MPMI Vol. 9, No. 5, 1996, pp. 401–408. Publication no. M-1996-0514-01R.

Characterization and Distribution of Tartrate Utilization Genes in the Grapevine Pathogen *Agrobacterium vitis*

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Received 2 January 1996. Accepted 15 March 1996.

***Agrobacterium vitis* is a common pathogen of grapevine. Most strains utilize tartrate, an abundant compound in grapevine. Strain AB3 carries two tartrate utilization (or TAR) regions: TAR-I (on the large pTrAB3 plasmid) and TAR-II (on the AB3 Ti plasmid). TAR-I and TAR-II were structurally and functionally analyzed and are similar to the TAR-III region from the tartrate utilization plasmid pTrAB4 of the nopaline-type *A. vitis* strain AB4 (Crouzet and Otten, J. Bacteriol. 1995, 177:6518–6526). The minimal tartrate utilization region of TAR–I contains four genes (*ttuA–ttuD*). The *ttuC* gene is homologous to the tartrate dehydrogenase gene from *Pseudomonas putida*. Outside the minimal region a second *ttuC*–like gene is found (*ttuC'*) which is transcribed and complements a *ttuC* mutant. Most grapevine isolates carry one or two of the three characterized TAR regions and show a considerable degree of polymorphism around these regions.**

Additional keywords: bacterial ecology, crown gall.

Crown gall on grape is mainly caused by *Agrobacterium vitis* (Kerr and Panagopoulos 1977; Süle 1978; Burr and Katz 1983; Burr et al. 1987; Ma et al. 1987; Ophel and Kerr 1990; Sawada et al. 1990). The *A. vitis* group consists of strains with octopine/cucumopine (o/c)-, nopaline- and vitopine-type Ti plasmids confined to specific chromosomal backgrounds (Szegedi et al. 1988; Paulus et al. 1989a, 1989b). The o/c isolates can be further divided into "small TA" (limited host range) and "large TA" (wide host range) strains. TA refers to one of the two T-DNAs of these strains. Grapevine isolates have been found to contain a specific polygalacturonase (Rodriguez-Palenzuela et al. 1991; McGuire et al. 1991). They also use L(+) tartrate as a carbon source (Süle 1978; Szegedi et al. 1988), an abundant compound in grapevine (Ruffner 1982). Both properties may be important host range factors.

We have demonstrated in an earlier study that 7 of 12 *A. vitis* isolates tested contained tartrate utilization genes on plasmids that can be transferred to *A. tumefaciens* recipients by conjugation in planta (Szegedi et al. 1992). Two DNA fragments encoding tartrate utilization (TAR regions) were cloned from the limited host range o/c strain AB3. TAR-I is located on a 245-kb plasmid, the AB3 tartrate utilization plasmid or pTrAB3. TAR-II is part of the 235-kb AB3 Ti plasmid (Szegedi et al. 1992).

Recently, the TAR-III region from the tartrate utilization plasmid (pTrAB4) of the nopaline *A. vitis* strain AB4 was cloned (Crouzet and Otten 1995). Since AB4, unlike AB3, carries only one TAR region, we started our investigation of tartrate degradation genes with TAR-III. A 7.2-kb region containing the TAR-III region was sequenced and analyzed by insertional mutagenesis (Crouzet and Otten 1995). Tar-III contains five genes (*ttuA–E*), four of which (*ttuA–D*) are required for tartrate utilization in the heterologous species *A. tumefaciens*. Based on functional studies and similarities with known genes, we have proposed that *ttuA* codes for a tartrate-sensitive LysR-like protein which regulates the transcription of *ttuB, C, D,* and *E*. The *ttuB* product allows entry of tartrate and the *ttuC* product (homologous to the tartrate dehydrogenase enzyme of *Pseudomonas putida*, Tipton and Beecher 1994) catalyzes the first step in tartrate degradation. This yields an unidentified toxic intermediate which slows growth unless further degraded by the product of *ttuD*. Finally, *ttuE* encodes a tartrate-inducible pyruvate kinase which is not required for tartrate utilization but may increase glycolysis. This paper describes the mutational analysis of the TAR-I and TAR-II regions from AB3, and the sequence of the TAR-I region. In the course of this work, a second *ttuC* copy was found which is closely linked to the minimal tartrate utilization region. The detailed analysis of the three TAR regions allowed us to identify similar regions in a large number of *Agrobacterium* isolates from grapevine.

RESULTS

Mutational analysis of the pTrAB3 TAR region (TAR-I).

The pTrAB3 TAR region (TAR-I) was cloned earlier as a 9.6-kb fragment into pMP2733, a spectinomycin-resistant broad host range IncW vector (Innes et al. 1988), yielding pPM405 (Otten et al. 1995, Fig. 1A). pPM405 was introduced into the cured *A. tumefaciens* strain UBAPF2 by electroporation. UBAPF2(pPM405) grew on minimal AB medium with 0.5% tartrate, while UBAPF2(pMP2733) did not.

UBAPF2(pPM405) was mutagenized with Tn*5-uidA*1. In a first step, 200 randomly chosen Km^R colonies were analyzed

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EMBL database accession number: U32375.

for position and orientation of Tn*5–uidA*1 inserts. This yielded 51 colonies with an insert in the TAR-I region. The other colonies either had an insert in the vector part (11 kb) or had an aberrant restriction pattern (possibly representing deletion derivatives) and were not further investigated. The 51 TAR-I region mutants were tested on ABT medium. Insertions between coordinates 500 and 6800 (for sequence, see below) led to impaired growth, and they defined a minimal tartrate utilization region of 6.3 kb. In a second step, several hundred Km^R colonies were tested on ABT and an additional 48 tartrate non-utilizing colonies were analyzed for Tn*5–uidA* insertions. Figure 1C shows the location and orientation of the 99 Tn*5-uidA*1 insertions, separated into two groups: insertions with the *uidA*1 gene oriented towards the right (R series) or the left (L series) with respect to the TAR-I map. The 99 mutants also were tested on AB minimal medium with glucose and tartrate and the pH indicator bromothymol blue which changes from yellow to blue when tartrate is utilized. Insertions within the cloned fragment yielded four phenotypes (Fig. 1D) which closely correlate with the open reading frames as determined by sequencing (Fig. 1B): i) Growth on ABT and blue colonies on ABTG (normal growth, insertions outside *ttuA-E*). ii) No growth on ABT and yellow colonies on ABTG (no tartrate utilization, insertions in *ttuA-C*). iii) No growth on ABT and blue colonies on ABTG (very weak tartrate utilization, insertions in *ttuD*). iv) Weak growth on ABT and blue colonies on ABTG (weak tartrate utilization, insertions in *ttuE*). Similar phenotypes have been described for TAR-III (Crouzet and Otten 1995).

Analysis of TAR-II region from pTiAB3.

AB3 carries a second TAR region (TAR-II) on its Ti plasmid. This region was cloned in pMP2733 as part of a 12.5-kb DNA fragment yielding pPM1105 (Fig. 2A, Otten et al. 1995) and confers tartrate utilization to UBAPF2. pPM1105 was mutagenized in the same way as pPM405. Growth tests on ABT and ABTG with 54 Tn*5-uidA*1 insertion mutants evenly distributed over the cloned fragment (not shown) yielded the same results for TAR-II as for TAR-I and TAR-III. Partial sequence analysis (L. Otten, unpublished) showed that the TAR-II region is about 95% homologous to the TAR-III region. This was confirmed by Southern hybridization: At normal stringency (see Materials and Methods) a probe covering the TAR-III region hybridized with all fragments of the TAR–II region, but only with the tartrate dehydrogenase gene of the TAR–I region (see below). We therefore sequenced only the more distantly related tartrate utilization region, the TAR–I region from pTrAB3.

Sequence of the pTrAB3 TAR region (TAR-I).

The sequence of the TAR-I region between coordinates 1 (0.2 kb to the right of the leftmost *Hin*dIII site of pPM405) and 11327 (2 kb to the right of the *Hin*dIII site of the pPM405 insert) was determined using subclones of pTr179 (Otten et al. 1995). The sequence (EMBL database accession number U32375) contains 12 putative open reading frames, called *ttuA-E*, ORFX, ORFY, *ttuC′*, and ORFZ1 to Z4 (coordinates and putative properties are summarized in Table 1). Most of the sequenced region could be aligned with the 7161-nt TAR-III sequence (Crouzet and Otten 1995; EMBL accession number U25634): coordinates 636-7372 of the TAR-I region cor-

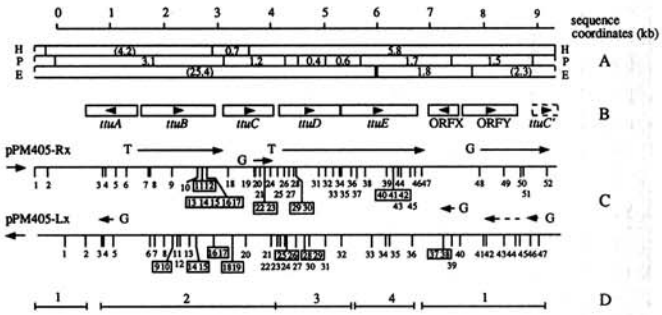

Fig. 1. Map and mutational analysis of pTrAB3 TAR region (TAR-I). From top to bottom: **A,** Sequence coordinates (in kb) and restriction map of pPM405 insert. Sizes of fragments are indicated in kb. H, P, and E: *Hin*dIII, *Pst*I, and *Eco*RI. **B,** Position of open reading frames (ORFs) as determined by sequencing. On pPM405, *ttuC′* (dashed box) is incomplete. **C,** Position of Tn*5-uidA1* inserts oriented towards the right (series pPM405-Rx) or left (series pPM405-Lx). G: *uidA* expression in AB medium with 0.2 mM X-Gluc and 0.5% glucose, 0.5% tartrate or both (tartrate-independent). T: idem with 0.5% tartrate or 0.5% glucose and 0.5% tartrate, but not in the presence of 0.5% glucose (tartrate–dependent). **D,** Phenotypes of different insertion mutants. 1: wild-type (normal growth on ABT and tartrate utilization on ABTG-BBT), 2: no growth on ABT, no tartrate utilisation on ABTG-BBT. 3: no growth on ABT, tartrate utilization on ABTG-BBT. 4: slow growth on ABT, tartrate utilization on ABTG-BBT.

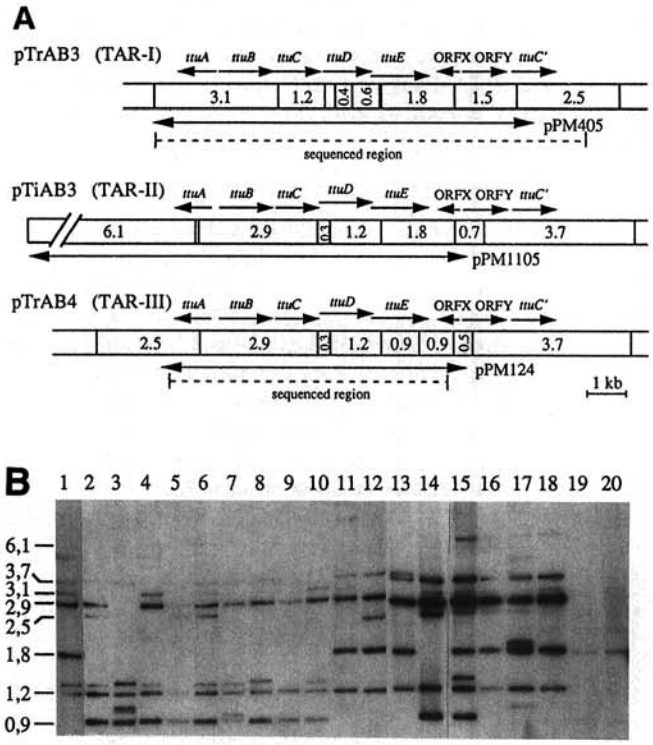

Fig. 2. *Pst*I maps of TAR-I, -II, and -III (sizes in kb). Positions of open reading frames (ORFs) of pTiAB3 and of ORFX, Y, and *ttuC′* of pTrAB4 are based on partial sequence data for pTiAB3 (unpublished) and on similarities between restriction maps for *Pst*I, *Hin*dIII, and *Eco*RI (not shown). pPM405 and pPM1105: Fragments used for Tn*5-uidA* mutagenesis. pPM124: probe used for hybridization experiments. **B,** Hybridization of total DNA of representative *A. vitis* isolates to the TAR region probe pPM124 from the TAR-III region of AB4. Lanes 1 to 20 correspond to patterns 1 to 20 of Table 4.

respond to coordinates 458-7161 of the TAR-III region. At the left end, homology ends 53 nt downstream of the *ttuA* stop codon. The overall DNA homology was found to be 72%. However, the DNA and protein homology values (Table 1) vary from gene to gene, with the lowest value being 60% and 61% respectively for *ttuA* (coding for a LysR-like protein), and the highest being 85% and 94% for *ttuC* (a putative tartrate dehydrogenase gene).

ttuA-E mutants of TAR-III have been analyzed by Crouzet and Otten (1995), and the functions proposed for these ORF's are summarized in Table 1.

The TAR-I sequence presented here extends an additional 4.5-kb to the right and 0.6 kb to the left compared to the TAR-III sequence. At the left, 95-512 are 62% homologous to the 3′ end of the *mcpA* gene of *Caulobacter crescentus*, which codes for a membrane protein involved in chemotaxis (Alley et al. 1992). At the right, the 7373-11327 region contains several ORFs: ORFX, ORFY, *ttuC′*, and ORFZ1 to ORFZ4 (Fig. 3A). The hypothetical translation product of ORFX has 33% homology to the ORF1 translation product from the unstable DNA locus of *Streptomyces ambofaciens* (X70975, Simonet et al. 1992), whose function is unknown. The hypothetical translation product of ORFY has 34.7% homology with the translation product of ORF3 from the *Pseudomonas* sp. strain P51 *tcb* cluster. The *tcb* cluster (M57629, van der Meer et al. 1991) codes for enzymes involved in the metabolism of chlorinated catechols, but the function of ORF3 is unknown. Insertions in ORFX or ORFY did not affect growth on tartrate or glucose.

To the right of ORFY the 8758-9840 region contains an ORF with 95.7% homology to *ttuC* from TAR-I (85.4% homology to *ttuC* from TAR-III). Homology starts only 10 nt before the start codon of *ttuC′* and ends 15 nt before the stop codon. The putative *ttuC′* translation product TtuC′ is therefore five amino acids shorter at the C-terminal end than TtuC. *ttuC′* is followed by four potential ORFs: ORFZ1 and ORFZ2 oriented in the same direction as *ttuC′*, while ORFZ3 and ORFZ4 are in the opposite direction. ORFZ1 to 4 lack homology to sequences in the data banks. Southern hybridization studies with a *ttuC*-specific probe demonstrated that pTiAB3 and pTrAB4 (for alignment with TAR-I see Fig. 2A) also carry a second *ttuC* copy at approximately the same position as in TAR-I.

Induction of TAR-I region genes by tartrate.

The induction of the different ORFs of pPM405 (TAR-I) was determined on solid minimal medium containing the colorless substrate X–Gluc and 0.5% glucose, 0.5% tartrate, or both. The *ttuA*, *ttuC*, ORFX, ORFY and *ttuC′* genes were expressed in the absence or presence of tartrate, whereas *ttuB*, *ttuD*, and *ttuE* were induced by tartrate (Fig. 1C). The induction levels of the different pTrAB3 genes on solid medium were very similar to those of the corresponding AB4 TAR–III genes (Crouzet and Otten 1995, not shown). Our data showed that *ttuA*, *ttuB*, *ttuC*, ORFX, and ORFY are independent transcription units.

Biological activity of *ttuC′*.

ttuC′ is transcribed and could code for an active tartrate dehydrogenase. This was tested by introducing *ttuC′* into pPM405-R20 (Fig. 1C). pPM405–R20 contains the minimal TAR–I region (*ttuA–ttuE*) with a Tn*5–uidA*1 insert in the *ttuC* gene and produces yellow colonies on ABTG-BBT medium. If the function of *ttuC′* is similar to that of *ttuC*, the new construct should enable growth on tartrate and lead to blue colonies on ABTG–BBT medium. To introduce *ttuC′* into pPM405–R20, the 768-nt *Not*I-*Bam*HI fragment from pTr179 (Otten et al. 1995, Fig. 3B) was first cloned into pBluescript, yielding pPM410. pPM410 was cut with *Not*I and ligated into the corresponding *Not*I site of pPM405-R20 in the correct orientation, yielding pPM411 (Fig. 3B). pPM411 can be considered as an extended pPM405-R20 derivative, the insert of which ends 117 bp downstream of the *ttuC′* stop codon.

pPM411 was introduced into UBAPF2 by transformation. UBAPF2(pPM411) produced blue colonies on ABTG-BBT medium, whereas UBAPF2(pPM405-R20) did not, showing that *ttuC′* restores the function of the mutated *ttuC* gene of pPM405-R20. However, the coloration was less intense than in the case of UBAPF2(pPM405) (results not shown). To test whether *ttuC′* enhances the tartrate utilizing capacity of the minimal TAR region (*ttuA-ttuE*) we extended pPM405 in the same way as pPM405-20. The resulting plasmid (pPM412) was introduced into UBAPF2 and its growth on tartrate and the rate of disappearance of tartrate was compared to that of UBAPF2(pPM405). The addition of *ttuC′* to the minimal TAR region did not notably increase growth nor tartrate utilization under the conditions tested (results not shown).

Table 1. Properties of different open reading frames (ORFs) sequenced in this study[a]

ORF	Coordinates	Size putative protein (number of AA, kD)	Proposed function and homology to TAR-III	Reference
L*	1-527	ND[b]	Membrane protein	This study
ttuA	583-1497	304, 34	LysR-like regulator (60, 61)	Crouzet and Otten 1995
ttuB	1579-2928	449, 50	Membrane protein (79, 89)	Crouzet and Otten 1995
ttuC	2954-4048	364, 40	Tartrate dehydrogenase (85, 94)	Crouzet and Otten 1995
ttuD	4156-5313	385, 42	Enzyme-degrading primary tartrate degrad a-tion product (68, 71)	Crouzet and Otten 1995
ttuE	5320-6744	474, 52	Pyruvate kinase	Crouzet and Otten 1995
X	7010-7525	171, 19	Unknown	This study
Y	7611-8651	319, 114	Unknown	This study
ttuC′	8768-9844	355, 39	Tartrate dehydrogenase	This study
Z1	9877-10344	155, 17	Unknown	This study
Z2*	10355-end	ND	Unknown	This study
Z3	9841-10557	238, 29	Unknown	This study
Z4	10379-11221	280, 34	Unknown	This study

[a] Asterisks indicate that ORF is incomplete. Between brackets: DNA and protein homology with TAR-III sequence from pTrAB4.
[b] Not determined.

Distribution of TAR regions in other *A. vitis* strains.

TAR-I, -II, and -III can be distinguished from each other by their characteristic *Pst*I fragments (Fig. 2A) and by their differences in DNA homology. Under standard hybridization conditions (see Materials and Methods), the TAR-III-derived probe pPM124 (covering *ttuA*-ORFX) hybridized to all *Pst*I fragments of the corresponding region of the highly homologous TAR-II region and to the *Pst*I fragments of TAR-I that contain *ttuC* and *ttuC'*. The distribution of the three different TAR regions among 108 different *Agrobacterium* isolates (Tables 2, 3, and 4) was studied by Southern analysis with pPM124 as a probe. The seven strains from sources other than grapevine did not hybridize to the probe, whereas 78 of 101 grapevine isolates did.

Among the grapevine isolates, 10 do not belong to *A. vitis*. Of these, *A. tumefaciens* biotype I strains 82143, 2654, 2655, and CG401 hybridized to the TAR probe (82143, 2654, and 2655 yielded the TAR-I pattern, CG401 the TAR-III pattern). As expected these strains grew on tartrate. *A. tumefaciens* biotype I strains CG628, AT4, 2946, and 2947, *A. radiobacter* isolate Ag125, and *A. tumefaciens* biotype II isolate PPI-1 did not hybridize. Of these nonhybridizing strains, Ag125 grew on tartrate, but its tartrate utilization system did not hybridize to our probes.

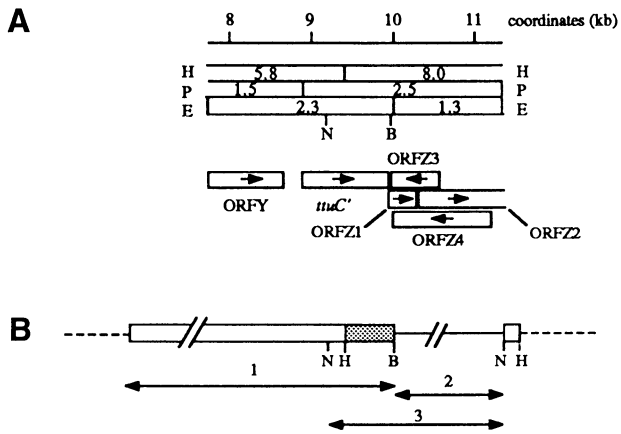

Fig. 3. A, Organization of the region containing gene *ttuC'*. **B,** Structure of enlarged TAR region pPM411 (pPM405-R20::pPM410 cointegrate). Gray: region added to minimal tartrate region of pPM405-R20. Dashed line: pMP2733. 1: pTrAB3 region cloned in pMP2733. 2: pBluescript vector. 3: pPM410 recombined into pPM405-R20. N, B, H, P, E: *Not*I, *Bam*HI, *Hin*dIII, *Pst*I, and *Eco*RI.

Among the 91 *A. vitis* strains, all from grapevine, 74 hybridized to the TAR probe. The negative strains are discussed below. The positive strains yielded 20 different hybridization patterns (Fig. 2B and Table 4) which were interpreted by reference to the TAR region maps.

Most of the bands could be attributed to one of the three TAR regions and are underlined in Table 4. Bands which did not correspond to the maps were further studied by hybridizing them to the 2.5- and 3.7-kb *Pst*I fragments of TAR-III (corresponding to the left and right part of the TAR-III region), hybridizing bands are indicated in Table 4 by the * and ° symbols, respectively. The results showed a considerable heterogeneity at the left end of the TAR region, and less so at the right end. The hybridization patterns obtained with the 3.7-kb *Pst*I fragment indicated the presence of additional *ttuC* copies in several strains. This was further tested by hybridizing total DNA of all strains with the 1.2-kb *Pst*I fragment of TAR-I. This probe hybridizes to each of the six *ttuC* genes of the three TAR regions. Additional *ttuC*-like sequences were found in patterns 6, 9, and 14, but their location is unknown. Earlier, Paulus et al. (1989b) demonstrated that Ti plasmid type and chromosomal background are strongly correlated in *A. vitis*. The correlation between the different TAR region types and the Ti plasmid types of the strains can be summarized as follows:

i) Almost all "small TA" o/c strains (25/26) hybridized to the TAR-III probe pPM124. Twenty-four showed the characteristic 1.2- and 2.5-kb bands of the TAR-I region which also hybridized strongly with the 1.2-kb *Pst*I fragment from TAR-I. In addition, they showed the characteristic bands of the TAR-II region, like AB3. NW263 only contains the TAR-II region from the Ti plasmid. AT6 has a complex pattern in which elements of all TAR regions are found. NW233 did not hybridize, although it grew on tartrate. Conversely, 2607 hybridized, but did not grow on tartrate.

ii) Of 30 "large TA" o/c strains, 22 hybridized: most (18/22) showed the TAR-III pattern of the nopaline strains, while four

Table 2. Strains of *Agrobacterium tumefaciens* and *A. radiobacter* used

Strain	Origin	Opine type	Tartrate utilization	Reference or source
A. tumefaciens biotype I, virulent				
82143	Grapevine	o/c	+	Michel et al. 1990
2654, 2655	Grapevine	o/c	+	Paulus et al. 1989a
CG628, AT4	Grapevine	nop	–	Bonnard et al. 1989
2946, 2947	Grapevine	nop	–	M. Ridé
CG401	Grapevine	oct	+	Bonnard et al. 1989
B6 (=NCPPB2437)	Apple	oct	–	Bonnard et al. 1989
45ASA	Chrysanthemum	nop	–	Bonnard et al. 1989
57SA	Soil	nop	–	Bonnard et al. 1989
A. radiobacter, avirulent				
Ag125	Grapevine		+	Knauf et al. 1983
DSM30147	ND[a]		–	Bien et al. 1990
K31	Peach		–	Bonnard et al. 1989
K188	Soil		–	Bonnard et al. 1989
1D1490	ND		–	Bonnard et al. 1989
A. tumefaciens biotype II, virulent				
PPI-1	Grapevine		–	Bonnard et al. 1989

[a] Not determined.

others showed the TAR-II pattern. Tm4, 2657, 2678, At2, H8/1, NW70, 550-22, and 550-27 did not hybridize. Among these strains, Tm4, At2, H8/1, NW70, and 550-27 did not grow on tartrate, whereas 2657, 2678, and 550-22 did. NW244 hybridized but did not grow on tartrate.

iii) Most nopaline strains (19/25) hybridized and showed the characteristic 860/870 bp double band of the TAR-III region (except for strain 2179, which showed a mixed I-II pattern). AT66, NI1, 339-6, 565-11, 565-58, and 565-59 did not hybridize, and none of them grew on tartrate.

Table 3. *Agrobacterium vitis* isolates (all from grapevine)

Isolates	References
o/c "small TA" type (tar–minus: Zw2, 2607)	
Zw2, B10/7, AB3, AT6, 2612, 2613, 2614, 2617, 2644, 2650, 2651, 2653, 2656, 2675, 2676, 2677, Ag57	Paulus et al. 1989a
2607, NW90, NW102	Paulus et al. 1989b
NW233, NW263	Otten and van Nuenen 1993
Ag63, Ag228	Knauf et al. 1983
NCPPB2562, NW103	Schulz et al. 1993
o/c "large TA" type (tar–minus: Tm4, NW70, NW244, At2, H8/1, 550-27)	
Tm4, K308, K305, 2608, 2616, 2618, 2641, 2645, 2646, 2647, 2648, 2649, 2652, 2657, 2678, 2679, 2680, 2686	Paulus et al. 1989a
NW12556	T. Schulz
2770, At2, H8/1, NW70, NW244	Paulus et al. 1989b
550-22, Ag82, 2948, 2949, NCPPB3554T	van Nuenen et al. 1993
550-27	M. Lopez
Nopaline type (tar–minus: AT66, NI-1, 339-6, 550-9, 565-11, 565-52, 565-58, 56 5-59)	
AB4, AT66, IS1.1, AT1, NI-1, EK2, 2179, 2609, 2643, 2673, 2674, BS-33-6, NW22, NW44, NW165, NW170, NW190, NW310, NCPPB1771	Otten and de Ruffray 1994
CG47	Bonnard et al. 1989
339-6, 565-11, 565-58, 565-59	M. Lopez
2610	M. Ridé
Vitopine type (tar–minus: none)	
S4, Sz1, Sz2, 2681	Paulus et al. 1989a
Bazzi, NW11, NW113, NW121, NW161	Paulus et al. 1989b
339-25	M. Lopez

[a] Non-tartrate degrading strains are indicated by "tar–minus" . T: *A. vitis* type strain.

Table 4. TAR region patterns of different *Agrobacterium vitis* isolates[a]

Pattern	Strains	Fragments	TAR region type
1	2179^{N}	1.2-1.3*-1.8-**2.9**°-**3.1**°-**3.7**°-5.2°*	I, II
2	2609^{N}, 2674^{N}, $NW310^{N}$	0.9-1.2-1.3*-2.5*-**2.9**°-**3.7**°	III
3	2673^{N}	0.9-1.0-**1.1**°-1.2-1.35*-**3.7**°	III
4	$NW22^{N}$, 2643^{N}, $IS1.1^{N}$, $AT1^{N}$, $EK2^{N}$, $BS–33–6^{N}$, 2649^{OL}	0.9-1.2-1.3*-**2.9**°-**3.1**°-5.2*	III
5	$NW44^{N}$, $AB4^{N}$, $NW190^{N}$, 2610^{N}	0.9-1.2-2.5*-**2.9**°-(**3.7**)°	III
6	$NW170^{N}$, $NW165^{N}$	0.9-1.2-1.3*-2.5*-**2.9**°-**3.1**°-**3.7**°	III
7	$CG47^{N}$	0.9-0.95-1.2-1.3*-**2.9**°-**3.7**°	III
8	2618^{OL}, 2646^{OL}, 2648^{OL}, 2616^{OL}, 2645^{OL}, 2647^{OL}	0.9-1.2-1.35*-**2.9**°-**3.7**°	III
9	$NW244^{OL}$	0.9-1.2*-**2.9**°-(**3.1**)°-**3.7**°	III
10	$K305^{OL}$, $K308^{OL}$, 2680^{OL}, 2686^{OL}, 2608^{OL}, 2679^{OL}, $NCPPB3554T^{OL}$, 2641^{OL}, 2652^{OL}	0.9-1.2-1.3*-**2.9**°-**3.1**°	III
11	2770^{OL}	1.2-1.8-**2.9**°-(3.1)-**3.7**°-8.0*	II
12	2948^{OL}, 2949^{OL}	1.2-1.8-2.4-**2.9**°-**3.7**°	II
13	$Ag82^{OL}$	1.2-1.8-**2.9**°-**3.7**°-4.0*	II
14	$NW12556^{OL}$	0.9-1.2-2.5*-**2.9**°-(**3.1**)°-**3.7**°	III
15	$AT6^{OS}$	0.9-**1.2**-1.3*-**1.8**-(**2.5**)°-**2.9**°-(3.1)-**3.7**°-(6.1)*	I, II, III
16	2617^{OS}, 2651^{OS}, 2656^{OS}, 2677^{OS}, $NW90^{OS}$, $NW102^{OS}$, $Ag57^{OS}$, $Ag63^{OS}$, $Ag228^{OS}$, $NW103^{OS}$, $NCPPB2562^{OS}$, $B10/7^{OS}$, 2650^{OS}, 2653^{OS}, $ZW2^{OS}$, $AB3^{OS}$, 2607^{OS}	**1.2**-1.8-(**2.5**)°-**2.9**°-(3.1)-**3.7**°-(6.1)*	I, II
17	2675^{OS}, 2676^{OS}, 2644^{OS}, 2613^{OS}, 2612^{OS}, 2614^{OS}	1.0-**1.2**-1.8°-1.85-(**2.5**)°-**2.9**°-(3.1)-**3.7**°-(5.0)*-(6.1)*	I, II
18	$NW263^{OS}$	1.2-1.8-**2.9**°-**3.7**°-(6.1)*	II
19	$BAZZI^{V}$	(**0.75**)°-(**1.65**)°	–
20	$Sz1^{V}$, $Sz2^{V}$, 2681^{V}, $NW11^{V}$, $NW113^{V}$, $NW121^{V}$, $NW161^{V}$	(0.75)-(**1.65**)°-(2.0)-(3.1)	–

[a] Southern blots with *Pst*I-digested DNAs from different *A. vitis* isolates were hybridized to the TAR-III probe pPM124 (Fig. 3A). The opine type of each strain is indicated as follows: N: nopaline, OL: octopine/cucumopine "large TA", OS: octopine/cucumopine "small TA", V: vitopine. Underlined fragments: fragments identified with the help of the TAR region maps (Fig. 3A). The same Southern blots were hybridized to the 2.5-kb *Pst*I fragment from the left end of TAR-III, yielding bands indicated by *, to the 3.7-kb *Pst*I fragment from the right end of TAR-III, yielding bands indicated by °, and to the 1.2-kb *Pst*I fragment from TAR-I (specific for the *ttuC*-like genes), yielding bands indicated by boldface. The TAR region type deduced from the hybridization patterns is indicated in the right column.

iv) Finally, eight of 10 vitopine strains showed homology to the 1.2-kb TAR-I probe, but none of the bands corresponded to the TAR region maps. S4 and 339-25 did not hybridize although both used tartrate.

DISCUSSION

DNA sequencing and mutational analysis showed that the tartrate utilization region of pTrAB3 (TAR-I) and pTiAB3 (TAR-II) are related to the earlier described TAR region from pTrAB4 (TAR-III). Sequence comparison shows that homology between TAR-I and TAR-III starts close to the 5′ end of *ttuA*. The right end of the common tartrate region remains to be determined.

The functional studies reported here indicate that the model derived from the study of TAR-III (Crouzet and Otten 1995) also applies to TAR-I and TAR-II. However, the utilization of tartrate is more complex than proposed in the earlier model. Sequence analysis of TAR-I, and hybridization analysis of TAR-II and TAR-III revealed a second *ttuC* copy (*ttuC′*) about 5 kb to the right of *ttuC*. *ttuC* (TAR-I) and *ttuC′* (TAR-I) are more closely related (95.7%) than *ttuC* (TAR-I) and *ttuC* (TAR-III) (85.4%). This indicates that the TAR-I *ttuC* duplication occurred after TAR-I and TAR-III had started to diverge. However, TAR-III and TAR-II also contain a *ttuC′* copy, at the same position as in TAR-I, suggesting that the *ttuC* gene was already duplicated in the ancestor TAR region. Gene conversion could have slowed down sequence divergence between the two copies. Earlier, we found evidence for gene conversion in a short repeat sequence in the T-DNA of AB4 (Otten and de Ruffray 1994). The remarkable variation in DNA homology between the genes of the TAR-I and TAR-III region may be due to recombination or gene conversion between the TAR-I region and the coresident TAR-III-like TAR-II region.

It is striking that the homology between *ttuC* and *ttuC′* is restricted to the coding region. There is at present no explanation for such an exact duplication. Whereas *ttuC* is surrounded by other genes involved in tartrate utilization (Crouzet and Otten 1995), *ttuC′* seems to be isolated. In spite of the lack of homology to the 5′ and 3′ region of *ttuC*, *ttuC′* is transcribed, both in the presence and in the absence of tartrate, and produces a functional protein as shown by *ttuC* complementation. However, *ttuC′* does not significantly increase the tartrate degradation capacity of the *ttuA-ttuE* system, at least under the conditions tested; its eventual role remains to be elucidated.

Hybridization of two TAR-specific probes against *Pst*I-digested DNA of 108 *Agrobacterium* isolates showed complex distribution patterns of the different TAR regions. Seventy-eight of 101 grapevine isolates hybridized, whereas 83 are tartrate utilizers. Among the 23 nonhybridizing strains, Ag125, NW233, 2657, 2678, 550-22, S4, and 339-25 degrade tartrate in the presence of glucose. The tartrate utilization system(s) of these nonhybridizing strains remain(s) to be isolated. In the case of the vitopine strain S4 the tartrate utilization system is located on a large plasmid that can be transferred to a cured *A. tumefaciens* recipient (Szegedi et al. 1992). Two strains (2607 and NW244) which hybridize but are unable to use tartrate may represent natural mutants.

In the case of the "small TA" o/c strains and the nopaline strains, the type of TAR region correlates well with the chromosomal background: most "small TA" o/c strains contain TAR-I and TAR-II, while most nopaline strains contain TAR-III. In the case of the "large TA" o/c strains the situation is more complex: whereas 18 strains have TAR-III-like genes, four carry a TAR-II-like region. It remains to be determined whether the TAR-II-like region of the latter strains is part of the Ti plasmid as in the case of the "small TA" o/c strain AB3. TAR-I and TAR-II of AB3 and TAR-III of AB4 are located on plasmids which can be transferred to a plasmid-less *A. tumefaciens* strain by conjugation in planta (Szegedi et al. 1992). It is interesting to note that three rare *A. tumefaciens* strains isolated from grapevine (82143, 2654, and 2655) carry a pTiAB3-like Ti plasmid with its TAR-II region but lack a pTrAB3-like plasmid. This suggests that under natural conditions the Ti plasmid and its TAR-II system can indeed be transferred to avirulent *Agrobacterium* strains and yield virulent pathogens which degrade tartrate and induce tumors. Thus, although *A. vitis* presently dominates in crown galls on grapevine, other *Agrobacterium* species should be considered as potential recipients of Ti plasmids that confer tartrate degradation. It remains to be studied whether tartrate utilization confers an important selective advantage on grapevine. Although the utilization of tartrate by most grapevine isolates suggests this to be the case, the correlation is not perfect. Competition studies with wild-type strains and tartrate nonutilizing mutants are needed to answer this question. Our studies provide a starting point for this since they now enable

Table 5. *E. coli* strain and plasmids used

Name	Properties	Reference
E. coli strains		
SB387	Tn*5-uidA*1 donor	Sharma and Signer 1990
Plasmids		
pBluescript	Cloning vector	Stratagene
pPM124	pTrAB4 TAR fragment cloned in pUC18	Crouzet and Otten 1995
Ab47	pTiAB3 TAR fragment cloned in pPM1016	van Nuenen et al. 1993
pTr179	pTrAB3 fragment cloned in pUC18	Otten et al. 1995
pPM403	*Bam*HI fragment with TAR-I region in pUC18	This study
pPM405	pTrAB3 TAR fragment cloned in pMP2733	Otten et al. 1995
pPM405-R1 to 52	pPM405 with Tn*5-uidA*1 insert	This study
pPM405-L1 to 47	pPM405 with Tn*5-uidA*1 insert	This study
pPM410	*Not*I-*Bam*HI fragment of pTr179 cloned into pBluescript	This study
pPM411	pPM405-R20::pPM410 cointegrate	This study
pPM412	pPM405::pPM410 cointegrate	This study
pPM1105	pTiAB3 TAR fragment cloned in pMP2733	Otten et al. 1995

us to introduce mutations within the wild-type strains with predictable effects on tartrate utilization.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Various *Agrobacterium* wild-type strains are listed in Tables 2 and 3. *E. coli* and plasmids are listed in Table 5.

Transformation of UBAPF2, Tn*5*-*uidA*1 mutagenesis, tartrate utilization and induction on different media.

Transformation of the cured C58-derived *A. tumefaciens* strain UBAPF2 (Hynes et al. 1985), has been described by Crouzet and Otten (1995). Tartrate utilization was tested on two media (Szegedi 1985): minimal AB medium (per liter: 3 g of K_2PO_4, 1 g of NaH_2PO_4, 1 g of NH_4Cl, 0.3 g of $MgSO_4.7H_2O$, 0.15 g of KCl, 2.5 mg of $FeSO_4.7H_2O$, adjusted to pH 6.5 and solidified with 1.5% agar) with 0.5% L(+)–tartrate (ABT) and AB medium with 0.5% L(+)–tartrate, 0.5% glucose and 25 mg of bromothymol blue per liter (ABTG–BBT). The latter medium allows growth of both tartrate degrading and nondegrading bacteria. However, in case of tartrate utilization the pH of the medium increases and its color changes from yellow to dark blue. All wild-type strains described in this study were tested for tartrate utilization on ABT and ABTG–BBT medium. The Tn*5* derivative Tn*5*-*uidA*1 was used for insertional mutagenesis of cloned DNA fragments and promoter analysis. This Tn*5* derivative allows transcriptional but not translational readthrough from the cloned region into a promoterless *uidA* (*gus*) gene situated at its IS50L end (Sharma and Signer 1990). Tn*5*-*uidA*1 was introduced into UBAPF2(pPM405) and UBAPF2(pPM1105) by conjugation with the Tn*5*-*uidA*1 donor strain SB387. Induction of Tn*5*–*uidA*1 was tested on solid AB medium with 0.2 mM X–Gluc and 0.5% tartrate, 0.5% tartrate plus 0.5% glucose or 0.5% glucose. For general molecular biology techniques see Sambrook et al. (1989).

DNA hybridization.

Hybridization of Southern blots was carried out in 50% formamide, 4× SSC (standard saline citrate), 10× Denhardt's solution, 0.2% SDS, and 1 mg/ml denatured calf thymus DNA, for 16 h at 42°C. These conditions correspond to standard hybridization conditions and permit the detection of sequences with similarities of more than 80%.

DNA sequence analysis.

DNA sequences were determined by the Sanger method (Sanger et al. 1977) using double-stranded DNA preparations of exonuclease III-shortened pKS Bluescript derivatives (Henikoff 1987) or subclones and analyzed using the UW8.0 version of the GCG sequence analysis software package (Devereux et al. 1987) on an Alpha Station computer. Nucleotide sequences were compared using the Bestfit program. Searches for sequence homology were done with the BlastN program.

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