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# The Kinetics of T-strand Production in a Nopaline-Type Helper Strain of *Agrobacterium tumefaciens*

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**The production of single-stranded DNA from the T-region in *Agrobacterium tumefaciens* is dependent on the induced cells being in an actively growing state. Once a culture approaches stationary phase, the cells of that culture are no longer inducible. After being induced, the cells continue producing single-stranded T-region derivatives from a nicked substrate well into the stationary phase of the culture, and the time of maximum T-strand production occurs approximately 24 hr after addition of the inducer. The data suggest that T-strand production will be greater in richer media where cell growth is greater. In the nopaline binary/helper combination used here, which contains a single left border from pTiT37 and *vir* region from pTiC58, maximum nicking of the border repeat occurs around 200–500 μM acetosyringone, but single-stranded DNA production from the nicked borders continues increasing to at least 1 mM acetosyringone. The data suggest that more than one single-stranded DNA molecule may be made from a given T-region under maximal induction conditions.**

*Additional keywords*: Plant transformation, T-DNA, crown gall, virulence.

*Agrobacterium tumefaciens*, the pathogenic bacterium responsible for the disease crown gall in plants, genetically engineers higher plant cells by transferring to them a defined segment of DNA (the T-region) from a plasmid (the Ti-plasmid) carried by the bacterium (Currier and Nester 1976; Chilton *et al.* 1977). The T-region is delimited by 23–25 base pair imperfect direct repeats (Zambryski *et al.* 1982; Yadav *et al.* 1982). A model has been proposed for T-region transfer (Stachel and Zambryski 1986) that likens the DNA transfer process to that which occurs in plasmid transfer during bacterial conjugation (Willetts and Wilkins 1984). A characteristic of this system is that a single-stranded DNA intermediate is formed from a nicked double-stranded DNA substrate. In *Agrobacterium* cells induced for transfer either by exposure to plant cells or monocyclic phenolic compounds of the type produced by wounded or metabolically active plant cells (Bolton *et al.* 1986; Stachel *et al.* 1986a; Janssens *et al.* 1986), specific nicking at the border sequences and production of T-region derived single-stranded molecules (T-strands) have been observed (Albright *et al.* 1987; Culianez-Macia and Hepburn 1988; Stachel *et al.* 1986b; Wang *et al.* 1987). The border nicking is done by two of the products of the *virD* locus (Yanofsky *et al.* 1986; Jayaswal *et al.* 1987). The production of single-stranded DNA is, by analogy with conjugative DNA transfer, thought to be the result of both plasmid (e.g., a gyrase) and host gene products (e.g., DNA polymerase III) (Willetts and Wilkins 1984).

We have shown (Culianez-Macia and Hepburn 1988) that nicking of the left border repeat of a nopaline-type Ti-plasmid is dependent on *vir*-region induction alone, but that single-stranded DNA production from this nicked substrate is dependent on the presence of an additional sequence, called overdrive, described by Peralta and Ream (1985) and Peralta *et al.* (1986). In this paper we demonstrate the kinetics of nick and single-stranded DNA production and present evidence to show that only actively growing bacteria are capable of being induced, but that once induced are capable of producing single-stranded DNA well into the stationary phase. We also show that maximum production of single-stranded molecules occurs at acetosyringone concentrations that significantly exceed those in conditioned culture media that have been reported (Stachel *et al.* 1986a) and that the inducer is not either fully taken up or metabolized by induced cells.

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## MATERIALS AND METHODS

**Plasmids and bacterial strains.** The helper strain C58-Z707 was derived from strain C58 by homogenotizing a plasmid construct, in which the T-region had been replaced by the NPT1 gene of Tn903 (Oka *et al.* 1981), into pTiC58 by using pNJ5000 (Grinter 1983) as the intermediate vector (Hepburn *et al.* 1985). The construction of pCW61 (9.14 kb; Fig. 1) has been described in detail elsewhere (Culianez-Macia and Hepburn 1988). The relevant portions of the plasmid are described in the results section. This binary vector was transferred to the helper strain C58-Z707 by the cointegrative transfer procedure described by Hepburn *et al.* (1985) by using pNJ1020 (53 kb) as the intermediate vector. pNJ1020 is similar to pNJ5000 except that pNJ1020 is stable in RecA+ backgrounds. Manipulations done in *Escherichia coli* used strains HB101, ED8767, and W3110.

**DNA enzymic manipulation**. Restriction endonucleases and DNA modification enzymes were purchased from Anglian Biotechnology or Bethesda Research Laboratories and were used in the buffers as described by the manufacturer. Because the total nucleic acid preparations from *A. tumefaciens* were relatively crude, restriction endonucleases were used in a 20- to 30-fold excess.

**DNA fractionation, blotting, and hybridization.** DNA samples were fractionated on 0.8 or 1.8% agarose gels (Seakem LE) in 200 mM glycine, 15 mM NaOH, 2 mM EDTA (final pH 9.0). Two to five μg of DNA was loaded per lane. Gels were blotted to Genescreen (New England Nuclear) in 25 mM phosphate buffer (pH 6.8). DNA fragments for use as probes were labeled either by nick translation (Maniatis *et al.* 1975) or by oligolabeling

(Feinberg and Vogelstein 1983) by using $\alpha^{32}$dCTP (ICN, 3,000 Ci/mmol). Hybridization and autoradiography were as described by Hepburn *et al.* (1985). To quantitate the hybridization signals, autoradiographs were scanned on a Beckman DU-8B spectrophotometer at 600 nm and the areas under the signal peaks determined. Autoradiographs were selected for scanning in which the hybridization signals were on the linear part of the emulsion signal response curve. In all cases, amounts of T-strands or nicked borders were corrected for variations in DNA load resulting from variations in sample concentration.

***Agrobacterium* growth and acetosyringone induction.** Starter cultures were grown with shaking overnight in the complete Difco medium, as described by Hepburn *et al.* (1985) containing 200 $\mu$g/ml of carbenicillin at 28° C. This is required to ensure maintenance of pCW61 as a cointegrate. In the absence of the antibiotic, decointegration and subsequent loss of pCW61 occurs in rapidly dividing cells. Induction of *Agrobacterium* cells was done essentially as described by Stachel *et al.* (1986b). Five ml of the starter culture was inoculated into 45 ml of Murashige and Skoog (MS) medium (Murashige and Skoog 1962) modified by the addition of 0.18 g/L of $K_2HPO_4$ (pH adjusted to 5.5 with HCl), filter-sterilized biotin to a final concentration of 1 mg/L and 200 $\mu$g/ml of carbenicillin, and incubated with shaking at 28° C for 3–5 hr. Unless stated otherwise, acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone; Aldrich) was added in a small volume of water to a final concentration of 100 $\mu$M and DNA extracted 18–24 hr later.

**DNA extraction and purification.** Plasmids were isolated from *E. coli* by the alkaline lysis technique of Birnboim and Doly (1979). *Agrobacterium* DNA was isolated as described by Stachel *et al.* (1986b).

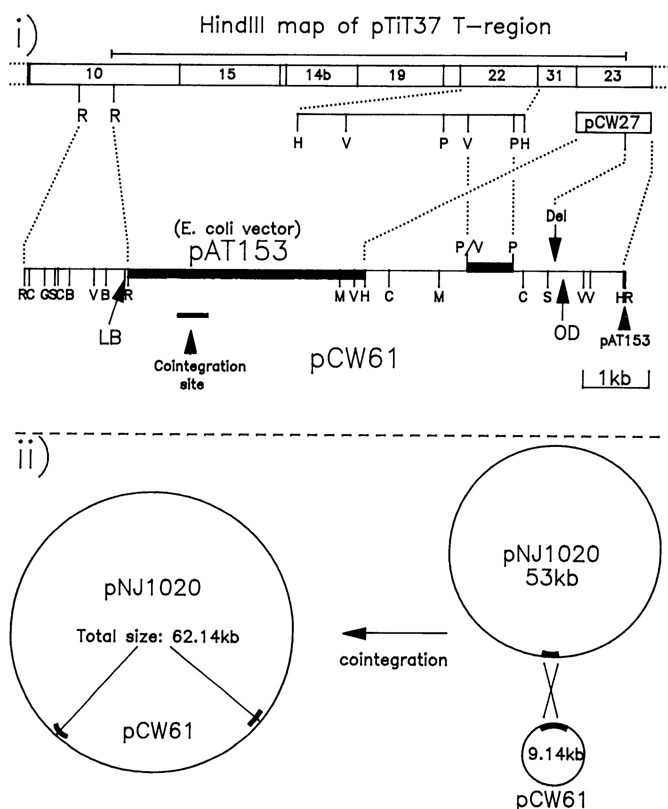



**Fig. 1.** Map of the binary vector pCW61. **i**, The fragments cloned into pAT153 to generate pCW61 were derived from the T-region of the *Agrobacterium tumefaciens* Ti-plasmid pTiT37, as shown. The numbering of the *Hin*dIII fragments of the T-region are given as the equivalent *Hin*dIII fragment numbers of pTiC58, determined by Depicker *et al.* (1980). The 0.69-kb segment of DNA derived from within the T-region (*Hin*dIII fragment 22) is not relevant to this work and will be discussed elsewhere. pCW27 was constructed as a deletion derivative of pCW11 (containing *Hin*dIII fragment 23) by the removal of 102 bases (marked Del) containing the right border repeat of the T-region (Hepburn *et al.* 1983; Hepburn and White 1985). The putative overdrive sequence of pTiT37 (marked OD; Peralta *et al.* 1986) is still present in this clone. The left border repeat of pTiT37 (LB) is contained within the 1.5-kb *Eco*RI fragment of pTiT37 *Hin*dIII fragment 10. **ii**, The scheme for cointegration of pCW61 into pNJ1020 is shown. The site of cointegration is between the portions of the $\beta$-lactamase gene (468 base pairs of homology) present in both pCW61 and pNJ1020. R = *Eco*RI; C = *Cla*I; G = *Bgl*II; S = *Sac*II; B = *Bcl*I; V = *Eco*RV; T = *Pst*I, M = *Bam*HI; P = *Pvu*II; H = *Hin*dIII.

## RESULTS

Figure 1 shows the structure of pCW61, the plasmid used in these experiments, and indicates the source of the various components derived from the T-region of pTiT37, the Ti-plasmid of *A. tumefaciens* strain T37. The *E. coli* vector was pAT153 (Twigg and Sheratt 1980). Into this vector was cloned a 1.5 kb *Eco*RI fragment containing the left border sequence of pTiT37 and a 3.23-kb *Hin*dIII fragment containing the presumed overdrive sequence of pTiT37 (Peralta *et al.* 1986) but lacking the right border sequence (through a 102-bp deletion; Fig. 1: Del), which lies approximately 70 bases to the left of the overdrive sequence (Hepburn and White 1985; Culianez-Macia and Hepburn 1988). The addition of 0.69 kb of DNA from within the T-region of pTiT37 is not relevant to this work and will be discussed elsewhere.

A cointegrate was formed between pCW61 and pNJ1020 in *E. coli* and was transferred to C58-Z707 by conjugation (Grinter 1983; Hepburn *et al.* 1985). Cointegration takes place by homologous DNA-directed, RecA-independent recombination between pNJ1020 and the vector component (pAT153) of pCW61. The site of recombination is within a 468-base pair sequence derived from the $\beta$-lactamase gene, part of which is common to both plasmids (Hepburn *et al.* 1985). The results on the specificity of border nicking and T-strand production that we have obtained by using this helper-binary cointegrate combination have been described in detail elsewhere (Culianez-Macia and Hepburn 1988). In summary, when C58-Z707 containing the binary cointegrate pCW61::pNJ1020 is incubated in plant tissue culture medium containing 100 $\mu$M acetosyringone, the left border sequence is nicked and a single-stranded copy of the whole of pCW61::pNJ1020 is produced. No double-stranded cleavage of the border is seen.

Figure 2 shows the kinetics of T-strand production over an 8-day period following induction with acetosyringone. The inducer was added after the culture had been growing for 4 hr, and samples were taken at the times indicated in Figure 2. T-strands can be seen as early as 6 hr and peak in concentration at 18–24 hr after induction. Even 8 days (192 hr) after induction, a small amount of T-strands can be seen. These kinetics are the same over a range of acetosyringone concentrations. The concentration used in the data presented here (100 $\mu$M) is that commonly used for *Agrobacterium* induction (Stachel *et al.* 1986a).

To determine how closely the production of T-strands correlates with nicking of the left border (which in turn has

been proposed as the first step in the process), the appearance of nicks was assayed in the same samples by digestion of total DNA with S1 nuclease as described by Stachel *et al.* (1986b). Treatment of nicked DNA with S1 nuclease converts the nicks to double-stranded breaks that can be seen as the appearance of two new fragments. In the data shown, this results in the generation of two fragments of 0.44 and 0.07 kb from the 0.51-kb *Eco*RI-*Eco*RV fragment that includes the border sequence, although only the 0.44-kb fragment can be seen on the gel. We have shown previously that this nicking occurs at the border sequence and that no double-strand cutting is seen in any of the induction conditions that we have used (Culianez-Macia and Hepburn 1988). As can be seen in Figure 3, the appearance of nicked molecules follows essentially the same kinetics as the production of T-strands. After 8 days (192 hr), the fraction of nicked molecules has dropped to 40% of the maximum value seen at 24 hr. This compares with only 5% of the maximum amount of T-strands still detectable at 192 hr. The probe used in this case was pCW61 itself.

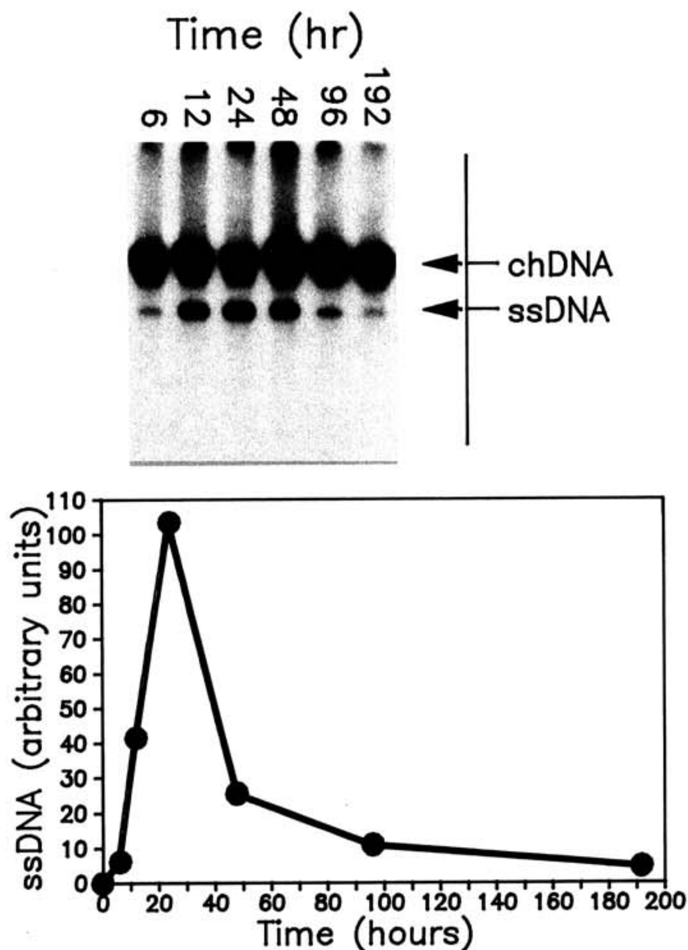



**Fig. 2.** Long-term kinetics of T-strand accumulation. Total DNA was extracted from cells sampled at various times after the addition of acetosyringone and fractionated on a 0.8% agarose gel. The gel was transferred to Genescreen and probed with $^{32}$P-labeled pCW61. The amount of T-strands (ssDNA) in each sample (given by arbitrary units) was determined by scanning autoradiographs of the hybridized filter and correcting for variations in DNA loadings. chDNA indicates the position of chromosomal DNA. The signal at this region results from hybridization of the probe (pCW61) to the linear form of the binary/cointegrate of approximately 62 kb that comigrates with the chromosomal DNA.

Comparable analyses were done on the same samples by using pNJ1020 as the probe (data not shown). A comparison of the data showed that decointegration and loss of the pCW61 component of the binary at longer culture periods was not responsible for any of the changes observed in the amount of T-strands or nicked border sequences. This was confirmed by the observation that the signal intensity of the 1.04-kb fragment (which derives from the pCW61 component of the binary cointegrate) in Figure 3 matches the sample loadings throughout the experiment.

These kinetics imply that effective induction is of limited duration and is followed by a loss of both T-strands and nicked borders but does not indicate the cause of this limitation. Loss or degradation of acetosyringone during long incubations could explain the observations as well as the possibility that the cells were no longer capable of being induced after a finite period of incubation in the medium. We therefore first tested whether there was any active acetosyringone still present after 8 days of incubation in the presence of the bacteria (Fig. 4). As reference controls, tracks 1 and 2 show the amount of T-strands after 18-hr and 192-hr incubation in acetosyringone, respectively. Eight-day-old cultures incubated with and without acetosyringone were filtered through 0.22 $\mu$M filters to remove the bacteria, and a fresh inoculum of bacteria was added. Fresh acetosyringone was then added to the medium lacking the inducer, and the bacteria were harvested after 18 hr. Figure 4, tracks 3 and 4, shows the amount of T-strands produced in the presence of preincubated and fresh acetosyringone, respectively. T-strands were present in the cells from both cultures, although the amount induced by the preincubated acetosyringone was less than that induced by the fresh acetosyringone, suggesting that a portion of the inducer had either been degraded or absorbed by the bacteria over the 8 days of preincubation. The apparent slight loss of inducer during prolonged incubation depends on exposure to bacteria because incubation of acetosyringone for 8 days in MS medium in the absence of the bacteria did not result in a noticeable loss of its capacity to induce the production of T-strands (Fig. 4, track 6). These data indicate that the ability to induce T-strand production after long incubation periods reflects an alteration in the bacteria rather than significant degradation or uptake of the inducer. This is confirmed by the observation that adding fresh acetosyringone to a noninduced 8-day-old culture did not induce any T-strand production (Figure 4, track 5). These data also indicate that the presence of T-strands at prolonged incubation times results from the persistence of molecules produced earlier in the incubation period. The same conclusion can be drawn about the persistence of nicked borders. The data also suggest that the cells are only competent to be induced for a limited time in culture.

Because these conclusions conflict with the observations of Stachel *et al.* (1986a), who showed that addition of extra inducer in the form of conditioned plant medium led to further induction of the *vir* gene used to assay induction, we tested the possibility that the crucial component of conditioned plant medium was the medium itself rather than the inducer it contained.

A 50-ml culture was initiated by the standard method (5 ml of an overnight starter culture in complete Difco medium was added to 45 ml of MS medium). After incubation for 24 hr, acetosyringone was added to 100 $\mu$M. Ten ml was

**Fig. 3.** Long-term kinetics of left border repeat nicking. The DNA samples from Figure 2 were digested with S1-nuclease and the restriction endonucleases *Eco*RI and *Eco*RV fractionated on a 1.8% agarose gel and transferred to Genescreen. The filter was probed with the *Eco*RI fragment from pCW61, which contains the border. This fragment and the expected products of digestion are shown in **ii**. The autoradiograph of the samples is shown in **i**. Autoradiographs of the gel were scanned and the amount of nicking of the border determined relative to the total amount of border fragment present in each sample. The quantitative data, after correction for variations in DNA loadings, are shown in **iii**.

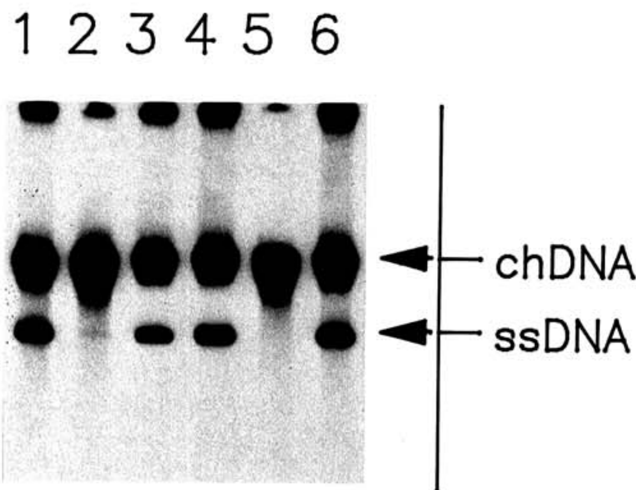



**Fig. 4.** Effect of acetosyringone age on induction. The lanes show the amount of T-strand (ssDNA) accumulation in cultures of bacteria containing acetosyringone treated in various ways: 1, 18-hr incubation (fresh cells) in acetosyringone (added 4 hr after inoculation); 2, 192-hr incubation (fresh cells) in acetosyringone; 3, Fresh cells incubated for 18 hr in the filter-sterilized medium from 2; 4, 18-hr incubation (fresh cells) with fresh acetosyringone (added at the time of inoculation); 5, 192-hr-old cells incubated (18 hr) with fresh acetosyringone; 6, 18-hr incubation (fresh cells) in 192-hr-old medium (no bacteria) containing acetosyringone. chDNA shows the location of chromosomal DNA. The hybridization signal to this location is explained in the legend to Figure 2.

removed at this time and diluted with 40 ml of fresh medium containing acetosyringone. After an additional 24 hr, a further 10 ml was removed from the original culture (then 48 hr old), diluted with 40 ml of fresh medium containing acetosyringone, and incubated for an additional 24 hr. DNA was extracted from the three cultures and assayed for the presence of T-strands. The experimental scheme and the results of the DNA assays are presented in Figure 5. There is no accumulation of T-strands in the 24 hr after adding acetosyringone to the 24-hr-old culture. Both diluted samples, however, show significant accumulation of T-strands in the presence of the inducer. Thus, T-strand production only occurs when the culture is diluted and does not correlate with the addition of inducer. We interpret these data as indicating that an effect of adding extra inducer can only be seen when the addition also involves diluting the bacteria with additional culture medium, as would be the case with adding conditioned plant medium as was done by Stachel *et al.* (1986a), but not with adding concentrated acetosyringone as we do in our standard protocols. The amount of T-strand production is significantly less than that observed when acetosyringone is added to a primary culture (cf. Fig. 2). This results from the limited growth that takes place following dilution of an MS culture into fresh MS. Considerably more growth takes place when a starter culture in complete Difco medium is

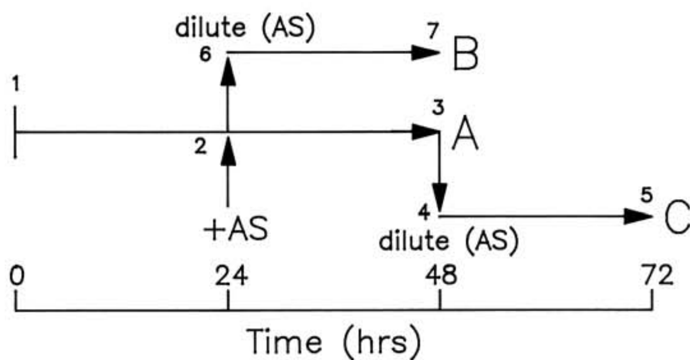
diluted into MS medium. This appears to result from the addition of nutrients in the complete Difco medium that are lacking in MS medium.

The linking of a second round of induction with the addition of fresh medium, coupled with our time course observations, suggests that induction may be related to the state of the cells. We therefore attempted to follow growth of the bacterial cultures both by determining the $A_{600}$ and by plating out diluted aliquots of the cultures during various induction experiments. Only the absorbance measurements gave useful data, because C58-Z707 is prone to clumping during the later stages of growth, which makes cell counting by plating relatively imprecise. From a comparison of several techniques, including DNA estimations, measurement of the absorbance at 600 nm with increasing time gave the most reliable, nondestructive, rapid estimate of the growth of the cells under different conditions. Figure 6 shows the absorbance data obtained for representative control and induction experiments. As can be seen, a control, untreated culture approaches stationary phase 18–20 hr after inoculation. Longer incubation (Fig. 6a) leads to as much as a 25% reduction in absorbance, as the cells clump and lyse to a certain extent. The lysis results from the presence of a lysogenic prophage in strain C58 that can become unstable when cultures are overgrown.

Addition of acetosyringone 5 hr after inoculation has only a slight effect on culture growth. The peak of nicking and T-strand production activities in such a culture would occur around hour 29, some time after the culture had reached stationary phase (cf. Figs. 2 and 3). If, however, the acetosyringone is present from the moment of inoculation, the growth of the culture is severely inhibited. In part, this may be due to slightly increased clumping in the presence of acetosyringone, but it also reflects a real inhibition in growth caused by the inducer. The data from the dilution experiment (see Fig. 5 for absorbance values at each stage) confirms that the appearance of T-strands following dilution is coupled with further growth and division of the cells and that incubation in acetosyringone significantly inhibits cell division. These data suggest that, although the cells may continue to synthesize T-strands for some considerable time after induction, they are only competent to be induced in the early stages of culture growth.

We confirmed this observation by adding acetosyringone to cultures at 2-hr intervals after inoculation and harvesting the cells for DNA extraction after an additional 18 hr. The data for T-strand and nick production during this experiment is presented graphically in Figure 7 and shows that the level of induction drops rapidly over the first 10 hr of culture growth. By this time, when the cells in the culture were in mid-log phase, only 2–3% of the initial induction level could be achieved. We therefore conclude that the capacity for induction is restricted to actively growing cells, but that once induced, the nicking and T-strand production systems continue operating for some considerable time. Although the ability to be induced drops off rapidly as the cultures approach stationary phase, the kinetics of induction do not alter significantly in that the peak of T-strand production occurs the same relative time after acetosyringone addition.

In these experiments, we used 100 $\mu$M acetosyringone to




| Sample | A600 |
|---|---|
| 1 | 0.11 |
| 2 | 0.98 |
| 3 | 1.02 |
| 4 | 0.25 |
| 5 | 0.38 |
| 6 | 0.22 |
| 7 | 0.28 |

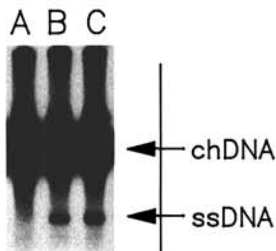



**Fig. 5.** Effect of culture inoculation and growth on induction. The flow diagram shows the culture times and the additions of either concentrated acetosyringone (AS) to a final concentration of 100 $\mu$M or fresh medium containing 100 $\mu$M acetosyringone. The culture was initiated by the addition of 5 ml of an overnight starter culture in complete Difco medium to 45 ml of MS medium. The dilutions involved adding 10 ml of the MS bacterial culture to 40 ml of fresh MS medium containing 100 $\mu$M acetosyringone. The numbers indicate when the $A_{600}$ values listed in the table were obtained. The times at which DNA samples were extracted for analysis are given by A, B, and C, and the results showing the T-strand accumulation are presented in the autoradiograph. Carbenicillin (200 $\mu$M) was present in all media. Total DNA was isolated from the cultures A, B, and C, fractionated on a 0.08% agarose gel and transferred to Genescreen. The blots were probed with $^{32}$P-labeled pCW61 to detect the amount of T-strands present.

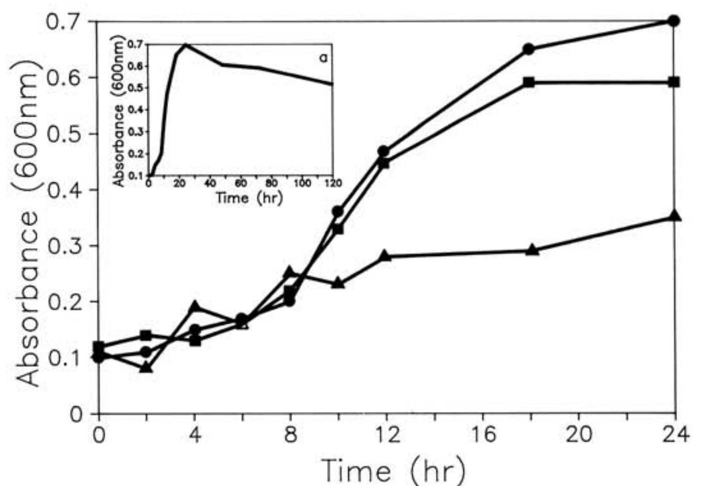



**Fig. 6.** Effect of acetosyringone on cell growth. The $A_{600}$ values were determined at various times after growth in the presence or absence of acetosyringone over a 24-hr period. ● = no acetosyringone; ■ = acetosyringone added after 5 hr; ▲ = acetosyringone added at time of inoculation. The insert (a) shows the long-term kinetics of growth determined from $A_{600}$ measurements.

induce the cells. This is the optimum concentration as described by Stachel *et al.* (1986a) for induction of *vir-lac* fusions. Because this concentration was determined by following the expression of specific *vir* genes rather than T-strand production, we determined the optimum concentration for our system by using both T-strand accumulation and border nicking to assay the effect of increasing concentrations of the inducer on the accumulation of the natural biological products of the system rather than on individual genes. Four-hr-old cultures were incubated in the presence of acetosyringone concentrations from 0.5 $\mu$M to 1,000 $\mu$M and assayed for T-strand production after a further 18 hr. The results are shown in Figure 8. The production of T-strands follows a biphasic curve and is still increasing at 1,000 $\mu$M (1 mM). The break point between the two slopes occurs at approximately 200 $\mu$M (Fig. 8a). In contrast, the frequency of border nicks shows an increase to between 200 $\mu$M and 500 $\mu$M and thereafter plateaus or decreases slightly (Fig. 8b). At the plateau, approximately 17% of the left borders show nicking in this experiment. Once again, although the absolute amounts of T-strand production and nicking vary from experiment to experiment, the kinetics (and relative amounts of the two parameters measured) are reproducible.

From these data it is clear that maximum induction of nicking occurs at an acetosyringone concentration slightly in excess of 200 $\mu$M. Even when nicking is maximum, however, the production of T-strands from the nicked substrate can be induced to even greater levels by higher acetosyringone concentrations.

## DISCUSSION

We have shown that the production of T-strands from an *Agrobacterium* border sequence in response to acetosyringone induction is clearly correlated with the presence of single-stranded nicks in the double helix at the border. Both processes peak in activity around 24 hr after exposure to acetosyringone. The time course of *virD* induction determined by Stachel *et al.* (1986a) shows that the expression of this specific *vir* gene reaches a maximum within 10 hr after inoculating the bacteria into induction medium. Because our data shows that the bacteria are only competent to be induced while they are actively growing, which, in MS medium, extends to mid-log phase (the first 8–10hr following inoculation), the continued accumulation of nicks at the border and T-strands beyond this time is likely to result from continuing activity of the *vir* gene products rather than additional gene expression. This, coupled with the observation that acetosyringone appears to inhibit cell division within a proportion of the cells, suggests that, once induced, these cells undergo a differentiation such that they no longer divide but enter a T-strand production phase. We have not as yet, however, been able to demonstrate that those cells whose growth is inhibited in the presence of acetosyringone are also those cells that are producing T-strands. Dilution of an induced or uninduced stationary phase culture results in renewed growth of some of the cells in the culture and consequently generates a further window of competence to induction. This interpretation contrasts with that of Stachel *et al.* (1986a), who concluded from direct assays of *virD* induction that the effect of dilution with additional conditioned medium was to effectively increase the inducer concentration. Because *virD*, the gene assayed by Stachel *et al.* (1986a), is known to encode the endonuclease responsible for nicking the border

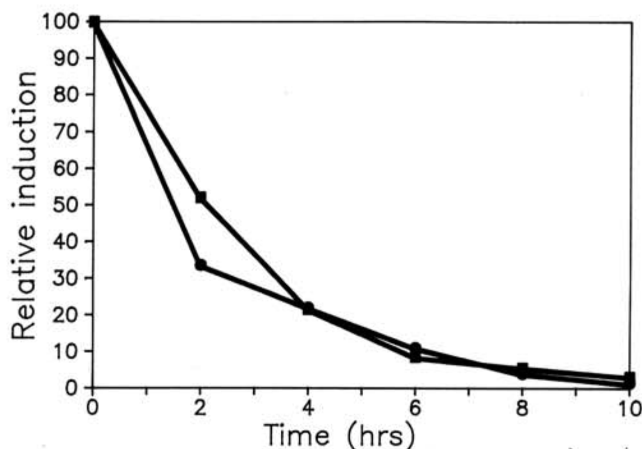
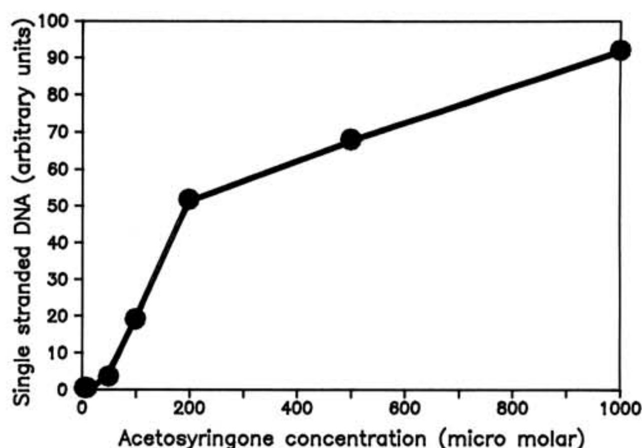



**Fig. 7.** Short-time course of acetosyringone induction. Acetosyringone was added to growing cells at the times indicated by the data points and total DNA extracted 18 hr later. Detection and quantitation of T-strands and nicked borders was as described in Figures 2 and 3. ● = T-strand accumulation; ■ = border nicking.



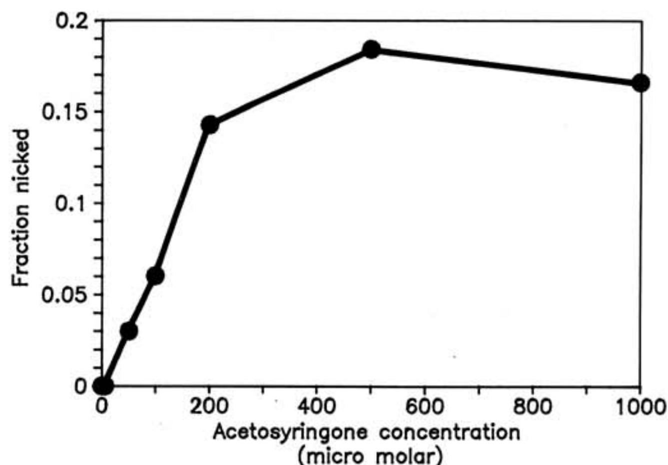




**Fig. 8.** Effect of acetosyringone concentration on induction. Cells were incubated for 18 hr in the indicated concentrations of acetosyringone added 4 hr after inoculation. Assay for and quantitation of T-strands (**a**) and nicked borders (**b**) was as described in Figures 2 and 3.

repeats (Yanofsky *et al.* 1986), the two assay systems can be directly related.

The growth of bacteria in MS medium is not as extensive as in our complete Difco growth medium (Hepburn *et al.* 1985), yet we observe some growth even in MS medium recovered from a stationary phase culture (Fig. 5). The absorbance data presented in Figure 5 also suggest that the initial growth in MS medium results, to a large extent, from carry-over of nutrients in the complete Difco medium added. When the MS culture is diluted into fresh MS as in the later stages of the experiment presented in Figure 5, growth is limited. Because we have shown that T-strand production is dependent on the growth of the cells, it is probable that higher levels of T-strand production may be obtained in media that allow more growth.

We have been unable to confirm the effect of pH on induction (Stachel *et al.* 1986a). In our system (MS containing potassium phosphate), the buffering is inadequate because the addition of a fresh inoculum to the MS medium raises the pH to 6.0–6.2. During incubation, the pH drops to 4.4 over the first 24 hr. By 10 hr, when the ability to be induced has been largely lost, the pH is 5.5–5.6. By the time the relative induction has dropped to 20% (Fig. 6b; 4 hr), the pH is 5.9. Because we see high levels of induction even when the pH of the medium is 6.0 or higher, we therefore conclude that in our system pH over the range 4.0–6.0 has little or no effect on T-strand production.

The effect of acetosyringone concentration suggests that the first limitation to the production of T-strands is the availability of nicked borders required to prime T-strand synthesis. This, in our system, was not saturated until the acetosyringone concentration had reached at least 200 $\mu$M. This does not, however, accurately reflect the natural system because the *vir* genes and the border are carried on separate plasmids. Because the binary/cointegrate origin of replication derives from RP4, the copy number is likely to be two to five times that of the Ti-plasmid (pTiC58-Z707) carrying the *vir* region. Probing the blots with Ti-plasmid derived sequences present only in pTiC58-Z707 showed that the relative concentration of the Ti-plasmid and the binary/cointegrate remains constant during the induction (data not shown), and hence both the kinetics and acetosyringone concentration curve data truly represent the interaction between *vir* gene products and T-region borders.

At acetosyringone concentrations in excess of 500 $\mu$M, all the available borders are apparently nicked. Residual intact borders are likely to be derived from cells that have not been induced. That the amount of T-stand production continues increasing at acetosyringone concentrations greater than that required to effect maximum border nicking suggests that nicking is not the only acetosyringone-inducible component of the T-strand production system.

Stachel and Zambryski (1986) have proposed that T-strands are produced by a mechanism analogous to conjugal transfer of plasmids (Willetts and Wilkins 1984). In such a model, T-strands would be released by displacement as a new strand is synthesized by using the 3′ end of the nicked border as a primer. An inevitable consequence of such synthesis would be repair of the nick at the border. The time of maximal T-strand production should therefore not be near the time of maximum nicking. That the two approximately coincide in our system suggests that nicking of the border is a continuous process. Because the frequency of nicked borders does in fact decrease at longer incubation times, it seems likely that multiple rounds of T-strand displacement also take place.

The data we have presented here show that the production of T-strands in *Agrobacterium* can only be induced in actively growing cells and that the process of induction leads to a partial reduction in cell division. This can, in part, explain the variability in induction efficiency with different media because cell viability will vary depending on the medium composition. Because in natural infections, induction would be coupled with binding to plant cells, it is not surprising that a consequence of induction is the cessation of cell division. It is not clear from our data, however, whether the inhibition of division results directly from the action of the inducer on the *vir* genes or whether chromosomal functions are also affected directly.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