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Research Note

Tartrate Utilization Genes Promote Growth of *Agrobacterium* spp. on Grapevine

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Crown gall on grapevine is mainly caused by *Agrobacterium vitis*, which metabolizes tartrate. Competition experiments between a tartrate-utilizing strain and its nonutilizing derivative showed that tartrate utilization confers a selective advantage on grapevine.

Agrobacterium vitis induces crown gall on grapevine (*Vitis vinifera*) (Panagopoulos and Psallidas 1973). Its narrow natural host range has been attributed to a chromosomally encoded polygalacturonase responsible for grapevine root necrosis (Burr et al. 1987b, Rodriguez-Palenzuela et al. 1991, McGuire et al. 1991). L(+)-tartrate degradation may be an additional host range factor since *A. vitis* and a few *A. tumefaciens* grapevine isolates utilize L(+)-tartrate in preference to glucose, whereas *A. tumefaciens* isolates from other hosts do not (Szegedi 1985; Gallie and Kado 1988). Tartaric acid is an abundant compound in grapevine (Ruffner 1982).

Three tartrate utilization (TAR) regions were cloned and partially sequenced. They carry the genes *ttuA-E* and *ttuC'* (required for tartrate utilization) and four open reading frames (ORFs) of unknown function (ORFX, ORFY, ORFZ1, and ORFZ2) (Crouzet and Otten 1995; Otten et al. 1995; Salomone et al. 1996).

To test the importance of tartrate utilization on grapevine, we constructed a TAR$^-$ mutant from a natural TAR$^+$ *A. tumefaciens* isolate from grapevine and compared its growth on grapevine with that of the parent strain, 2655. Strain 2655 carries a pTi plasmid with a TAR region that was probably acquired from an AB3-like *A. vitis* strain by natural plasmid transfer (Salomone et al. 1996).

The *ttuC*-ORFY region of pTi2655 was deleted by double recombination with the suicide plasmid pKC7 (a pBR322 derivative; Rao and Rogers 1979) containing two fragments from the pTiAB3 TAR region, yielding pPM420 (Fig. 1). pPM420 was introduced into 2655 by triparental mating with the mobilizing strain GJ23, *A. tumefaciens* 2655, and NM522(pPM420). An *A. tumefaciens* exconjugant with a pTi2655::pPM420 cointegrate (AJ19) was selected on minimal medium containing neomycin (400 μg/ml). A double recombinant (AJ16) was isolated by screening AJ19 for the loss of tartrate utilization. Ti plasmid structures of AJ16 and AJ19 were checked by Southern analysis. Growth of 2655, AJ16, and AJ19 was the same in liquid yeast extract broth (YEB) medium with or without tartrate (data not shown). AJ19 was used as a TAR$^+$ control strain because it allowed selection on kanamycin after extraction of bacteria from nonsterile plant material.

Strains AJ16 and AJ19 were grown on YEB medium and washed, diluted, and mixed in various proportions shortly before infection of *Vitis vinifera* cv. Gamay plants. Shoot fragments (2 to 3 cm long) were sterilized and put apical side down on Murashige and Skoog medium (Murashige and Skoog 1962) with 0.7% agar. The basal end was infected with 2 μl of a bacterial suspension.

AJ16 and AJ19 induced tumors of similar size. After 4 weeks, tumors were excised, crushed, and resuspended in 1 ml of 10 mM $MgSO_4$. After dilution, bacteria were plated on minimal AB medium containing 0.5% tartrate (ABT medium; Szegedi 1985) and kanamycin (100 μg/ml). On this medium, TAR$^-$ colonies are smaller than TAR$^+$ colonies. A relative increase of the TAR$^+$ population was found in 10 out of 14 cases, without a clear correlation with absolute numbers or initial ratios (Table 1). Thus, tartrate utilization genes favor growth of *Agrobacterium* spp. in grapevine tumors.

To exclude artefacts due to in vitro conditions, we also infected intact grapevine plants in the greenhouse. *Nicotiana rustica* plants were used as a control. Stems were wounded by a small incision with a scalpel at different positions and infected with 2 μl of a bacterial suspension. The small volume assured that most bacteria were absorbed at the wound site. AJ19 and AJ16 formed tumors on grapevine after 6 weeks and on *N. rustica* after 4 weeks (not shown). On grapevine, the percentage of TAR$^+$ strains in the mixed population increased by 3, 13, and 14%, in *N. rustica* the proportion of TAR$^-$ and TAR$^+$ bacteria varied by less than 2% (Table 1). Bacterial multiplication occurred in each case (about 12 generations on grapevine stem fragments and about five generations on *N. rustica* and grapevine plants), showing that changes in proportion did not result from differential survival.

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Growth was also tested in grapevine bleeding sap. Bleeding sap is an exudate of the xylem and rich in organic and inorganic nutrients. Besides tartrate, it contains amino acids (mainly glutamine), other organic acids (malate and citrate), and sugars (Andersen and Brodbeck 1989a, 1989b; Glad et al. 1992). Equal amounts of 2-day-old cultures of AJ16 and AJ19 were suspended in M9 salt solution (Sambrook et al. 1989) and mixed 1:1, yielding a 5.10^6 cells per ml suspension. Aliquots of 5 ml of autoclaved bleeding sap from *V. vinifera* cv. Bluefrankish (obtained from wounded canes during spring) were inoculated in three replicates with 20 µl of bacterial suspension. Samples (5 µl) were removed each 24 h and counted after dilution and plating on ABT medium. Strong bacterial growth was observed after 24 h. The results (Table 2) show a relative increase of the TAR$^+$ population with time. Although the TAR$^-$ mutant does not grow on tartrate as a sole carbon source, it multiplies on grapevine in vitro and in vivo, albeit less efficiently. Since 2655-induced tumors contain octopine and cucumopine (Paulus et al. 1989), tartrate is not expected to constitute the sole carbon source.

Tartrate may confer a selective advantage under noninfectious conditions, as suggested by the bleeding sap experiments. It is remarkable that even with high levels of organic acids, sugars, and amino acids in bleeding sap, growth can be increased through the use of tartrate. Nonsymptomatic, systemic infection by *Agrobacterium* spp. is common on grapevine (Burr et al. 1987a). Induction of tumors and use of opines may serve as a second selection step favoring growth of *A. vitis*.

Opine-producing plants favor growth of opine-utilizing *Agrobacterium* spp. (Guyon et al. 1993; Oger et al. 1997; Savka and Farrand 1997), demonstrating the importance of opines for the growth of this pathogen. Our studies show for the first time that *Agrobacterium* spp. can also take advantage of an organic compound made by nontransformed host cells.

Table 1. Competition experiments between TAR$^+$ and TAR$^-$ strains in plant tissues

Host	No. of bacteria in inoculum (CFU)	TAR$^+$ bacteria in inoculum (%)	TAR$^+$ bacteria in tumor extract (%)
Grapevine stem fragments (in vitro)	5.10^2	6.9	11.8 ± 4.6
		16.6	42.5 ± 16.0
		24.9	31.0 ± 9.9
	3.10^3	4.3	15.8 ± 8.8
		10.4	27.6 ± 8.4
		13.3	33.0 ± 9.9
	3.10^4	4.9	10.7 ± 4.9
		8.8	16.7 ± 1.5
		19.4	19.7 ± 4.3
		26.6	28.0 ± 0.9
		46.5	51.9 ± 3.0
	3.10^5	7.0	19.2 ± 5.5
		15.2	26.0 ± 5.0
		20.2	31.7 ± 4.5
N. rustica plants (greenhouse)	3.10^3	42.5	45.1 ± 9.4
	3.10^4	47.5	46.5 ± 7.6
	3.10^5	47.4	48.8 ± 4.0
Grapevine plants (greenhouse)	3.10^3	40.6	54.8 ± 6.1
	3.10^4	40.9	53.7 ± 15.8
	3.10^5	46.7	50.5 ± 4.8

Table 2. Competition experiments between TAR$^+$ and TAR$^-$ strains in grapevine bleeding sap

Days	TAR$^+$ bacteria (%)
0	42.3 ± 3.2
1	46.3 ± 7.1
2	48.6 ± 6.5
3	62.3 ± 4.2
4	70.0 ± 7.0

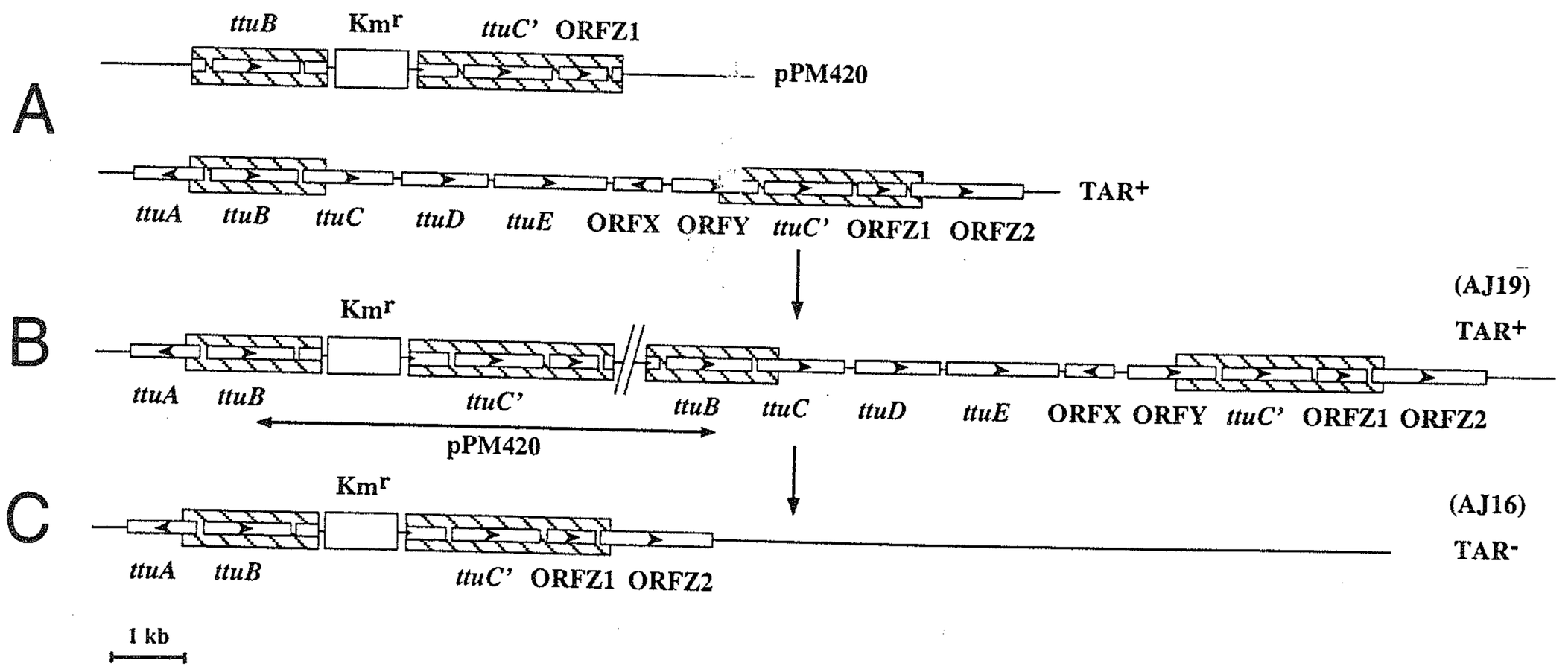

Fig. 1. Construction of the TAR$^-$ derivative of strain 2655. The first recombination between pPM420 and pTi2655 over *ttuB* (A) yields AJ19 (B). AJ16 (C) results from a second, intramolecular recombination. White boxes represent the kanamycin resistance gene. Hatched boxes correspond to the common regions between pPM420 and pTi2655.

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Research Note

Multicopy Vectors Carrying the *Klebsiella pneumoniae nifA* Gene Do Not Enhance the Nodulation Competitiveness of *Sinorhizobium meliloti* on Alfalfa

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It has been reported that *Sinorhizobium meliloti* strains harboring IncQ and IncP multicopy vectors containing constitutively expressed *Klebsiella pneumoniae nifA* exhibit an increase in nodulation competitiveness on alfalfa (J. Sanjuan and J. Olivares, Mol. Plant-Microbe Interact. 4:365-369, 1991). In our efforts to understand the mechanisms involved, in this work, we have found that the observed enhancement on nodulation competitiveness by IncQ derivatives carrying *K. pneumoniae nifA* was not dependent on the plasmid-borne *nifA* activity but on the sensitivity of nonresistant strains to the streptomycin carried over from growth cultures. Furthermore, it was also determined that the *nifA* of *K. pneumoniae* on an IncP vector does not have an effect on competitiveness.

Leguminous crops have been inoculated with *Rhizobium* and *Bradyrhizobium* spp. since the end of the last century with the aim to improve legume yields. However, the development of highly efficient rhizobial inoculants has been hampered by the inability of the released strains to compete for legume nodulation against the well-adapted indigenous population. This is known as "the competitiveness problem," which has been extensively reviewed (Maier and Triplett 1996; Toro 1996). The competitive ability of rhizobial strains is affected by many environmental and genetic factors that complicate efforts to overcome this problem. Nevertheless, one strategy that could be pursued is to introduce genes that give the recipient bacteria an advantage over the indigenous population. Some genes of interest include those that produce and confer resistance to antibiotics such as trifolitoxin (Robleto et al. 1997), or those involved in improving nodulation efficiency, such as the *nfe* genes (Soto et al. 1994; Chun and Stacey 1994). Additionally, genes that give a metabolic advantage such as rhizopine synthesis and catabolism genes (*mos/moc*) (Murphy et al. 1995) or the proline dehydrogenase *putA* gene (Jiménez-Zurdo et al. 1995), could be considered. Finally, the regulatory gene *nifA* has been proposed to play a role in competitiveness in *Sinorhizobium meliloti* (Sanjuan and Olivares 1991). Sanjuan and Olivares (1991) reported that multicopy plasmids (pCK1 and pCK3) containing the *nifA* gene of *Klebsiella pneumoniae* enhance the competitive ability of *S. meliloti* strains. These authors hypothesized that this positive effect on *S. meliloti* nodulation competitiveness might be explained by the presence of *nifA*-regulated, *nfe*-analogous genes. More recently, Herrera-Cervera et al. (1997) reported that the reduced nodulation competitiveness shown by an *S. meliloti* GR4 RecA$^-$ derivative strain could be enhanced by the presence of plasmid pCK3. Furthermore, it has been reported that pCK3 in *S. meliloti* has a positive effect on mycorrhiza formation (Tobar et al. 1996). The mechanism by which these multicopy vectors carrying *K. pneumoniae nifA* enhance *S. meliloti* competitiveness and mycorrhiza formation remains unknown. Therefore, the potential use (if any) of *K. pneumoniae nifA* gene as a genetic tool to enhance the competitiveness of *S. meliloti* still needs to be elucidated.

Here, in contradiction to published results (Sanjuan and Olivares 1991; Herrera-Cervera et al. 1997), we report that multicopy vectors carrying *K. pneumoniae nifA* do not enhance the nodulation competitiveness of *S. meliloti* on alfalfa.

Bacterial strains and plasmids used and constructed in this work are listed and described in Table 1. *Escherichia coli* and *S. meliloti* strains were routinely grown as described by Jiménez-Zurdo et al. (1995). Antibiotics were used as required at the following concentrations (μg ml^{-1}): tetracycline (10), ampicillin (200), kanamycin (50 for *E. coli* and 180 for *Sinorhizobium* strains), spectinomycin (100), streptomycin (50 for *E. coli* and 250 for *Sinorhizobium* strains), chloramphenicol (50), and gentamycin (30).

To test the effect of *K. pneumoniae nifA* on *S. meliloti* competitiveness, plasmid pDIL120, an analogous construction to pCK1, was made by cloning the wild-type *nifA* gene, preceded by a constitutive kanamycin resistance gene promoter, into the IncQ plasmid pMP77 (Fig. 1 and Table 1). As a control, a frameshift mutation was induced in the *nifA* gene to give pDIL119 (Fig. 1 and Table 1). The *nifA* activity produced by these constructions was determined with a plasmid carrying a P1 (*nifH* promoter)-*lacZ* fusion as a reporter in *E. coli* cells, as described by Better et al. (1985). The results indicated that the *nifA* activity exhibited by pDIL120 is similar to that of pCK1 while pDIL119 lacks this activity (data not shown).

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