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Transfer of Nitrogen Fixation Genes from a Bacterium with the Characteristics of Both *Rhizobium* and *Agrobacterium*

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Strain T1K, reported to be *Rhizobium trifolii* strain T1 carrying the drug resistance plasmid R1-1*drd*, was able to transfer a cluster of *nif*$^+$ genes to *Escherichia coli* K-12. Additional genetic material, resembling the *gal-chlA* region of *E. coli*, was also transferred from strain T1K. The segregation pattern of these transferred genes suggested that they were on a plasmid. Although strain TIK was able to nodulate red and white clover, it also formed very slow-growing galls on tomato stems and shared many physiological properties with *Agrobacterium tumefaciens*, to which it seemed more closely related than to *R. trifolii*. The *R. trifolii* hybrid T1(R1-19*drd*), constructed by conjugation, did not share any of these properties with *A. tumefaciens*. Thus, strain T1K appears to be a bacterium with properties of both *A. tumefaciens* and *R. trifolii* and with the capacity to transfer *nif*$^+$ genes and other functions which it may have "cloned" from another bacterium such as *Klebsiella*.

In 1973, Dunican and Tierney reported transformation of *Rhizobium trifolii* strain T1 with the drug resistance plasmid R1-19*drd* (9). Plasmid R1-19*drd* was first transferred by conjugation from its *Escherichia coli* host to a strain of *Pseudomonas aeruginosa*. The DNA was extracted from this hybrid and was then used to transform *R. trifolii* T1. After selection for kanamycin resistance (determined by the R1-19 plasmid), a hybrid, T1K, was obtained. This hybrid had also acquired the other drug resistances determined by the R1-19 plasmid, ampicillin and chloramphenicol (9).

After irradiation of T1K with ultraviolet light to promote recombination between R1-19*drd* and nif^+_{Rt} genes (nitrogen-fixing genes from *R. trifolii*), T1K was used as the donor in conjugation experiments with recipient *Klebsiella aerogenes* strain 418 *rif* (10). Dunican reported successful cotransfer of the R1-19 plasmid and nif^+_{Rt} genes, which were directly selected for in the *K. aerogenes* recipient. In two experiments, transfer frequencies of about 10^{-5} for Km^r and 10^{-6} to 10^{-7} for Km^r *nif*$^+$ were obtained (10).

Dunican et al. concluded from these results that *R. trifolii* does possess all the necessary genetic material for nitrogen fixation, and that such genes may be located on a resident plasmid in *R. trifolii* strain T1 (8).

Although *K. aerogenes* is closely related to *E. coli*, it has been much less widely studied and is therefore less well genetically characterized than *E. coli*. Since *nif*$^+$ genes could be transferred from T1K to *K. aerogenes*, it seemed probable that similar transfer could be obtained with *E. coli* recipients with the advantage that in this background the control of nif^+_{Rt} genes could be studied by methods similar to those used for nif^+_{Kp} genes (nitrogen-fixing genes from *K. pneumoniae*) (28).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1, and bacteriophages and bacteriocins are listed in Table 2. One particular strain of *E. coli* K-12, strain KS650 (deleted in the *gal-chl* region), was chosen as a recipient to test whether *nif*$^+$ genes could be transferred from strain T1K and expressed in this background. Three separate isolates of strain T1K were obtained from Dunican, and all behaved similarly on all occasions.

Media and growth conditions. Luria broth with glucose (LBG) and eosin-methylene blue medium (EMB) have been described previously (20). Minimal medium (MM) was that of Davis and Mingioli (5). Nitrogen-free medium (NFM) was that of Cannon et al. (4). The *Rhizobium* medium used was Bergersen's modified medium (BMM) (2).

Antibiotics were added to media as freshly prepared, filter-sterilized solutions. Octopine was kindly provided by J. Schell since nopaline was unavailable.

All cultures were grown at 30°C.

Tests for nitrogen fixation. Cultures were grown to mid-log phase in rich media, washed in saline phosphate buffer (4), and either plated on solid NFM and incubated under continuously flowing 99% N_2-1% CO_2 (15), or inoculated (0.25 ml) into 10 ml of NFM in anaerobic Pankhurst tubes (3, 22) with or without 100 μg of Casamino Acids per ml.

TABLE 1. *Bacterial strains used*

Bacterium	Strain	Characteristics[a]	Source
R. trifolii	T1	Prototroph; nodulates white, red, and subterranean clovers effectively	E. A. Schwinghamer
	T1K	Nodulates white clover effectively and red clover ineffectively	L. K. Dunican
K. aerogenes	418 *rif*	*rif* recipient used by Dunican in matings with T1K	F. C. Cannon
K. pneumoniae	M5a1	*nif*$^+$ prototroph	F. C. Cannon
K. pneumoniae	Δ17	Δ*nif* Δ*his* Δ*shu* mutant of M5a1	R. C. Valentine
E. coli K-12	KS650	Δ(*gal-chl*) *his*	M. Gottesman
	SA291	Δ(*gal-chlA*) *his str*	D. Dykhuizen
	J53(R1-19)	Carries R1-19 Km^r Ap^r Cm^r plasmid used by Dunican to transform T1	N. Datta
A. tumefaciens	B6S3	*oct*$^+$ prototroph	J. Schell
	C58	*nop*$^+$ prototroph	J. Schell

[a] Genetic symbols: *gal* (ability to ferment galactose), *chl* (defective in nitrate reductase and thus resistant to chlorate), *uvrB* (resistance to ultraviolet irradiation), *attλ* (attachment site for phage λ), *nif* (nitrogen fixation), *his* (histidine biosynthesis), *oct* (ability to use octopine), *nop* (ability to use nopoline), Δ (deletion). The deletion of strain KS650 was shown to extend into *chlA*.

TABLE 2. *Bacteriophages and bacteriocins used*

Phage or bacteriocin	Host bacterium	Source
Bacteriophage		
λ$^+$	*E. coli*	B. G. Rolfe
P1	*E. coli*	B. G. Rolfe
φ80	*E. coli*	B. G. Rolfe
T4	*E. coli*	B. G. Rolfe
φT10	*R. trifolii* strain T1	E. A. Schwinghamer
Tr8	*R. trifolii*	E. A. Schwinghamer
GS1	*A. tumefaciens*	J. Schell
GS2	*A. tumefaciens*	J. Schell
GS5	*A. tumefaciens*	J. Schell
GS6	*A. tumefaciens*	J. Schell
GS18	*A. tumefaciens*	J. Schell
ψ	*A. tumefaciens*	J. Schell
Bacteriocin		
S1005	*A. tumefaciens*	J. Schell
K84	*A. tumefaciens nop*$^+$	A. Kerr
T24	*R. trifolii*	E. A. Schwinghamer

Nitrogenase activity was measured as reduction of acetylene to ethylene by Pankhurst tube cultures, by the method of Tubb and Postgate (29).

Phage sensitivity and lysogeny experiments. Sensitivity to phages was tested by spotting the phage suspensions onto test bacteria poured in soft agar on LBG or BMM plates. Zones of clearing and single plaques for diluted phage suspensions indicated sensitivity of the bacterium to the phage.

Bacteriocin sensitivity was determined in a similar manner, with clear zones indicating sensitivity to the particular bacteriocin.

Lysogeny of *E. coli* K-12 strains and hybrids by phage λ was determined by the method of Miller (20).

Nodulation and gall formation tests. Nodulation of white, red, and subterranean clovers was tested by the method of Vincent (32).

Gall formation on *Datura*, tomato (*Lycopercison esculentum* cultivar Grosse Lisse), peas, French beans, wheat, maize, and sorghum was tested by dipping a sterile needle into a washed overnight culture of the test bacterium and then stabbing the needle through the stem of the test plant. Gall formation was first visible after about 2 weeks, and the galls continued to grow from then on.

Conjugation between T1K and *E. coli* K-12 strain KS650. T1K and *E. coli* K-12 strain KS650 were grown to mid-log phase in LBG at 30°C. The donor strain, T1K, was irradiated with ultraviolet light (80% kill) and mixed with recipient KS650 in the ratio 1 part T1K (3×10^8 cells/ml)-9 parts KS650 (3×10^8 cells/ml)-9 parts fresh broth. The mixture was incubated without shaking at 30°C for 4.5 h. The cells were washed twice in saline phosphate buffer, plated on NFM with biotin (2 μg/ml), and incubated under a nitrogen atmosphere for 5 days at 30°C. Presumptive nitrogen-fixing clones, which arose at a frequency of about 10^{-7}, were picked and purified by restreaking twice on similar medium.

RESULTS

Characteristics of hybrids. Conjugation experiments between strain T1K and *E. coli* K-12 strain KS650 yielded nitrogen-fixing hybrid clones at a low frequency (about 10^{-7}) on NFM under anaerobic conditions (27). Two examples of these hybrid clones which were picked for more detailed analysis were hybrids RB95 and RB96. The criteria for classifying these hybrids as *E. coli* rather than *R. trifolii* are listed in Table 3.

Like several other T1K × KS650 hybrids tested, both hybrids RB95 and RB96 grew well on solid and liquid NFM under N_2; both reduced acetylene at rates comparable to that of the standard strain *K. pneumoniae* M5a1 (Table 4). None of these properties was observed for recipient strain KS650, so it was concluded that *nif*$^+$ genes could be transferred from strain T1K to *E. coli* K-12 and give full expression, in the absence of any plant material.

On further examination of these hybrids, it was found that whereas KS650 had required biotin for growth, they no longer needed biotin. Since the *bio* gene is deleted in KS650, it could not have reverted in the hybrids and therefore could only have been acquired from strain T1K during conjugation. Other genes deleted by the same mutation that removed the *bio* gene in strain KS650 are *gal chlD pgl attλ bio uvrB chlA*. Thus, hybrids RB95 and RB96 were examined to see whether any of these other deleted genes had also been acquired by conjugation. Both hybrids were found to be *gal*$^+$ *chl*$^+$ *bio*$^+$ and *uvr*$^+$. For hybrids RB95 and RB96 to regain sensitivity to chlorate, they had to acquire functions equivalent to the *chlD*$^+$ and *chlA*$^+$ loci from strain T1K.

TABLE 3. *Characteristics of donor and recipient strains*

Characteristic	Bacterial strain			
	T1K	*E. coli* KS650	Hybrids	
			RB95	RB96
Excessive production of polysaccharides	+	−	−	−
Urease activity	+	−	−	−
Sensitivity to phages: λ, φ80, T4, P1	−	+	+	+
Indole formation	−	+	+	+
Fermentation of:				
Glucose	−	+	+	+
Melibiose	−	+	+	+
Growth on NFM under N_2	−	−	+	+
Acetylene reduction	−	−	+	+
Resistance to drugs:				
Kanamycin (20 μg/ml)	+	−	+	+
Chloramphenicol (12.5 μg/ml)	+	−	+	+
Ampicillin (50 μg/ml)	+	−	+	+
Streptomycin (250 μg/ml)	+	−	+	+

Since the genes acquired by hybrids RB95 and RB96 correspond so closely to the chromosomal region deleted in recipient KS650, tests were carried out for another locus in this region (the integration site for phage λ). If the λ integration site (*attλ*) is present, lysogens of *E. coli* K-12 strain HfrH can be made at a frequency of about 61%. However, in KS650, which is derived from HfrH but deleted for *attλ*, the frequency of lysogeny is only 0.05% (Table 5). When tested for ability to lysogenize phage λ, both hybrids RB95 and RB96 had greatly increased frequencies of lysogeny of 15 and 27%, respectively (Table 5), indicating that they had also acquired an *attλ* site from strain T1K. Thus a completely unrelated species (supposedly *R. trifolii*) possessed an attachment site for a bacteriophage specific for *E. coli*.

Hence, a region equivalent to the *gal-chlA* chromosomal region deleted in KS650 and amounting to about 15 genes in length, had been acquired in hybrids RB95 and RB96 from strain T1K by conjugation.

Other genes acquired from strain T1K were those determining resistance to the drugs ampicillin, streptomycin, chloramphenicol, and kanamycin. Resistance to a variety of other drugs, including tetracycline, erythromycin, and rifampin was not observed. These acquired resistances correspond to those of strain T1K and are presumably carried on the plasmid R1-19 (9).

Another characteristic of hybrids RB95 and RB96 was their inability to grow on rich media at 42°C. Whereas KS650 grows well at both 30 and 42°C, strain T1K can only grow at 30°C. Incubation at 42°C has a bacteriostatic effect on T1K: if a plate is incubated for several days

TABLE 4. *Expression of nitrogen fixation genes from T1K in E. coli K-12 hybrids RB95 and RB96*

Determination	T1K	*E. coli* K-12 KS560	Hybrids		*K. pneumoniae* M5a1[a]
			RB95	RB96	
Growth on solid NFM[b]	0	0	2	2	2
Growth in liquid NFM[c]	0	0	45	43	60
Acetylene reduction (nmol of C_2H_4/min per mg of protein)	<0.01	<0.01	38.0	40.1	51.0

[a] *K. pneumoniae* strain M5a1 is included as an *nif*$^+$ standard strain.
[b] Colony diameter (millimeters) after 5 days of incubation under N_2 at 30°C.
[c] Turbidity in Pankhurst tubes (nephelometer units) after 19 h of incubation.

at 42°C, there is no growth, but on further incubation at 30°C normal growth occurs. Both RB95 and RB96 behaved like T1K in this respect, although on each occasion tested a few colonies per plate grew at 42°C, as though segregation of the gene or genes responsible for 42°C inhibition was taking place.

Segregation of transferred genes in hybrids RB95 and RB96. Further examination of the ability to grow at 42°C showed that segregation of the "heat inhibition" marker was dependent on the previous growth condition and on the particular hybrid. For example, if RB96 was streaked on NFM and single colonies from this medium were then tested for their ability to grow at 42°C, all colonies were inhibited; i.e., they had the heat inhibition marker. If, however, this hybrid was grown first on EMBgal, then almost all colonies tested grew at 42°C, indicating loss or segregation of the heat inhibition marker.

Since segregation of the heat inhibition marker was occurring, hybrids RB95 and RB96 were examined for segregation of other genes acquired from strain T1K.

Hybrids were grown in liquid NFM in Pankhurst tubes to select for those cells still *nif*$^+$, and then these cells were plated onto different media under various growth conditions. Both hybrids RB95 and RB96 showed segregation of all known acquired genes from donor strain T1K. The frequency of loss of individual acquired markers depended on the previous growth medium on which the hybrid was plated (Fig. 1). This segregation was so frequent that the genes in question were most probably located on a plasmid rather than incorporated into the bacterial chromosome.

To test whether genes were permanently lost by segregation, *gal* colonies of RB96 that had arisen from a *gal*$^+$ culture were restreaked several times onto EMBgal media at 30°C. On no occasion did any *gal*$^+$ revertant colonies grow, indicating permanent loss of the *gal*$^+$ genes by segregation.

Characteristics of plasmid R1-19*drd*. It seemed strange that an organism, supposedly a strain of *R. trifolii*, should possess a piece of DNA with a possible gene order similar to that of the phylogenetically distant bacterium *E. coli*. One possibility was that the plasmid R1-19*drd* already carried the *gal-chlA* region from *E. coli* K-12 before it was transferred into strain T1 of *R. trifolii*. This possibility was eliminated by mating plasmid R1-19*drd* from the original *E. coli* K-12 strain J53 used by Datta (9) into strain SA291, another *E. coli* K-12 strain deleted between the *gal* and *chlA* loci. However, there was no evidence for cotransfer of any of the *gal-chlA* region by plasmid R1-19*drd*, although the plasmid was transferred to this recipient strain as detected by transfer of drug resistance markers (Table 6).

TABLE 5. *Frequency of lysogeny by phage λ in E. coli K-12 and hybrids*

E. coli K-12 host	No. of lysogens/100 infected cells
HfrH	61
KS650	0.05
RB95	15
RB96	27

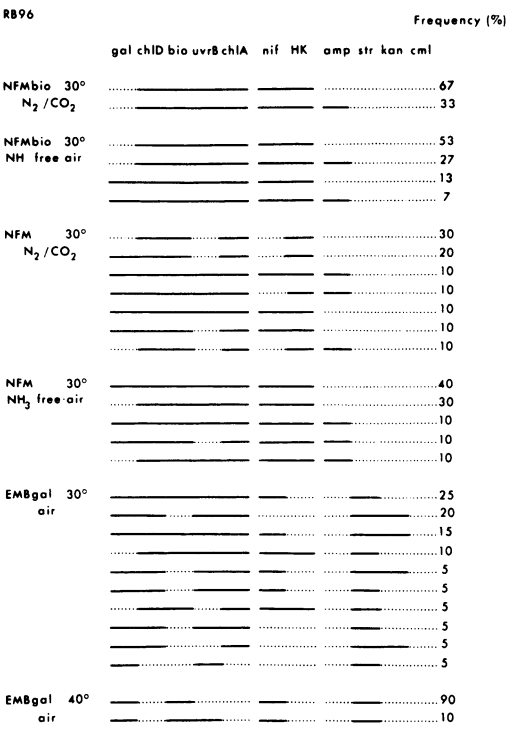

FIG. 1. *Segregation of genes in hybrid RB96. RB96 was first grown in liquid NFM and then streaked onto different media under various conditions as described in the figure. Single colonies from each medium were then tested for the presence of each of the genes listed in the figure. Between 40 and 100 single colonies were picked from each medium and resuspended in small amounts of saline phosphate buffer. Drops of each suspension were then spotted onto media to test for presence or absence of genes. All plates were incubated at 30°C except for those testing for the heat inhibition marker, which were incubated at 42°C. Symbols: solid line, gene present; dotted line, gene absent. amp, Ampicillin; str, streptomycin; kan, kanamycin; cml, chloramphenicol; HK, heat killing or inhibition.*

Retransfer of Nif$^+$ phenotype from hybrid RB96. A conjugation experiment was set

TABLE 6. *Properties of a Δ(gal-chlA) strain of E. coli K-12, SA291, containing drug resistance plasmid R1-19drd*

Property	SA291	SA291 (R1-19*drd*)
Fermentation of galactose	−	−
Sensitivity to chlorate	−	−
Growth without biotin	−	−
Resistance to ultraviolet light	−	−
Resistance to drugs:		
Kanamycin (20 μg/ml)	−	+
Chloramphenicol (12.5 μg/ml)	−	+
Ampicillin (50 μg/ml)	−	+

up between hybrid RB96 and deletion strain Δ17 to check whether a cluster of *nif*$^+$ genes had been transferred to KS650 from strain T1K, or whether there was complementation between genetic material transferred from strain T1K and genes of the *E. coli* chromosome. Strain Δ17 is a derivative of *K. pneumoniae* M5a1, deleted for the *his-nif* gene cluster-*shu* region of the chromosome (26). Since this strain cannot revert to Nif$^+$, any Nif$^+$ exconjugants from the mating experiments with hybrid RB96 must be derived by transfer of *nif*$^+$ genes from the donor.

Strains RB96 and Δ17 were grown to mid-log phase in LBG at 37°C, mixed and incubated statically for 7 h at 37°C, washed in saline phosphate buffer, and plated on NFMhis with 0.5% citrate as sole carbon source (*K. pneumoniae* but not *E. coli* can use citrate as sole carbon source [17]). Nif$^+$ exconjugants were obtained at a frequency of about 10^{-5}, indicating a transfer of a *nif*$^+$ cluster of genes from hybrid RB96.

Nodulation of T1K × KS650 hybrids. Eleven hybrids from the T1K × KS650 cross, including RB95 and RB96, were tested for nodulation by the method of Vincent (32). Strain T1K nodulated white clover effectively and red clover ineffectively, did not nodulate subterranean clover, and could be reisolated from white clover nodules. Strain KS650 and all the hybrids tested did not nodulate any of the three species of clover, so that either the genes responsible for nodulation were not transferred from strain T1K or else they could not be expressed in the background of *E. coli* K-12 strain KS650.

Properties of strain T1K. Because strain T1K apparently possessed genes similar to those on the *E. coli* chromosome, in particular, a lambdoid phage attachment site, this strain was investigated in detail.

R. trifolii strain T1 requires biotin for growth and is unable to grow on a wide range of media used for enterobacteria, e.g., LBG, EMB, MM, and NFM. Strain T1K, however, no longer required biotin for growth and was able to grow on all media tested, including LBG, EMB, and MM (Table 7). It was unable to grow on NFM under N_2 (the growth conditions used for the conjugation experiment with KS650) but did grow on NFM plates under 99% N_2-1% CO_2 or under air. *Agrobacterium tumefaciens* strains B6S3 and C58 behaved like strain T1K on NFM under the various atmospheres, whereas *R. trifolii* strain T1 did not grow on NFM under any condition. However, since no acetylene reduction by strain T1K, or any *A. tumefaciens* strain, could be detected, the mucoid translucent growth under these conditions was presumably due to scavenging of trace amounts of nitrogen from the agar. Moreover, strain T1K did not grow in liquid NFM under air, N_2, or 99% N_2-1% CO_2 (neither did strain T1 nor any *A. tumefaciens* strain).

Thus strain T1K differs greatly from strain T1 in its ability to grow on various media, and it is difficult to see how a simple transformation of strain T1 with R1-19*drd* plasmid DNA could lead to such radical alterations.

To check whether strain T1K was really equivalent to strain T1 containing the plasmid R1-19*drd*, a conjugation experiment to transfer plasmid R1-19*drd* into strain T1 was attempted. *E. coli* K-12 strain J53 (R1-19*drd*) was grown in LBG at 37°C to mid-log phase and was mixed 1:1 with strain T1 grown in BMM at 30°C to mid-log phase. One part BMM and one part LBG was added to the mating mixture, which was then incubated without shaking at 30°C for 7 h. The cells were washed in saline phosphate buffer and plated on *Rhizobium* minimal medium (16) plus kanamycin (20 μg/ml) and incubated at 30°C for 3 days. Recombinants arose at a frequency of 2×10^{-6}, and several were

TABLE 7. *Comparison of R. trifolii strain T1 with strains T1K and T1 (R1-19drd)*

Property	T1	T1K	T1 (R1-19*drd*)
Growth on:			
BMM	+	+	+
LBG	−	+	−
EMBgal	−	+	−
MM	−	+	−
NFM	−	+	−
Requirement for biotin	+	−	+
Resistance to drugs:			
Kanamycin (20 μg/ml)	−	+	+
Chloramphenicol (12.5 μg/ml)	−	+	+
Ampicillin (50 μg/ml)	−	+	+

picked and purified on the same medium. Four potential strain T1(R1-19*drd*) colonies were further tested, and their properties are summarized in Table 7. Except for resistance to drugs determined by plasmid R1-19*drd*, strain T1(R1-19*drd*) colonies retained all the characteristics of strain T1 and did not acquire any properties characteristic of strain T1K.

Thus, unless the R1-19*drd* plasmid picked up some DNA from *E. coli* or *P. aeruginosa* before being used to transform *R. trifolii* strain T1, strain T1K could not have arisen as a T1(R1-19*drd*) derivative.

Strains T1, T1(R1-19*drd*), and T1K were tested for sensitivity to phages Tr8 and T10. Phage Tr8 is a general *Rhizobium* phage, whereas T10 is specific for strain T1 (25). Strains T1 and T1(R1-19*drd*) were sensitive to both of these phages, but strain T1K was not (Table 8). Because strain T1K appeared similar to *A. tumefaciens* on morphological and physiological criteria, it was tested for sensitivity to several *Agrobacterium* phages (31). Unlike strains T1 and T1(R1-19*drd*), which were resistant to all the *Agrobacterium* phages, strain T1K was sensitive to phage GS18, forming clear plaques (Table 8). Phage GS18 was thought to be specific for strain Kerr 14, a nopaline-utilizing strain of *A. tumefaciens* known to have at least one "silent" plasmid besides the tumor-inducing plasmid (31; Schell, personal communication).

Although strain T1K effectively nodulated white clover and ineffectively nodulated red clover, it appeared to be much more closely related to *A. tumefaciens* than to *R. trifolii*. For this reason, strain T1K was tested for its ability to form crown gall on a range of plants and for other *Agrobacterium*-like properties.

It was found that strain T1K induced very slow-growing galls when stabbed through stems of tomato plants, but did not induce galls on *Datura*, peas, French beans, or wheat, maize, and sorghum (Fig. 2). Strain T1K could be reisolated from galls on tomato by surface sterilizing a piece of gall tissue in 5% sodium hypochlorite, crushing the tissue in sterile water, and plating on LBG or BMM. The cells retained sensitivity to phage GS18 and could still effectively nodulate white clover.

Octopine-utilizing strains of *A. tumefaciens* grow well on octopine as sole carbon and nitrogen source. Nopaline-utilizing strains can give rise to mutants constitutive for nopaline utilization; such mutants can also use octopine as sole carbon and nitrogen source (19). Since strain T1K gave rise to octopine-utilizing mutants at a low frequency above a background of octopine nonutilizers, this is indicative of strain T1K being a nopaline-utilizing strain.

TABLE 8. *Sensitivity of T1, T1K, and T1 (R1-19drd) to phages*

Phage	Specific for bacteria:	T1	T1K	T1 (R1-19*drd*)
Tr8	*R. trifolii*	+	−	+
T10	*R. trifolii* strain T1	+	−	+
GS1	*A. tumefaciens*	−	−	−
GS2	*A. tumefaciens*	−	−	−
GS3	*A. tumefaciens*	−	−	−
GS5	*A. tumefaciens*	−	−	−
GS6	*A. tumefaciens*	−	−	−
GS18	*A. tumefaciens*	−	+	−
λ*vir*, λ⁺	*E. coli*	−	−	−
φ80	*E. coli*	−	−	−
P1	*Enterobacteriaceae*	−	−	−

Another test for nopaline-utilizing *A. tumefaciens* strains is their sensitivity to agrocin 84 (11, 18, 31). Strain T1K was found to be sensitive to agrocin 84 at a level similar to that for strain C58, a nopaline-utilizing strain of *A. tumefaciens*. Strain T1K was also sensitive to agrocin S1005, another bacteriocin specific for *A. tumefaciens* (11).

R. trifolii strain T1, unlike strain T1K, did not give rise to octopine-utilizing mutants, did not induce galls on any similar test plants, and was not sensitive to agrocin S1005, although it was very slightly sensitive to agrocin 84.

DISCUSSION

These findings show that genetic information coding for a Nif⁺ phenotype (ability to grow on NFM under N_2 and a capacity to reduce acetylene) can be transferred at a low frequency from strain T1K to *E. coli* K-12. Since this Nif⁺ phenotype can be retransferred to a *nif* deletion mutant of *K. pneumoniae*, a cluster of *nif*⁺ genes was transferred from strain T1K to *E. coli* strain KS650 rather than there being a complementation between some *nif* genes from strain T1K and some genes from *E. coli*. The same situation could also apply to the hybrids which occurred in conjugation experiments between strain T1K and *K. aerogenes* strain 418 *rif*ʳ described by Dunican and Tierney (10).

It was also found that additional genetic material, resembling the *gal-chl*A region of *E. coli*, was cotransferred with the *nif*⁺ genes from strain T1K to *E. coli* K-12. When examined, both hybrids RB95 and RB96 showed segregation of all their known acquired genes. However, which genes were lost depended on the previous growth medium on which the hybrids were grown. Drug resistance markers were most frequently lost, whereas the *bio* genes were usually retained. The segregation pattern indicates that these ac-

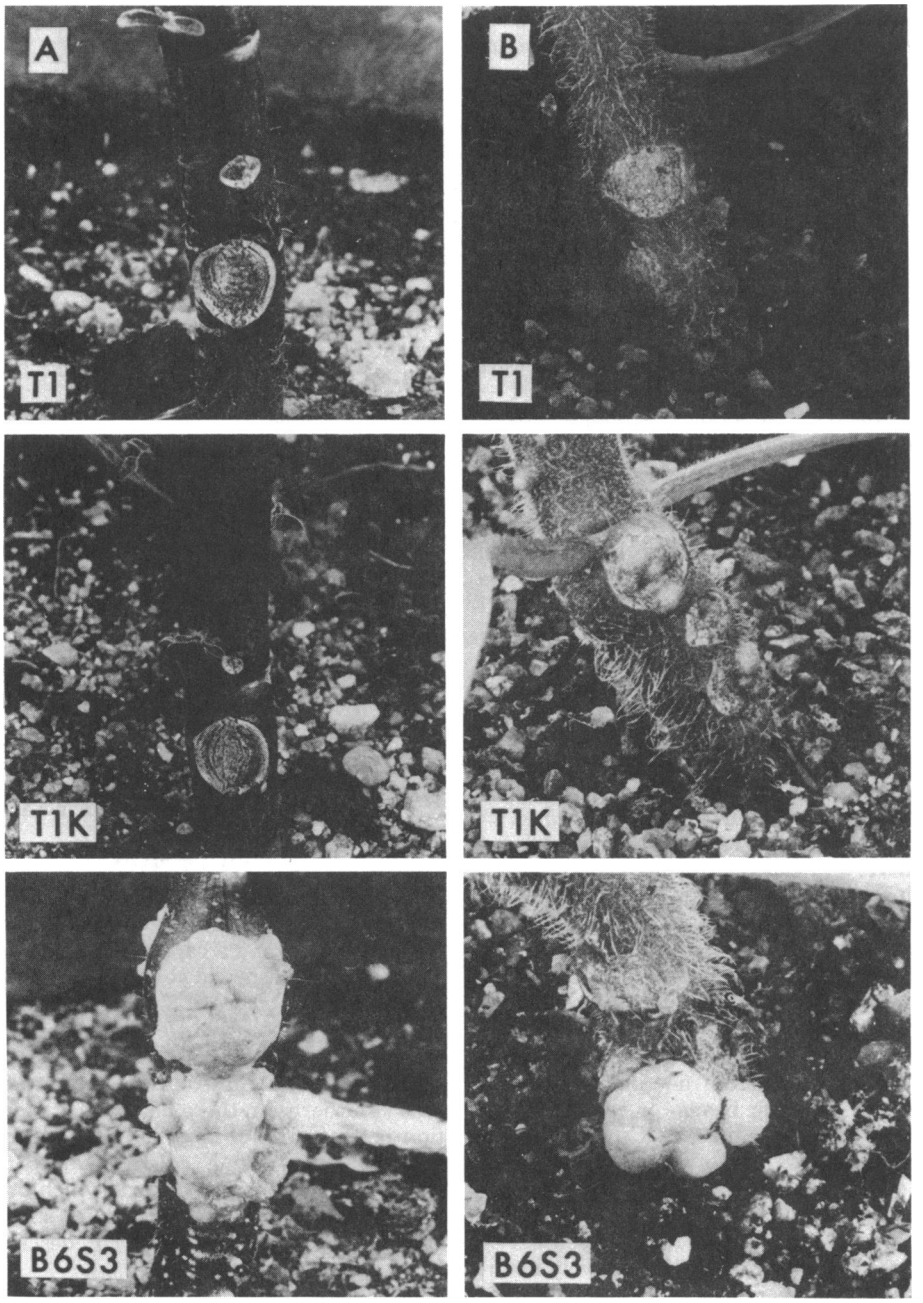

FIG. 2. *Induction of galls on (A) Datura and (B) tomato by R. trifolii strains T1 and T1K, and A. tumefaciens strain B6S3. T1 did not induce galls to form on Datura or tomato; T1K induced very slow-growing galls on tomato only. B6S3 induced galls on both Datura and tomato.*

quired genes were on a plasmid and suggests a gene order similar to that of the *gal-chl*A region of *E. coli*. It is conceivable that strain T1K has somehow "cloned" a cluster of *nif*$^+$ genes and other functions from another organism such as *Klebsiella*. Such an explanation would account for the other genes corresponding to the *gal-chl*A region of the *E. coli/Klebsiella* chromosome in strain T1K.

The findings described here show that strain

T1K is not a derivative of *R. trifolii* strain T1, but that it is a very interesting bacterium having characteristics of both *Agrobacterium* and *Rhizobium*. Many comments on the similarities of the genera *Rhizobium* and *Agrobacterium* have been made, and it has even been suggested that the fast-growing group of rhizobia should be amalgamated with *Agrobacterium* to form a single genus, *Rhizobium*, leaving the slow-growing rhizobia in a separate genus, the *Phytomyxa* (7, 13, 14, 21). *Agrobacterium* and the fast-growing rhizobia share many characteristics not found in the slow-growing rhizobia, such as similar guanine-cytosine ratios and utilization of the same carbohydrates and nitrogen sources (6, 13, 14, 34), as well as sharing a common phage (24). The main distinguishing characteristic is that whereas *Agrobacterium* forms galls and related diseases on stems of a wide range of dicotyledonous plants, *Rhizobium* forms nodules on a restricted range of legumes. The ability to form crown gall (and possibly also to nodulate) is believed to be coded for by genes carried on extrachromosomal plasmids in these bacteria (12, 19, 31), and loss of the plasmid leads to loss of ability to form crown gall (30, 33). Strain T1K, however, appears to be a bacterium bridging the gap between the fast-growing rhizobia and the agrobacteria. It is thought to have at least one plasmid (8) and may contain others. It is conceivable that the so-called silent plasmids found in many strains of *A. tumefaciens* at one stage in evolution allowed such strains to nodulate. Although plasmids of *Rhizobium* have not been examined in great detail, it is also possible that similar silent tumor-inducing plasmids could exist in some strains. A recent report has shown that it is possible to construct a gall-forming, nodulating hybrid bacterium of *R. trifolii* by transfer of the tumor-inducing plasmid from *A. tumefaciens* (16).

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