GENETIC TRANSFER OF NITROGEN FIXATION FROM *RHIZOBIUM TRIFOLII* TO *KLEBSIELLA AEROGENES*

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SUMMARY: The transfer of genes controlling N_2-fixation from *Rhizobium trifolii* to *Klebsiella aerogenes* 418, a strain incapable of fixing nitrogen, is reported. This intergeneric transfer was mediated by an F-like R-factor which had previously been transferred into the *R. trifolii*. The Nif operon was transferred at a frequency of 10^{-7} or higher. The *K. aerogenes* hybrids reduced acetylene at rates similar to a naturally occurring N_2-fixing strain, *K. pneumonia* M5a1. Acetylene reduction was supressed in the presence of oxygen or combined nitrogen. While the results presented are consistent with the general conclusion that Nif genes were transferred from *R. trifolii* to *K. aerogenes* the possibility that the transfer of genetic material from *R. trifolii* permitted the expression of latent Nif genes in *K. aerogenes* must remain an alternate consideration. This report is the first evidence that *Rhizobium* contains genetic information specifying functions involved in nitrogen fixation.

Biological nitrogen fixation has been historically assigned to two main bacterial groups - the *Rhizobium* group which fixes nitrogen in symbiotic association within nodules on the roots of specific legumes, and the free-living nitrogen fixing group which fix N_2 independently of any such association. Fixation of N_2 by members of the genus *Rhizobium* has never been demonstrated in laboratory-grown cultures of these organisms and it has been remarked that the genetic contribution of the legume, if any, towards the production of nitrogenase in *Rhizobium* bacteroids remained to be resolved (9).

Recent studies have shown that intraspecific transfer of genes controlling N_2-fixation in *Klebsiella pneumonia*, viz. the Nif operon can be accomplished using transduction (15) or conjugation mediated by an R-factor (6). Interspecific transfer of the Nif operon from *K. pneumonia* to *Escherichia coli* has also been accomplished (7). No suitable genetic system has been available to study the Nif operon in *Rhizobium*.

We now report genetic experiments employed to examine if the nitrogen-

fixing genes (Nif) of Rhizobium trifolii, whose expression has never been observed in laboratory-grown cultures, could be transferred to, and expressed in, a strain of Klebsiella aerogenes which was initially incapable of fixing N_2. A preliminary report of the transfer of Rhizobium Nif genes into K. aerogenes and the ability of the hybrid K. aerogenes strains to fix N_2 is presented.

MATERIALS AND METHODS

The R. trifolii T1K strain used contained an R-factor R1-19drd which carried resistances to the antibiotics kanamycin and chloramphenicol (8). K. aerogenes 418 was a non N_2-fixing strain obtained from F.C. Cannon, University of Sussex. Spontaneous mutants of this strain with resistance to 50 ug/ml. and 500 ug/ml. rifampicin (Merck) were isolated. These mutants were used as recipients in the conjugation experiments.

R. trifolii was grown on mannitol yeast extract medium (MSY) (14). The Klebsiella strains were grown and maintained on nutrient agar or broth (Oxoid). The mating procedure described by Datta et. al. (5) was used to transfer the R-factor from R. trifolii T1K to the Rif^r mutants of K. aerogenes 418. Prior to preparing the mating mixture the donor strain R. trifolii T1K was irradiated with UV (50% kill) and the mating mixture was incubated at 27°C for five hrs. Transfer of the R-factor was detected by plating the conjugation mixture on nutrient agar plates containing rifampicin (Rif) 20 ug/ml. and kanamycin (Kan) 20 ug/ml. Burk's medium (12) containing the same antibiotics was used to select for K. aerogenes 418 Rif^r hybrids which had acquired the R-factor and Nif genes by plating the conjugation mixture on this medium and incubating the plates for 3 days at 27°C in a nitrogen atmosphere. Presumptive N_2-fixing hybrids of K. aerogenes 418 Rif^r appeared as large mucoid colonies on this medium. The colonies were replica-plated on the same medium, purified on the same medium and transferred to Hino and Wilson's (HW) medium (11) in 50 ml. flasks with or without 1 mg/ml. of yeast extract (Oxoid). The flasks were fitted with Subba seals, flushed with N_2 and incubated on a rotary incubator shaking at 30°C. After good growth had occurred in the

flasks the atmosphere was replaced with argon containing 0.5 atmospheres of acetylene (British Oxygen) and the flasks were incubated in a shaking water bath at 30°C. Ethylene production was measured at 1 and 3 hrs. on a Pye-Unicam 104 GLC apparatus with a Poropak R column at an oven temperature of 60°C.

K. pneumonia M5a1, an indigenous N_2-fixing Klebsiella strain obtained from F.C. Cannon, A.R.C. Unit for Nitrogen Fixation, University of Sussex, was used as a comparative strain.

RESULTS

The donor strain R. trifolii T1K contained an R-factor R1-19drd which was transferred into the strain by bacterial transformation from Pseudomonas aeruginosa G-23 (R1-19drd) (8). This R-factor in this strain of R. trifolii mediated the transfer of resistance to kanamycin and chloramphenicol. In conjugation experiments R. trifolii and K. aerogenes 418 Rif^r were grown separately and a mating mixture was prepared. The R. trifolii was irradiated with UV prior to preparing the mating mixture. After 5 hrs. incubation the mixture was placed on two selective media - nutrient agar plus rifampicin and kanamycin to select for the transfer of the R-factor and Burk's medium with the same antibiotics to select for transfer of the R-factor and Nif genes. Table 1 shows data from two experiments and approximately similar transfers were found in three other experiments. These results show that the transfer of Nif genes as assessed in this instance by growth of the K. aerogenes hybrids on N-free agar, occurred at a frequency of approximately 10^{-7} and that the transfer was mediated by the R-factor. Transfer frequencies of this magnitude were only found when the donor strain was irradiated with UV. A lower frequency was observed when the donors had no pre-treatment with UV. Hybrid colonies were absent when UV-irradiated R. trifolii T1 (without the R-factor) was mated with K. aerogenes 418 Rif^r. When twenty colonies were selected from plates designed to determine R-factor transfer, were purified and examined for presence of both Kan and Cm, it was found that both resistances on the R-factor had been transferred.

TABLE 1

TRANSFER OF NIF GENES AND AN R-FACTOR BETWEEN *RHIZOBIUM TRIFOLII* AND *KLEBSIELLA AEROGENES*

Expt.	Strain Donor	Recipient Strain	Characters selected in hybrids	Frequency of transfers
1.	*R. trifolii* T1K	-	Nif, Kan^r	$<10^{-8}$
	-	*K. aerogenes* 418 Rif^r1	Nif, Kan^r	$<10^{-8}$
	R. trifolii T1K	*K. aerogenes* 418 Rif^r1	Nif, Kan^r	2×10^{-7}
	R. trifolii T1K	*K. aerogenes* 418 Rif^r1	Kan^r	4×10^{-5}
2.	*R. trifolii* T1K	-	Nif, Kan^r	$<10^{-8}$
	-	*K. aerogenes* 418 Rif^r2	Nif, Kan^r	$<10^{-8}$
	R. trifolii T1K	*K. aerogenes* 418 Rif^r2	Nif, Kan^r	3×10^{-6}
	R. trifolii T1K	*K. aerogenes* 418 Rif^r2	Kan^r	1.2×10^{-5}

Strain 418 Rif^r1 and strain 418 Rif^r2 are resistant to 50 ug/ml. and 500 ug/ml. respectively. Selection for Nif, Kan^r was on Burk's medium and incubated in an N_2 atmosphere, and selection for Kan^r was on nutrient agar with the antibiotics added as outlined in methods. The frequency of transfer was calculated in relation to the number of donors present in the mating mixture.

Large colonies growing on Burk's medium presumed to be Nif hybrids of *K. aerogenes* were purified on the same medium and inoculated into flasks of HW N-free medium and grown anaerobically in an atmosphere of N_2. When ten or more such isolates from any one conjugation were studied for the ability to grow in HW N-free liquid medium, it was usually found that not all such flasks showed good growth, some isolates failing to grow in this medium. In subsequent experiments duplicate flasks were set up with 1 mg/ml. yeast

TABLE 2

ACETYLENE REDUCTION BY K. AEROGENES 418 NIF$^+$ HYBRIDS AND CONTROL STRAINS

Bacterial Strain	n mol C_2H_4 Formed/min/mg dry wt.
K. pneumonia M5a1	5.9
K. aerogenes 418 Rif^r	0
K. aerogenes 418 hybrid B1	3.9
418 hybrid B2	0.88
418 hybrid B3	0.2
418 hybrid B4	10.0
418 hybrid B5	1.5
418 hybrid B6	0.325
418 hybrid B7	3.0
418 hybrid B8	2.75

extract added HW medium. The establishment of broth cultures from hybrid colonies in this medium was almost 100%.

Cultures of the hybrid K. aerogenes grown in the HW medium were examined for nitrogenase activity. Acetylene reduction by a series of these hybrids is shown in Table 2. K. pneumonia M5a1, a much studied N_2-fixing Klebsiella strain was used as a comparative strain and was cultured under identical conditions. Several of the K. aerogenes hybrids showed nitrogenase activity at a level comparable with strain M5a1. Hybrids capable of fixing N_2 as measured by acetylene reduction were found in five conjugation experiments.

These hybrids failed to reduce acetylene when incubated under aerobic conditions indicating that this nitrogenase has the extreme oxygen sensitivity observed in all other comparable nitrogen-fixing systems. These hybrids did not reduce acetylene in the presence of 100 ug/ml. N as $(NH_4)_2SO_4$ indicating that, as in other systems, the nitrogenase is suppressed by ammonia.

The levels of nitrogenase activity shown in Table 2 were measured on cultures grown for two days in HW medium containing 1 mg/ml. yeast extract. The Nif hybrids were found to be generally unstable and many lost the ability to reduce acetylene after a few transfers in HW medium. When the Nif hybrids were cultured on nutrient agar plates their ability to reduce acetylene was lost even more rapidly. Several stable Nif hybrid strains were obtained following daily transfer of the cultures in HW liquid medium containing 1 mg/ml. yeast extract using large inocula (5-10%) and these fixed N_2 after 12 transfers. The levels of nitrogenase activity of three hybrids transferred daily is shown in Table 3. Nitrogenase activity was higher than the levels shown in Table 2 presumably due to the enrichment of N-fixing organisms in the culture. In general, the hybrid strains showed improved acetylene reduction following serial transfer. Strain M5a1 showed lower levels of acetylene reduction. This strain was not cultured serially.

Initially all the Nif hybrids contained an R-factor. During subsequent sub-culturing the R-factor was unstable. Maintenance of the R-factor was not found to be necessary to maintain Nif genes. Strains B1 and B2 in Table 2 lost the R-factor during the serial transfer. Very few stable hybrid strains continued to maintain the R-factor and the Nif genes unless cultured under selective conditions necessary to maintain both these genetic properties, i.e. on N-free media with kanamycin added.

The parent and hybrid *K. aerogenes* strains reacted similarly in the series of biochemical tests suggested for the identification of *Enterobacteriaceae* by Cowan and Steele (3). The hybrid strains all had the same degree of rifampicin resistance as the parent strains used as recipients in the conjugation.

TABLE 3

ACETYLENE REDUCTION BY *K. AEROGENES* NIF^+ HYBRIDS AND *K. PNEUMONIA* M5a1 GROWN OVER TWELVE TRANSFERS

Transfer	Strain	n mol C_2H_4 Formed/min/mg dry wt.
Initial	M5a1	3.9
	418 Nif^+ hybrid A1	2.9
	418 Nif^+ hybrid A2	18.0
	418 Nif^+ hybrid A3	8.8
Second	M5a1	4.5
	418 Nif^+ hybrid A1	8.25
	418 Nif^+ hybrid A2	4.5
	418 Nif^+ hybrid A3	11.0
Fourth	M5a1	3.8
	418 Nif^+ hybrid A1	9.75
	418 Nif^+ hybrid A2	10.5
	418 Nif^+ hybrid A3	16.4
Eighth	M5a1	Not done
	418 Nif^+ hybrid A1	10.5
	418 Nif^+ hybrid A2	18.2
	418 Nif^+ hybrid A3	17.2

H.W. medium used and cultures were transferred daily using large inocula (5%).

Recipient *K. aerogenes* strains resistant to 50 and 500 ug/ml. rifampicin retained their respective resistance levels when the hybrids were examined subsequently. *K. aerogenes* 418 was naturally resistant to 50 ug/ml. amphicillin. This level of resistance was also observed in the *K. aerogenes* hybrids. Additional experiments were conducted to exclude the possibility of

the Nif genes originating in E. coli or P. aeruginosa, both hosts of the R-factor prior to its transfer into R. trifolii. The R-factor was transferred from E. coli to K. aerogenes 418 at a frequency of 2×10^{-5} on nutrient agar incorporating rifampicin and kanamycin. A representative selection of the K. aerogenes which had received the R-factor were tested for nitrogen-fixation using the acetylene reduction procedure. No acetylene reduction could be detected. No Nif hybrids were observed when the mating mixture was plated on Burk's N_2-deficient medium. From this it is evident that the Nif genes did not arise in the E. coli. It also shows that the presence of the R-factor in K. aerogenes 418 was not activating latent Nif genes. The transfer of Nif genes from P. aeruginosa, containing the R-factor, to K. aerogenes was also examined by plating a mating mixture of these two species on Burk's medium followed by anaerobic incubation. No Nif hybrids were observed, indicating that P. aeruginosa did not donate the Nif genes.

DISCUSSION

Our results are consistent with the interpretation that Nif genes can be transferred from R. trifolii to K. aerogenes which acquires the ability to fix N_2 as indicated by the acetylene reduction method and that this transfer is mediated by an R-factor. However, since the genetic nature of the K. aerogenes 418 with respect to Nif genes is unknown we recognise that the transfer of genetic material from R. trifolii permitting the expression of latent Nif genes in K. aerogenes must also be a possible alternate explanation of our results. K. aerogenes 418 has never been shown to reduce acetylene in our laboratory or in the ARC Unit of Nitrogen Fixation, University of Sussex, where it has been used extensively as a negative control in acetylene reduction tests (Cannon personal communication). Neither E. coli or P. aeruginosa were capable of reducing acetylene or transferring the ability to fix N_2 when used in R-factor transfers with K. aerogenes. The specific acetylene reducing activity was low in hybrids when grown for the first time in HW medium when compared with a known N_2-fixing strain K. pneumonia M5a1. The hybrids grew more slowly than M5a1 and were stimulated when a low

concentration of yeast extract was added to the medium. When hybrids were serially transferred daily the specific acetylene reducing activity increased. The failure of the recipient strain to reduce acetylene and the failure of the Nif hybrids to reduce acetylene under aerobic conditions or in the presence of combined nitrogen is evidence that genetic material for nitrogen fixation has been transferred from R. trifolii to K. aerogenes. It is clear from this study that the R-factor is required to mediate this transfer since no hybrids were found using the wild-type R. trifolii. Several reports have shown that R-factors can be transferred to species of Rhizobium by conjugation (4,5). These reports have used RP4, an R-factor indigenous to Pseudomonas. In this report the F-like R-factor, R1-19drd was transferred into R. trifolii by transformation from P. aeruginosa (8) which we considered to be more useful because this factor was derepressed and could be transferred to a very wide range of host bacteria. R1-19drd is unstable in many hosts other than Escherichia coli and dissociates into distinct replicons (10). It remains to be determined if R1-19drd in R. trifolii is present as a single closed circular structure or is present in the dissociated form. The latter seems initially to be likely since this R-factor in R. trifolii T1K contains only two of its original five resistance determinants, suggesting that the complete R1-19drd is not present in R. trifolii T1K.

We are as yet unclear as to how the R-factor mediates the transfer of the Nif genes. R-factors are known to mediate the transfer of chromosomal genes from donor strains at low frequency compared with the transfer of the R-factor itself (13). In the R-factor mediated transfer of Nif genes in this report the frequency of Nif gene transfer occurred at a higher frequency than would be expected for genetic material located on a chromosome. The fact that ultraviolet light treatment of the R.trifolii donor prior to the conjugation was necessary for obtaining good transfer of Nif genes cannot be explained at this time. However, it may be noted that an R-factor in Salmonella typhimurium dissociates into the transfer factors and the

resistance factors and this dissociation is stimulated by ultraviolet light (1). Further studies are in progress to explain the role of UV treatment of the R. trifolii T1K which has been shown to be necessary to obtain efficient transfer of Nif genes into K. aerogenes.

Instability was noted in hybrids which contained the Nif genes. Instability was also found in many Nif hybrids obtained in intergeneric matings between K. pneumonia M5a1 and Escherichia coli (7). In contrast, the naturally occurring nitrogen-fixing strain K. pneumonia M5a1 remained stable during many transfers in our laboratory even when cultured on nutrient agar.

We interpret these results as indicating that genes required for nitrogen fixation can be transferred using an R-factor from R. trifolii to K. aerogenes. Our results also indicate that R. trifolii contains genetic material for N_2-fixation, a point which was presumed since the initial discovery of the Rhizobium legume symbiosis in 1888 (2). This technique also allows for the first time a biochemical comparison of the same nitrogenase enzymes in vivo in nodule tissue with those synthesized under asymbiotic conditions in hybrid strains. The genetic transfer of Nif genes also permits other intergeneric matings between symbiotic and asymbiotic genera which will offer interesting possibilities of generating new strains with the increased potential to fix N_2.

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