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Temperature Affects the T-DNA Transfer Machinery of *Agrobacterium tumefaciens*

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Early studies on *Agrobacterium tumefaciens* showed that development of tumors on plants following infection by *A. tumefaciens* was optimal at temperatures around 22°C and did not occur at temperatures above 29°C. To assess whether this inability to induce tumors is due to a defect in the T-DNA transfer machinery, mobilization of an incompatibility group Q (IncQ) plasmid by the T-DNA transfer machinery of *A. tumefaciens* was tested at various temperatures. Optimal transfer occurred when matings were performed at 19°C, and transfer was not seen when matings were incubated above 28°C. Transfer of the IncQ plasmid was dependent upon induction of the *virB* and *virD* operons by acetosyringone but was not dependent upon induction of the *tra* genes by octopine. However, alterations in the level of *vir* gene induction could not account for the decrease in transfer with increasing temperature. *A. tumefaciens* did successfully mobilize IncQ plasmids at higher temperatures when alternative transfer machineries were provided. Thus, the defect in transfer at high temperature is apparently in the T-DNA transfer machinery itself. As these data correlate with earlier tumorigenesis studies, we propose that tumor suppression at higher temperatures results from a T-DNA transfer machinery which does not function properly.

Seventy years ago, A. J. Riker discovered that development of crown gall tumors on plants following infection by *Agrobacterium tumefaciens* is sensitive to elevated temperatures (37). *Lycopersicon esculentum* (37), *Vinca rosea* (9, 46), and *Kalanchöe diagremontiana* (11, 12) all failed to produce tumors when infected with *A. tumefaciens* at temperatures greater than 29°C. The size of tumors produced on plants infected at temperatures below 29°C increased as the incubation temperature decreased, with the largest tumors developing on plants kept at 22°C (37). Later experiments established that suppression of tumor development at higher temperatures was due to the inability of *A. tumefaciens* to provide the tumor-inducing principle rather than to effects on the plant (10, 11, 13).

This tumor-inducing principle has now been well characterized. *A. tumefaciens* causes tumors on plants by transferring a segment of DNA, the T-DNA, from the bacterial tumor-inducing (Ti) plasmid to the nucleus of the plant cell (57). Processing and transfer of the T-DNA depend on the *vir* genes, whose expression is controlled by the VirA-VirG two-component regulatory system (22). At temperatures greater than 32°C, the *vir* genes are not expressed because of a conformational change in VirA which inactivates the protein (26). However, the structure of the transcriptional activator VirG is unaffected by incubation at this temperature. Indeed, inhibition of *vir* gene expression at 32°C can be overcome by the expression of a mutant form of VirG ($VirG^c$) which activates transcription even in the absence of VirA. Expression of $VirG^c$, however, does not confer the ability of *A. tumefaciens* to induce tumors at 32°C (26). Thus, high temperature must affect a later stage in the virulence pathway.

One major event of tumorigenesis which occurs after *vir* gene induction is the processing of the T-DNA region of the Ti plasmid. The resulting T-DNA molecule consists of a single-stranded copy of the T-DNA region of the Ti plasmid with the VirD2 protein covalently bound to its 5′ end (57). This T-DNA is then transferred to the plant cell either separately or in association with the single-stranded DNA-binding protein VirE2 (57).

The machinery responsible for transfer of the processed T-DNA and the VirE2 protein is encoded by the 11 genes of the *virB* operon and *virD4* (25). The protein products of these 12 genes are essential for efficient tumorigenesis (5, 21, 41, 43, 48, 51), and most have been localized to the membrane fraction of *A. tumefaciens* (3, 4, 17, 33, 40, 41, 47, 48). Deduced amino acid sequences of these proteins showed that the VirB and VirD4 proteins are homologous to membrane proteins necessary for conjugative transfer of the broad-host-range plasmids RP4, R388, and pKM101 (27, 30, 35). These sequence similarities provide the framework for a conjugative model for T-DNA transfer (30).

The conjugative model is supported by the observation that *A. tumefaciens* can mediate the conjugative transfer of the IncQ group plasmid RSF1010 (2, 14). RSF1010 is a nonconjugal plasmid in that it does not carry all the genetic information to promote its own transfer into other bacteria (53). However, RSF1010 can utilize a transfer machinery encoded by another conjugative plasmid present in the same host for its transfer into other bacteria (53). Interestingly, *A. tumefaciens* can transfer RSF1010 into both plant cells (14) and other strains of *A. tumefaciens* (2) in the absence of a coresident conjugative plasmid. These observations suggest that RSF1010 utilizes the T-DNA transfer machinery for its entry into plant cells and other bacteria. Indeed, mobilization of RSF1010 to other strains of *A. tumefaciens* requires at least three genes known to be essential for T-DNA transfer: *virB4*, *virB11*, and *virD4* (2, 21, 28, 41). Recently, we have shown that all 11 *virB* genes and *virD4* are essential for RSF1010 mobilization between strains of *A. tumefaciens*, showing that RSF1010 and T-DNA share the same transfer machinery (20a).

Mobilization of an IncQ plasmid between *Agrobacterium* strains was used in this study to monitor the function of the T-DNA transfer machinery at various temperatures. This assay is advantageous in this study as effects of temperature on either

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
Agrobacterium tumefaciens		
A136	C58 chromosome, pAtC58, Rif^r	52
A348	C58 chromosome, pTiA6NC, pAtC58, Rif^r	39
LBA4404	Ach5 with pAch5, Δ*tra* Δ*occ* ΔT_L ΔT_R Rif^r	23
MX243	A348 *virB1*::Tn*3*HoHo1, pPH1JI, Cb^r Gm^r	43
UIA5	C58 chromosome, pTi$^-$, pAtC58$^-$, Rif^r	18
UIA143	C58 chromosome, pTi$^-$, *recA143* Ery^r	20
At11067	A348 *virB1*::Tn*3*HoHo1, MX243 (43) cured of pPH1JI, Cb^r	21
At11068	A348 Δ*virA*::Ω fragment, Sp^r Str^r	16a
At12506	A348 *virD4*::Tn*3*HoHo1, MX367 (43) cured of pPH1JI, Cb^r	21
At12507	A348 *virH*::Tn*3*HoHo1, MX231 (43) cured of pPH1JI, Cb^r	This study
At12508	A348 *virG*::Tn*3*HoHo1, MX363 (43) cured of pPH1JI, Cb^r	This study
At12509	A348 *virC*::Tn*3*HoHo1, MX379 (43) cured of pPH1JI, Cb^r	This study
At12510	A348 *virF*::Tn*3*HoHo1, MX546 (7) cured of pPH1JI, Cb^r	This study
At12511	A348 *virD2*::Tn*3*HoHo1, MX334 (43) cured of pPH1JI, Cb^r	21
At12515	A348 *chvE*::Tn*3*HoHo1, MX1 (19) cured of pPH1JI, Cb^r	This study
At12516	A348 Δ*virE2*::Ω fragment, Sp^r Str^r	This study
Escherichia coli		
S17.1	294 *rec* RP4-2(Tc::Mu, Km::Tn*7*)	42
TG1	*supE hsdD5 thi-1* d(*lac-proAB* F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15])	Amersham Corp.
Plasmids		
pSW108	*virE* operon in pUC7, Ap^r	54
pUC19Ω	Ω fragment in pUC19, Ap^r Sp^r Str^r	6
pKJF82	pSW108 with Ω fragment at *Stu*I-*Hin*dIII, Ap^r Sp^r Str^r	This study
pIB50	IncP *virB*::*lacZ virE*::*cat* Km^r	1
pML122	IncQ Km^r Gm^r	29
pML122ΔGm	IncQ Km^r	This study

[a] Abbreviations: Ap, ampicillin, Cb, carbenicillin; Ery, erythromycin; Gm, gentamicin; Km, kanamycin; Nal, nalidixic acid; Rif, rifampin; Sp, spectinomycin; Str, streptomycin.

T-DNA processing or on the plant are eliminated. Using this system, we show that transfer of the IncQ plasmid pML122 is sensitive to high temperature. We further demonstrate that the T-DNA transfer machinery is affected by the temperature shifts.

MATERIALS AND METHODS

Media, antibiotics, and growth conditions. For growth of *A. tumefaciens*, Luria-Bertani (LB) (38) or MG/L (16) medium was used. Matings were done on CIB agar (21) with 2% glucose. Media were supplemented with the following, in micrograms per milliliter: acetosyringone, 40; carbenicillin, 100; DNase I, 1; erythromycin, 150; gentamicin, 100; kanamycin, 100; octopine, 100; rifampin, 20; spectinomycin, 100; and streptomycin, 200. For growth of *Escherichia coli*, LB medium (38) was supplemented with the following antibiotics, in micrograms per milliliter: ampicillin, 100; gentamicin, 10; kanamycin, 25; spectinomycin, 50; and streptomycin, 12.5.

Construction and characterization of *A. tumefaciens* strains. The bacterial strains used in this study are listed in Table 1. *A. tumefaciens* strains carrying Tn*3*HoHo1 insertions were cured of pPH1JI by either electroporation or nonselective passage followed by screening for gentamicin sensitivity. Newly constructed strains were characterized by Southern analysis, by Western blotting (immunoblotting), and for tumorigenesis as previously described (21).

A deletion of the *virE2* gene was created by double homologous recombination following electroporation of pKJF82 into *A. tumefaciens* A348. The resulting strain, *A. tumefaciens* At12516, has an omega fragment insertion beginning at the *Stu*I restriction site located 33 nucleotides into the *virE2* gene which eliminates 1,055 bp of *virE2* and blocks downstream transcription and translation. At12516 is avirulent, does not produce VirE2 protein, and can be complemented for virulence by providing the *virE* operon in *trans* (data not shown).

Plasmid constructions. Plasmids used in this study are listed in Table 1. Plasmid DNA was manipulated by standard recombinant methodology with techniques previously detailed (21). Plasmids were transferred into *A. tumefaciens* either by mating with *E. coli* S17.1 (42) or by electroporation (16).

The 2.0-kb omega fragment consists of the spectinomycin and streptomycin resistance gene, *aadA*, flanked by short inverted repeats containing transcriptional and translational termination signals (36). To construct the suicide vector pKJF82 for deletion of the *virE2* gene, the omega fragment was isolated from pUC19Ω as a *Sma*I-*Sma*I fragment. The fragment was ligated into pSW108, which had been digested with *Stu*I and *Hin*dIII and treated with Klenow fragment to create blunt ends.

pML122ΔGm was created by digesting pML122 with *Bgl*II and *Sac*II, treating with Klenow fragment, and religating the plasmid. Following transformation into *E. coli* TG1, kanamycin-resistant cells were screened for gentamicin sensitivity. The resulting plasmid was then transformed into *E. coli* S17.1.

Conjugation assay. Cultures of donor and recipient strains were grown overnight in MG/L with antibiotics at 28°C. Cultures were diluted into CIB broth without antibiotics to an optical density at 600 nm of about 0.25 and were incubated at 28°C with shaking for 6 h. Donor and recipient cells were mixed together at a ratio of 7 to 1 in a 1.5-ml microcentrifuge tube to a combined optical density at 600 nm of 0.25. Five microliters of the mating mix was spotted in the center of a 35-mm petri dish containing 4 ml of CIB agar. Plates were inverted and incubated at various temperatures for 3 days. Bacteria were recovered by washing the surface of the agar three times with 0.9% NaCl to yield a pooled volume of 1 ml. Recovered bacteria were plated onto LB agar supplemented with the appropriate antibiotics to select for transconjugants. Plates were incubated at 28°C for 4 to 5 days, and colonies were counted.

Spontaneous reversion of donors to Ery^r varies with the strain but is always less than one revertant per assay. For plates with fewer than 20 transconjugants, colonies were patched onto MG/L agar with rifampin (50 μg/ml) to check for reversion of donors to Ery^r. Reversion of UIA143 to Gm^r (100 μg/ml) has never been seen although transconjugants selected for gain of pML122 on gentamicin were checked for the concurrent gain of Km^r in some assays.

Mating plate induction assay. Cultures of *A. tumefaciens* were prepared as described for the conjugation assay. Cultures grown in CIB broth were diluted to an optical density at 600 nm of 0.22, and 5 μl was spotted onto several 4-ml CIB agar plates. After 3 days, bacteria were recovered as described for the conjugation assay and were assayed for β-galactosidase activity (32).

RESULTS

Mobilization of the IncQ plasmid pML122 at pH 5.3 is temperature sensitive. To assess the effect of temperature on the function of the T-DNA transfer machinery, mobilization of pML122, a gentamicin- and kanamycin-resistant derivative of the IncQ plasmid RSF1010, was assayed. The same mixture of preinduced wild-type *A. tumefaciens* A348 cells and recipient UIA143 cells was spotted onto mating plates, which were then

TABLE 2. Transfer of pML122 by *A. tumefaciens* A348 at various temperatures[a]

Temp (°C)	No. of recovered bacteria/ml		No. of recovered transconjugants	Conjugation frequency	
	Donor (10^5)	Recipient (10^5)		No. of transconjugants/ input donor (±SD)	No. of transconjugants/ recovered recipient (±SD)
15	118	1,110	436	2.0×10^{-4} (±0.2)	3.9×10^{-6} (±0.4)
19	309	4,160	5,620	2.6×10^{-3} (±0.5)	1.4×10^{-5} (±0.3)
22	216	4,590	2,350	1.1×10^{-3} (±0.3)	5.2×10^{-6} (±1.7)
25	97	3,450	1,170	5.3×10^{-4} (±1)	3.5×10^{-6} (±1.1)
28	38	2,190	4	2.0×10^{-6} (±1)	2.0×10^{-8} (±0.9)
31	17	1,380	0	$<4.5 \times 10^{-7}$ (±0)	$<7.3 \times 10^{-9}$ (±0.8)

[a] Five microliters of a mating mix of 4.3×10^8 cells of preinduced donor A348(pML122) per ml and 3.6×10^7 cells of preinduced recipient UIA143 per ml was spotted onto CIB + acetosyringone agar, and plates were incubated at the indicated temperatures. Following 3 days of mating, cells were recovered and plated onto LB agar with erythromycin and gentamicin to select transconjugants, LB agar with rifampin to select donors, and LB agar with erythromycin to select recipients. Data shown are the means and standard deviations of triplicates from a single experiment. A total of five independent experiments were performed with similar results.

incubated at temperatures between 15 and 31°C. The number of transconjugants recovered from matings incubated at lower temperatures was markedly greater than when matings were incubated at higher temperatures. Analysis of the conjugation frequency (transconjugants per input donor) at the various temperatures showed that transfer of pML122 between strains of *A. tumefaciens* was most efficient on mating plates incubated at 19°C (Table 2). The transfer frequency then declined at least 50% with every 3°C temperature increase up to 25°C. Then, between 25 and 28°C, the frequency of transfer declined by 2 orders of magnitude. At temperatures above 28°C, mobilization of pML122 was undetectable. Thus, transfer of pML122 by *A. tumefaciens* was shown to be extremely sensitive to temperature, particularly in the narrow range between 25 and 28°C.

Effects of temperature on the growth and survival of both the donor and the recipient strain could affect the transfer frequency at different temperatures. For the donor and recipient cells, the number of viable bacteria recovered after 3 days of incubation on CIB agar was greatest at 19 and 22°C, respectively, indicating that *A. tumefaciens* survives better at lower temperatures when grown under inducing conditions (Table 2). However, the conjugation frequency was greatest at 19°C even when the frequency was calculated as transconjugants per recovered recipient, showing that fluctuations in the growth and viability of the recipient at various temperatures did not alone affect the transfer frequency (Table 2). Fluctuations in the donor survival, however, may have influenced the transfer frequency. The number of recovered viable donors decreased above 22°C, suggesting that the number of donors available to form mating pairs late in the course of mating was drastically altered by the temperature shifts. This difference could have significantly contributed to the observed decrease in the transfer frequencies at higher temperatures, particularly since transfer occurs over the entire 3-day period (data not shown). Between 19 and 25°C, the number of recovered viable donors decreased threefold. This decrease may have contributed to the observed fivefold decrease in the transfer frequency, although the extent of that contribution is not known. A similar decrease in recovered viable donors was observed between 25 and 28°C, but the decrease in the transfer frequency was greater than 250-fold. Thus, although variation in the number of viable bacteria may in part account for the decrease in transfer between 19 and 25°C, it cannot account for the significant decrease which occurs between 25 and 28°C. To address this finding, numerous other possible factors which could have contributed to the decreased frequency at 28°C were studied.

Transfer of pML122 at low temperatures occurs by conjugation. *A. tumefaciens* can be transformed by naked plasmid (24). However, the increased recovery of transconjugants at lower temperatures was not the result of the activation of an alternate DNA uptake pathway. Under the conjugation assay conditions, the recipient UIA143 was not transformed by cesium chloride-purified pML122 DNA (data not shown). Further, A348 mobilized pML122 at 19°C in the presence of 1 μg of DNase I per ml (data not shown). These data show that mobilization of pML122 between strains of *A. tumefaciens* must be via a conjugative mechanism which is temperature sensitive.

***vir* gene requirements for conjugal transfer.** Transfer of pML122 via the T-DNA transfer machinery requires induction of the *vir* genes by acetosyringone (2) (Table 3). To establish which *vir* genes are essential for transfer from A348 at both 19 and 28°C, strains with either deletions or Tn*3*HoHo1 insertions in *virA-H* and *chvE* (Table 1) were tested for mobilization of pML122 at both temperatures. Donor strains with a mutation in *virA*, *virG*, or *chvE* were unable to mobilize pML122, reconfirming that *vir* gene induction is essential for IncQ plasmid transfer (data not shown). Likewise, donor strains with muta-

TABLE 3. Temperature-sensitive mobilization of pML122 in the presence of various inducing compounds[a]

Donor strain	Mating media[b]	Temp (°C)	Conjugation frequency[c]
A348 (pML122)	CIB + AS	19	2.0×10^{-3}
		28	7.1×10^{-6}
	CIB − AS	19	$<7.1 \times 10^{-7}$
		28	$<7.1 \times 10^{-7}$
	CIB + Oct	19	$<7.1 \times 10^{-7}$
		28	$<7.1 \times 10^{-7}$
	CIB + Oct + AS	19	1.9×10^{-3}
		28	5.0×10^{-6}
LBA4404 (pML122)	CIB + AS	19	2.3×10^{-3}
		28	3.1×10^{-6}
	CIB − AS	19	$<8.3 \times 10^{-7}$
		28	$<8.3 \times 10^{-7}$

[a] Donor strains and recipient UIA143 were preinduced in either the presence or the absence of acetosyringone. Mating mixes were spotted to the indicated medium, and plates were incubated at the indicated temperatures. Following 3 days of mating, cells were recovered and plated onto LB agar with erythromycin and gentamicin to select transconjugants. Data shown are the means of triplicates from a single experiment. Two independent experiments were performed with similar results.

[b] Abbreviations: AS, acetosyringone; Oct, octopine.

[c] Expressed as number of transconjugants per input donor.

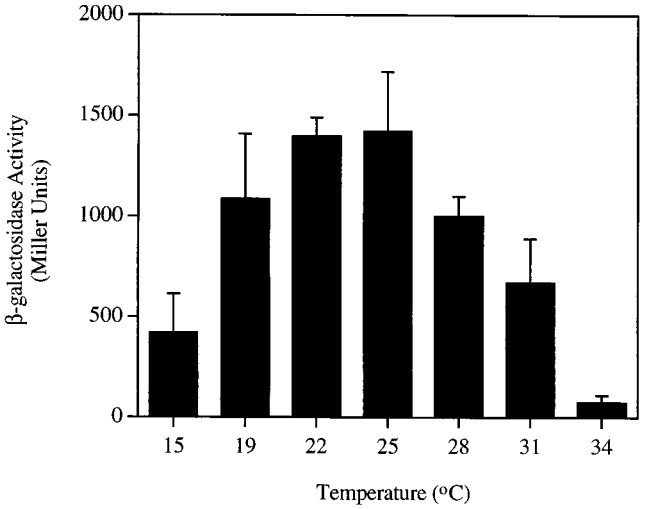

FIG. 1. A348(pIB50) was preinduced in CIB with acetosyringone and kanamycin. Five microliters of cells at 2.8×10^8/ml was spotted onto 21 plates of CIB agar with acetosyringone and kanamycin, and three plates were incubated at each of the indicated temperatures. After 3 days, bacteria were recovered and assayed for β-galactosidase activity. Data shown are the mean of triplicates ±1 standard deviation.

tions in either the *virB* or the *virD* operons did not transfer pML122, showing that these previously identified elements of the T-DNA transfer machinery were essential for transfer of pML122 (data not shown). Other *vir* genes, *virC*, *virE2*, *virF*, and *virH*, were not essential for transfer (data not shown). These data demonstrate that there are no differences in the genetic requirements for transfer at 28 and 19°C, suggesting that the mechanism of transfer is the same at both temperatures.

Induction of *vir* genes is optimal at 25°C. Since the T-DNA transfer proteins encoded by *virB* and *virD4* are critical for transfer, alterations in their induction level may affect the frequency of transfer. Thus, we examined the effect of various temperatures on *vir* gene induction. The reporter plasmid pIB50 was first introduced into A348. The resulting strain was treated under conjugation assay conditions at various temperatures between 15 and 34°C, and the level of β-galactosidase activity produced from *virB*::*lacZ* was measured. At all temperatures, induction of *vir* genes was dependent upon acetosyringone (data not shown). In the presence of acetosyringone, the optimal temperature for *vir* gene induction was 25°C (Fig. 1). β-Galactosidase production at 19°C, the optimal temperature for transfer, was nearly identical to β-galactosidase production at 28°C, a temperature at which transfer occurs at only a very low frequency. Further, β-galactosidase production at 19°C was only slightly greater than the activity at 31°C, the temperature which is nonpermissive for transfer. Thus, under the conjugation assay conditions, *vir* gene induction at different temperatures does not correlate with IncQ plasmid transfer; therefore, it cannot account for the significant effect of temperature on the transfer of pML122.

Mobilization of IncQ plasmids does not depend on the *tra* genes. Octopine-stimulated conjugation of the Ti plasmid between cells of *A. tumefaciens* is optimal between 23 and 27°C and is sensitive to temperatures above 30°C (45). This sensitivity is due to the lack of *tra* gene induction by homoserine lactone (34, 55, 56). Since A348 has *tra* genes on its Ti plasmid, we considered the possibility that mobilization of pML122 may require some of the Tra proteins and that the observed increase in the transfer frequency at lower temperatures was due to the increased expression of the *tra* genes. This possibility is unlikely for several reasons. First, transfer of pML122 was inhibited at temperatures at which the Ti plasmid is transferred (45) (Table 2). Second, transfer of pML122 was not stimulated by the addition of octopine to the plates either in the presence or in the absence of acetosyringone (Table 3). Finally, *A. tumefaciens* strains with Ti plasmids having the *tra* region deleted mobilize IncQ plasmids (2) (Table 3), and this transfer is temperature sensitive (Table 3). Thus, we conclude that the *tra* genes are not required for IncQ plasmid transfer between bacteria at any temperature.

Alternate pathways for mobilization of IncQ plasmid pML122 are functional only at neutral pH. Thus far, we have established that *virB* and *virD4*-dependent transfer of pML122 is temperature sensitive. It is possible that the block to transfer at higher temperature is due to factors such as an inability of pML122 to be processed properly or the inability of *A. tumefaciens* to conjugate due to membrane alterations or attachment defects. To examine whether *A. tumefaciens* has a nonspecific defect in its ability to conjugate, mobilization of pML122 by pathways other than the *virB* and *virD4*-dependent transfer pathway was tested. IncQ plasmids can be mobilized both by the large plasmid pAtC58, a plasmid resident in most strains of *A. tumefaciens* derived from C58, and by an uncharacterized pathway in the plasmidless strain UIA5 (18). Transfer via these pathways is independent of *virB*, *virD4*, and the *tra* genes, since the Ti plasmid-cured strains A136 and UIA5 were able to transfer pML122 and this transfer was independent of acetosyringone (Table 4). However, transfer of pML122 via these pathways occurred only when matings were performed on media adjusted to neutral pH, not on media of acidic pH (Table 4).

When matings were performed at pH 7.0, pML122 was transferred from the plasmidless strain UIA5 at elevated tem-

TABLE 4. Effect of temperature on mobilization of pML122 via three alternative pathways present in different strains of *A. tumefaciens*[a]

Donor strain	Plasmid(s)[b]	Conjugation frequency[c]			
		pH 5.3 + AS (19°C)	pH 5.3 − AS (19°C)	pH 7.0 − AS (19°C)	pH 7.0 − AS (28°C)
A348	pTiA6, pAtC58	3.4×10^{-3}	$<3.8 \times 10^{-7}$	1.5×10^{-5}	2.9×10^{-4}
A136	pAtC58	$<6.8 \times 10^{-7}$	$<5.6 \times 10^{-7}$	6.7×10^{-5}	8.6×10^{-4}
UIA5	None	$<5.6 \times 10^{-7}$	$<5.2 \times 10^{-7}$	4.7×10^{-6}	1.4×10^{-4}

[a] Donor strains and recipient UIA143 were preinduced in CIB broth as indicated. Mating mixes were spotted onto CIB agar and incubated as indicated. Transconjugants were selected on LB agar with erythromycin and gentamicin. Data shown are the mean of triplicates from a single experiment. Two independent experiments were performed with similar results. AS, acetosyringone.

[b] All strains have pML122.

[c] Expressed as number of transconjugants per input donor.

TABLE 5. pPH1JI-dependent mobilization of pML122 at 19 and 28°C[a]

Donor strain	Relevant genotype	Conjugation frequency[b]		
		pH 5.3 + AS (19°C)	pH 5.3 − AS (19°C)	pH 5.3 − AS (28°C)
A348 (pML122)	Wild type	2.5×10^{-3}	$<2.7 \times 10^{-7}$	$<2.7 \times 10^{-7}$
At11067 (pML122)	*virB1*::Tn*3*HoHo1	$<7.7 \times 10^{-7}$	$<3.6 \times 10^{-7}$	$<3.6 \times 10^{-7}$
MX243 (pML122ΔGm)[c]	*virB1*::Tn*3*HoHo1, pPH1JI$^+$	2.1×10^{-3}	1.2×10^{-3}	8.8×10^{-4}

[a] Donor strains and recipient UIA143 were preinduced in CIB broth as indicated. Mating mixes were spotted onto CIB agar and incubated as indicated. Transconjugants were selected on LB agar with erythromycin and kanamycin. Data shown are the mean of triplicates from a single experiment. Two independent experiments were performed with similar results. AS, acetosyringone.

[b] Expressed as number of transconjugants per input donor.

[c] pML122ΔGm was used in MX243 to allow gentamicin selection for maintenance of pPH1JI.

peratures. The frequency of transfer via this uncharacterized pathway was increased 30-fold when the temperature was raised from 19 to 28°C (Table 4). Similarly, transfer of pML122 mediated by the large plasmid pAtC58 was stimulated by increasing the temperature. In both the presence and the absence of a Ti plasmid, transfer of pML122 was stimulated 10- to 20-fold when the matings were incubated at 28 compared with 19°C (Table 4). Whether this increased activity is in part due to activation of the same pathway as is present in the plasmidless strain UIA5 could not be determined from our data. In all cases, though, transfer of IncQ plasmids by these alternative pathways exhibited a different temperature preference profile compared with the *virB* and *virD4*-dependent pathway.

Mobilization of the IncQ plasmid pML122 by the IncP plasmid pPH1JI. IncQ plasmids can also be mobilized by members of the IncP group of conjugative plasmids (53). At11067 has a Tn*3*HoHo1 insertion at *virB1*, thereby disabling the *virB*-dependent mobilization of pML122 at pH 5.3 (Table 5). MX243 is the same strain carrying the IncP plasmid pPH1JI. MX243 transferred pML122 at pH 5.3 both in the presence and in the absence of acetosyringone (Table 5), showing that pML122 can be transferred between strains of *A. tumefaciens* by pPH1JI. However, transfer of pML122 by pPH1JI occurred at a similar frequency regardless of the incubation temperature. Thus, pPH1JI-dependent transfer of IncQ plasmids at acidic pH shows a different temperature preference profile from both the *virB* and *virD4*-dependent pathway and the other poorly understood transfer systems functional at pH 7.0.

Temperature affects only the T-DNA transfer machinery. Although *A. tumefaciens* A348 transfers pML122 poorly via the *virB* and *virD4*-dependent pathway at 28°C (Table 2), A348 is capable of processing and transferring pML122 at 28°C at both neutral (Table 4) and acidic (Table 5) pH. Thus, the block to transfer of IncQ plasmids via *virB* and *virD4*-encoded transfer apparatus is not due to a general defect in the ability of *A. tumefaciens* to conjugate at higher temperatures. In these experiments, the optimal temperature for conjugation varied with the transfer machinery provided, showing that it is function of the transfer machinery which is most affected by changes in temperature. Thus, the inhibition of transfer of IncQ plasmids from A348 at elevated temperatures via the *virB* and *virD4*-dependent pathway must be due to adverse effects on the function of the VirB-VirD4 transfer machinery.

DISCUSSION

In classical experiments performed in 1926, A. J. Riker showed that development of tumors on plants following infection by *A. tumefaciens* was optimal at temperatures around 22°C and was absent at temperatures greater than 28°C (37). The most obvious decrease in tumorigenesis occurred in the narrow temperature range between 25 and 28°C (11). In the subsequent 70 years, the molecular basis for tumor suppression with increasing temperature has not been elucidated. To gain insight into the possible mechanism, we have examined the effect of temperature on the T-DNA transfer machinery by using mobilization of an IncQ plasmid between bacteria as a quantitative assay for function. Previous studies have clearly established that IncQ plasmids can utilize the same machinery for transfer as the T-DNA (2, 14, 21, 50). To date, this machinery is known to consist of at least 12 proteins, VirB1 to VirB11 and VirD4, all of which are required for the transfer of IncQ plasmids (2, 20a, 21). We have shown that the ability of this machinery to mobilize an IncQ plasmid is strongly inhibited by incubation at temperatures above 25°C. Further, we have shown that this temperature sensitivity is not due to differences in bacterial viability, inability of pML122 to be processed, alterations in *vir* gene induction, or transfer via the Ti plasmid conjugation pathway. Indeed, we have shown that *A. tumefaciens* can successfully mobilize IncQ plasmids at higher temperatures when pathways other than the VirB-VirD4 pathway are provided. Thus, the defect in transfer at high temperature is apparently in the T-DNA transfer machinery itself.

Although this impairment of the T-DNA transfer machinery was shown by assaying for IncQ plasmid transfer, T-DNA transfer is also affected by temperature. Recently, it has been reported that T-DNA can be mobilized from *A. tumefaciens* into *Saccharomyces cerevisiae* (15). Similar experiments performed in our laboratory have also demonstrated transfer of T-DNA to *S. cerevisiae*. In these studies, the frequency of T-DNA transfer was increased by decreasing the cocultivation incubation temperature from 28 to 22°C (33a).

Since the optimal temperature for mobilization of IncQ plasmids between bacteria and for T-DNA transfer into *S. cerevisiae* correlates so well with early studies on tumorigenesis (37), we propose that the primary cause of tumor suppression by high temperature is a T-DNA transfer machinery which does not function efficiently.

The nature of this defect in the T-DNA transfer machinery at the molecular level is currently unknown. Evidence presented in this paper shows that induction of the *vir* genes is sufficient at temperatures which limit transfer. Also, studies on the membrane localization of the VirB and VirD4 proteins were carried out on bacteria grown at 28°C, showing that these proteins are present at higher temperatures (3, 4, 17, 33, 40, 41, 47, 48). Thus, the components essential to construct an active complex are presumably not limiting at higher temperatures. Therefore, either the ability to assemble a functional transfer apparatus or the stability of the completed apparatus is likely to be the source of the temperature sensitivity.

Early studies of the nature of tumor suppression by temperature favored an instability model. In 1950, A. Braun examined tumorigenesis within narrow temperature ranges and then performed thermodynamic calculations based on the observations. From these calculations, he postulated that the inability of *A. tumefaciens* to induce tumors on plants at high temperatures was due to rapid protein denaturation of a complex structure (11). However, at that time, the components of the structure were not identified, but we now postulate that this structure is the T-DNA transfer machinery which is encoded by the *virB* and *virD4* genes.

The VirB-VirD4 transfer machinery is probably a highly complex structure, although its structure has not been elucidated. However, evidence from homologous systems suggests that the VirB and VirD4 proteins assemble into a sex pilus or a pilus-like structure (27). The VirB proteins and VirD4 are similar to proteins essential for pilus biogenesis and for conjugative transfer of broad-host-range plasmids (27, 30, 35). VirB2 is 52% identical in amino acid sequence to the pilin subunit of the F plasmid (40). Although these sequence homologies are compelling, a sex pilus on induced *A. tumefaciens* has not been identified (29a). If a sex pilus is essential for T-DNA transfer, it is possible that the pilus is absent or unstable at 28°C, the standard laboratory growth temperature for *A. tumefaciens*.

Loss of pilus structure has already been implicated as the cause of temperature-sensitive conjugation of two classes of self-transmissible plasmids (8, 44). IncH1 plasmid conjugation between cells of *E. coli* is optimal at 26°C (44), even though the optimal temperature for both growth of *E. coli* and mating bridge formation is 37°C (49). However, at 37°C, *E. coli* cells carrying IncH1 plasmids do not have pili and are thus unable to conjugate (31). The reason for the lack of pili at the higher temperature has not yet been identified, but this case demonstrates that elevated temperatures can have a profound effect on pilus biogenesis and transfer functions.

Another example is provided by the IncT group of plasmids. *E. coli* cells carrying plasmids of the IncT group have pili at 37°C, but they are short and rigid, allowing conjugation of IncT plasmids at 37°C only on solid agar (8). By contrast, at 26°C, the bacteria have long, flexible pili and can conjugate at high frequency on both solid and liquid media. In this case, high temperature affects the superstructure of the sex pilus, thereby decreasing transfer efficiency. These two temperature-sensitive conjugative systems illustrate that temperature can exert a drastic effect on both the presence and the structure of a conjugative pilus. In light of these studies and our finding that T-DNA transfer is temperature sensitive, we are currently attempting to identify pili on cells of *A. tumefaciens* grown at 19°C.

Regardless of the precise cause of the temperature effect, the implications of the effect of even small temperature increases on T-DNA transfer are potentially quite broad. Protocols for the genetic engineering of plants should carefully consider the incubation temperature during cocultivation to optimize cell viability and T-DNA transfer. On the basis of results presented here, it is conceivable that decreasing temperatures of cocultivation may lead to a significant increase in the transformation frequency and potentially to the genetic transformation of recalcitrant plants.

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