	21.7

F - Values:
(a) Ethylene = 11.07**
(b) Nodules = 3.43** (Scored in test tubes only)
(c) Dry weight = 5.40**
** = P < 0.01

Fig. 1: Growth of *E. coli* UNF514 on NFDM with and without recombinant plasmids. Top: UNF514 on a plate with NFDM containing standard amounts of histidine and tryptophan. Bottom Left: UNF514 (pHN20) with tryptophan. Bottom Right: UNF514 (pHN20) with histidine and tryptophan. Although UNF514 bearing the recombinant plasmids grows well on minimal medium (AB) at 37^o C, it can do so only at 30^o C on NFDM indicating that the *A. tumefaciens nif* system also functions at a lower temperature as in most other diazotrophic organisms.

Fig. 2: Effect of *A. tumefaciens nif* $^{+}$ genes on nitrogen fixing ability of *S. meliloti.* One week old seedlings were infected with (a) no bacteria, (b) *nif* K^{-}, (c) wild type and (d) *nif* K^{-} (pSRK9). Plants were eight weeks old when photographed; best plants from each treatment were chosen for the photograph.

Fig. 3: Effect of pHN20. Left group were infected with *S. meliloti nif* K^{-} and right-hand side group were infected with the same strain but bearing the recombinant plasmid, pHN20.

The work reported here was repeated several times both in the United Kingdom and in Iran with other Rhizobium species as well; results (not yet published) were either similar or even better particularly with *R. Japonicum.*

In view of the fact that none of the 'authentic' probes from well characterized *nif* systems were able to hybridize visibly with the Agrobacterium system, one might question whether one is justified to describe the genetic factors in *A. tumefaciens* as nitrogen fixation genes! For the time being that has to remain as an unanswered question. We suggest the name 'nitrogen fixation' genes should be considered as tentative until full molecular characterization of the system is available. It might be pointed out that in some cases similar functions could be carried out by genes that have structural features which cannot be identified by probes form previously characterized genes. A good example is lactose utilization in *A. tumefaciens* itself! Several strains of the bacterium can utilize lactose as the sole carbon source. But in some such as C58 (used in the present study) utilization is carried out without producing β-galactosidase and the gene concerned does not hybridize with an *E.coli lac* probe; on the other hand, the reaction is carried out by an entirely different enzyme, P- β-galactosidase. [15]. The gene concerned works just like the one (and cross hybridizes with) described in Gram positives such as *Lactobacillus casei* (see in 16). Just to add an interesting codocil to the problem, *E. coli lac* operon expresses perfectly well in *A. tumefaciens* C58 and *vice versa.*

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