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On the isolation of TI-plasmid from Agrobacterium tumefaciens.

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ABSTRACT

An efficient lysis method for *Agrobacterium* cells was developed, which allows a reproducible isolation of the tumor inducing (TI)-plasmid. The lysis method is based on the sensitivity of this bacterium to incubation with lysozyme, n-dodecylamine,EDTA, followed by Sarkosyl, after growth in the presence of carbenicillin.

We also present a procedure for the isolation of the TI-plasmid on a large scale, that might be used for the mass isolation of other large plasmids which like the TI-plasmid, can not be cleared with earlier described procedures.

The purity of the plasmid preparations was determined with DNA renaturation kinetics, which method has the advantage that the plasmid need not to be in the supercoiled or open circular form.

INTRODUCTION

When virulent *Agrobacterium tumefaciens* cells are inoculated into wounded dicotyledonous plants, crown galls arise. They have a tumorous character as a result of permanent alterations in the cell, leading to non-selflimmiting growth also in the absence of the inciting bacterium. Recently it has been demonstrated that the tumour inducing capacity of this bacterium is determined by a large plasmid (1, 2, 3). For further studies it is essential to develop a lysis procedure allowing a reproducible and large scale isolation of this plasmid.

Agrobacterium cells are not sensitive to the lysozyme-EDTA-detergent lysis procedures described by Marmur and others (4, 5). A relatively long treatment with proteolytic enzymes at elevated temperatures is needed (1, 6). This might result in loss of plasmid as suggested (7) and often lysis is not complete, as indicated by not entirely clear lysates. Even when lysis seems to be complete, flocky material is found, sedimenting in sucrose gradients or floating in CsCl-dye buoyant density gradients, mostly between the chromosomal and the TI-plasmid band. Because of this, TI-plasmid can not be isolated free from chromosomal DNA. Also procedures for the isolation of the plasmid on a large scale are seriously hampered by incomplete lysis.

Therefore we worked out a new lysis procedure, based on an enrichment procedure for *Agrobacterium* mutants presented by Klapwijk *et al* (8). A large scale isolation procedure for the high molecular weight TI-plasmid will be described.

MATERIALS AND METHODS

Bacterial strains. The strains used in the experiments are listed in table I. Bacteria were cultured at 29°C, in a New Brunswick G 24 environmental incubator shaker at 250 rpm.

Media. The minimal medium of Vogel and Bonner (9) was used, supplemented with 50 mg/l $FeSO_4.7H_2O$, 50 mg/l $MnSO_4.H_2O$, 1% glucose and 0.1% yeast extract (Difco). In this medium, the generation time for most *Agrobacterium* strains is about 1 h, resulting in a high labeling efficiency of DNA.

Buffers. The buffers used were :

TES buffer: 0,01 M Tris-HCl(pH 8,0), 0,05 M NaCl and 0,05 M EDTA. This buffer was autoclaved before use.

Lysozyme buffer : 400 µg/ml lysozyme(Egg-white,grade I,Sigma U.S.A.) in TES-buffer, saturated with n-dodecylamine (10) (Merck-Schuchardt).

Lysing mixture : 3% Sarkosyl NL 97 (Geigy,Switzerland), 1N NaOH in TES buffer in the case of alkaline lysis (11) and 10% Sarkosyl in TES-buffer in the case of neutral lysis.

Na-Phosphate buffers(NaPB): equimolar mixtures of Na_2HPO_4 and NaH_2PO_4(pH 6,8).

Growth and labeling conditions. Cells were grown overnight and then used to inoculate fresh medium to a density of 2-4x10^8 cells/ml. ^{3}H-Thymidine (the Radiochemical Centre, Amersham; 30 C/mM) was added very slowly continuously during growth, by means of a peristaltic pump, to a final concentration of 10 - 20 µC/ml for low labeling experiments and 50 - 500 µC/ml for high labeling experiments. Bacteria were grown for about 4 h to a density of 3x10^9 cells/ml. With this procedure specific activities of 4x10^6 cpm/µg DNA could be obtained. However, this is strongly dependent upon the strain used. Subsequently 500 µg/ml (or more when indicated) carbenicillin (Pyopen,Beecham S.A.Belgium) was added to the culture and growth prolonged for 1½ h, while shaking was reduced to 150 rpm. Cells were harvested by centrifugation and lysed according to one of the following procedures.

Lysate preparation.

a. Alkaline lysis. Cells grown in the presence of carbenicillin were rigorously resuspended in 0,35 times the original culture volume TES buffer, by means of a disposable syringe. 0.35 volume lysozyme buffer was added and

the mixture was incubated for 0.5 h on ice, while unnecessary shaking was prevented (11). At the end of the incubation period 0.3 volume alkaline lysing mixture was added while the suspension remained on ice. A clear lysate was obtained within a few minutes.

b. Neutral lysis. Cells, grown in the presence of carbenicillin were resuspended in 0.45 times the original culture volume TES buffer, by means of a disposable syringe and 0.45 volume lysozyme buffer was added subsequently. The mixture was incubated under conditions as described for the alkaline lysis. At the end of the incubation period, 0.1 - 0.2% diethyl pyrocarbonate (DEP) was added to inhibit nuclease activity (12), followed by 0.1 volume neutral lysing mixture. Also with this procedure a clear lysate was obtained within a few minutes.

Gradients.

a. Alkaline sucrose gradients. The alkaline lysate (1-5 ml) was sheared for 2 min. in a testtube (1.6 cm diameter) on a Vortex mixer at maximum speed (Vortex-Genie,Wilten,Holland). 1 ml was layered on a 5-20% linear sucrose gradient containing 1 M NaCl and 0.3 N NaOH in TES buffer. Autoclaved sucrose stock solutions were used. Gradients were centrifuged for 25 min. at 4°C and 40,000 rpm, using a SW41 rotor in a Beckman Spinco LIIB preparative ultracentrifuge. Alternatively 3 ml sheared lysate was layered on top of a 30 ml 5-20% gradient and centrifuged for 80 min. at 4°C and 25,000 rpm in a SW27 rotor. Fractions of 250 µl were collected after puncturing the bottom of the tube. Dependent upon the specific activity 20 - 100 µl were spotted on Whatmann 3 MM paperstrips and dried. The DNA was precipitated by incubation of the strips in cold 5% TCA for 15 min. after which the strips were rinsed with cold 2% TCA followed by a rinse with cold ethanol(-20°C). The strips were dried at 80°C and the radioactivity determined in toluene - 0.4% PPO in a liquid scintillation analyser.

b. Neutral sucrose gradients. The neutral lysate was sheared as described for the alkaline lysate and 1 ml was applied on a 5-20% linear sucrose gradient in TES buffer. The gradient was centrifuged for 45 min. at 4°C and 40,000 rpm in a SW41 rotor. Alternatively 3ml lysate was centrifuged on a SW27 gradient for 135 min. at 4°C and 25,000 rpm. Fractionation and counting were performed as in a.

c. Dye buoyant density equilibrium centrifugation. This was performed according to Radloff *et al.*(13) and Hudson *et al.*(14). Centrifugation was carried out for 50-70 h in either a fixed angle rotor 50 Ti or 60 Ti at 20° C

and 38,000 rpm. The neutral lysate was sheared for 15-30 sec. with a vibromixer (A.G.für Chemie Aparatenbau, Switzerland) at maximum speed. Volumes up to 100 ml could effectively be sheared. Ethidium bromide (puriss.Serva, Germany) or Propidium di-iodide (A grade, Calbiochem, Switzerland) was added to a final concentration of 400 µg/ml and the density was adjusted to 1.56 g/ml (0.94 g CsCl per ml lysate). Fractionation and radioactivity measurements were performed as in a. The DNA was freed from dye by dialysis against Dowex-50 (15) (type WX2, Serva).

Large scale isolation after alkaline lysis. The alkaline lysate was sheared for 2 min. with a vibromixer at maximum speed. 30 ml of the sheared lysate was poured into a SW27 centrifuge tube and a 3 ml 20% alkaline sucrose cushion, without 1 M NaCl, was pumped underneath the lysate. The tube was centrifuged for 2 h at 4°C and 25,000 rpm. The first 30 ml. freed from plasmid were taken out. The 3 ml sucrose cushion, together with the resuspended pellet, containing the plasmid, were layered upon a 10-20% alkaline sucrose gradient containing 1 M NaCl. This gradient was centrifuged, fractionated and counted as described.

Large scale isolation after neutral lysis. The neutral lysate was sheared for 15-30 sec. with a vibromixer at maximum speed. 25 ml of the sheared lysate was poured into a SW27 centrifuge tube and 5 ml 10% sucrose and 2.5 ml CsCl with a density of 1.75 g/ml were successively pumped underneath the lysate. The tube was centrifuged for 270 min. at 4°C and 25,000 rpm. The first 27.5 ml were taken out and the remaining 5 ml from each of the gradients - containing most of the plasmid, but also much chromosomal DNA - were pooled. The density was adjusted to 1.50 g/ml CsCl and propidium di-iodide was added to a final concentration of 400 µg/ml. Centrifugation was carried out as described. The gradient still contained much chromosomal DNA, visible as a fluorescent band on illumination with long wave UV-light. This band was generally first removed by aspiration and thereafter the gradient was punctured at the bottom and fractionated and radioactivity determined as described.

Determination of DNA renaturation kinetics. The DNA solutions were dialysed extensively against distilled water. Samples of 2-6 ml were bubbled through with N_2 for 5 min. and sonicated for 15-20 sec. at 20 Kc/sec. with an amplitude of 8 µm using a MSE-Mullard ultrasonic desintegrator. Subsequently they were evaporated to dryness in small conical centrifuge tubes in a vacuum oven at 30-40°C.The dry DNA was dissolved in 0.12 M NaPB to the desired concentration by placing the tubes at 40°C for 15 min. The DNA solution was centrifuged for 5 min. at 2,000 rpm and 1-50 µl samples were put into capilla-

ries (Micropipettes, Brand, Germany), which were sealed by melting both ends. The sealed capillaries were placed for 10 min. in boiling water in order to denature the DNA, and then transferred immediately into a waterbath set at 66°C. Two capillaries were taken at zero time from the boiling water and chilled quickly in ice/salt. For the others, at different times, renaturation was stopped by chilling the capillaries in cold water. The DNA solution was blown out into 1 ml 0.15 M NaPB and the capillary was washed repeatedly. Duplex assay was performed on hydroxyl apatite (Biogel HTP DNA grade, Biorad, U.S.A.) as described by Chilton *et al.*(16) but at 60°C. The kinetics of renaturation were plotted according to the method of Britten and Kohne (17).

Determination of the DNA concentration. DNA concentration was determined by a modified reaction of Burton (18) as described by Richards (19). This procedure gives an A_{600} value of 0.049 ± 0.004 per µg DNA.

Preparation of samples for electron microscopy and contourlength measurements. Samples were prepared and contourlength measured as described earlier (1).

TABLE I : Source and properties of the *Agrobacterium* strains used in this investigation.

Strain	Properties	Origin
A6	virulent	U.S.A. (32)
B6S3	Ω-phage sensitive derivative of strain B6,virulent	Laboratorium voor Genetica, Gent, Belgium (33)
C58	virulent	U.S.A.(34)
C58-C9	plasmid cured derivative of strain C58,non virulent	Laboratorium voor Genetica, Gent, Belgium
C58-RP4	strain C58, containing RP4, virulent	Laboratorium voor Genetica, Gent, Belgium
LBA 601	strain C58-C9, containing the plasmid of strain B6S3, by co-transfer with RP4,virulent	Biochemisch Laboratorium, Leiden, The Netherlands(Klapwijk)
ID135(A0)	virulent, ATCC 27912	U.S.A.(35) This strain and its derivative were kindly provided by Dr.C.I.Kado.
ID135-10a	plasmid cured derivative of strain ID135, ampicillin resistant, non virulent	

RESULTS.

a. The lysis procedure. Several attempts were made to lyse *Agrobacterium* cells without the use of proteolytic enzymes. They are all summarized in table II. Non-reproducible results were obtained with the Freifelder procedure (1, 11). In this procedure neither carbenicellin nor lysozyme were used. Since *Agrobacterium* cells are insensitive to lysozyme, we investigated whether treating the cells in different ways might allow lysozyme to digest the cellwall. Cells were disrupted by grinding with alcoa with a mortar and pestle, as described by Marmur (4). After incubation with lysozyme (200 µg/ml) lysis was not achieved but clear lysates were obtained on subsequent incubation with pronase B for 1 h at 37°C. The lysates did not show flocky material in density gradients. However, the DNA was sheared so vigorously that it was not possible to isolate TI-plasmid without contamination with chromosomal DNA. An other attempt to enhance the working of lysozyme was made, by washing the cells with 0.5 M

TABLE II : The lysis and plasmid isolation procedures tested in this investigation.

Lysis procedure	lysis after addition of Sarkosyl	lysis after incubation with Pronase	Plasmid detected	Remarks
1.The original procedure of Zaenen *et al.*(1)	No	Yes	Yes	Not generally applicable.Flocky material sometimes present.
2.The alkaline procedure of Freifelder (11)	sometimes	Not necessary	sometimes	Frequently bacteria clotted together after addition of alkaline Sarkosyl.
3.Incubation with lysozyme after grinding the cells with alcoa (4)	No	Yes	Yes	Plasmid heavily contaminated with too extensively sheared chromosomal DNA No flocky material
4.Incubation with lysozyme after a wash with 0,5M NaCl and 0,5M sucrose (20)	No	Yes	Yes	Flocky material was present, although less than in procedure 1
5.As 4,but with doubling of the lysozyme concentration	No	Yes	No	Enhancement of the flocky material
6.Growing with 500 µg/ml Carbenicillin followed by an incubation with 200 µg/ml lysozyme,half saturated with n-dodecylamine	Yes	Not necessary	Yes	Always clear lysates without flocky material. Incubation with Pronase did not improve the yield of the plasmid

NaCl followed by 0.5 M sucrose (20). On treatment with lysozyme, EDTA and Sarkosyl the washed bacteria did not lyse. They only lysed after subsequent incubation with pronase B. Although less flocky material was obtained in the gradients, it was still not completely eliminated. A 2-4 times higher concentration of lysozyme only resulted in an increase in flocky precipitates, as could be expected (21). These precipitates did contain considerable amounts of DNA. According to Klapwijk (8) *Agrobacterium* cells become sensitive to lysozyme after growth in the presence of 500 µg/ml carbenicillin. Inspired by this, we came to the procedures as described in materials and methods. We found that, in the case of *Agrobacterium* cells n-dodecylamine considerably enhanced the lysis (10). Clear lysates were always obtained.
Further incubation of neutral lysates with pronase B did not improve plasmid-yield. The *Agrobacterium* cells did not become sensitive enough to lysosyme-EDTA-Sarkosyl treatment after growing with penicillin or ampicillin.

b.The isolation of TI-plasmid. In the former section a neutral and alkaline lysis procedure has been presented (fig.1) which both lead to the reproducible

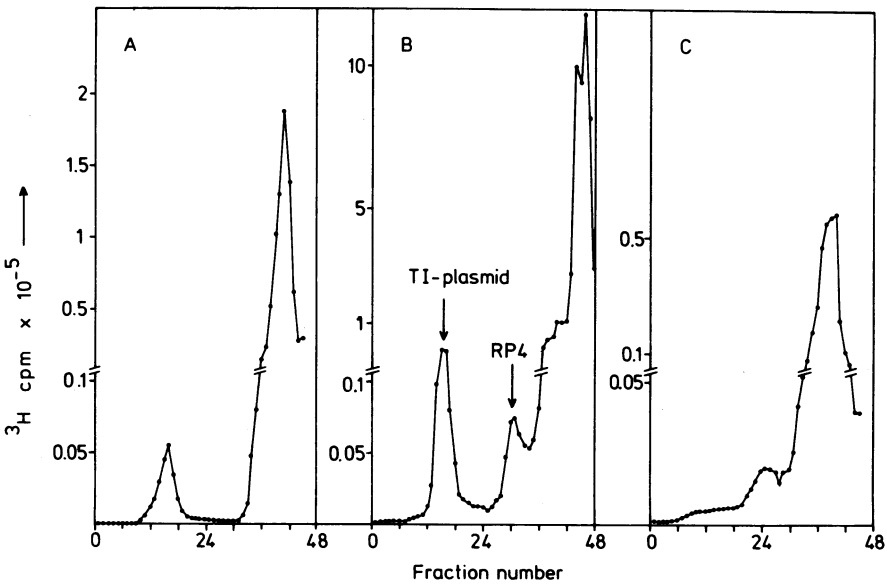

Fig.1 : The separation of TI-plasmid from chromosomal DNA on sucrose gradients. a : The TI-plasmid from strain B6S3 on an alkaline sucrose gradient. b : The TI-plasmid from strain LBA 601 on an alkaline sucrose gradient, resulting also in a separation of RP4. c : The TI-plasmid from strain B6S3 on a neutral sucrose gradient.

isolation of pure plasmid. The alkaline procedure has two advantages. Nuclease activity is inhibited throughout the procedure and the distance between TI-plasmid and chromosomal DNA in the alkaline sucrose gradient is larger than in neutral gradients. As a result, the plasmid preparations are very pure, which is a prerequisite for the DNA renaturation experiments that we wanted to perform. Because of this the alkaline procedure using 1 ml cultures labeled with 0.1 mC/ml ^{3}H-thymidine is preferred to look for the presence of TI-plasmid in *Agrobacterium* strains.

However, the method leads to irreversibly denatured DNA which might be a disadvantage for certain types of experiments. As an alternative the neutral procedure can be used (fig.1c) but than one has to avoid nuclease activity during preparation by adding 0.2% DEP (12). In this case also purification in CsCl-dye buoyant density gradients can be applied (fig.2).We tested these isolation procedures for several strains, including strain A6, which usually lyses very badly (table III).

Also strain LBA 601 was lysed, a strain constructed in this laboratory, which in addition contains RP4, a plasmid that carries among others, resistance against carbenicillin (fig.1b). In this case the concentration of carbenicillin had to be raised to 10 mg/ml. We also tested strains ID135(A0) and ID135-10a about which some controversy exists (1, 35). Also in this case the correlation between tumorinducing capacity and the presence of the TI-plasmid exists.

Table III also shows the percentage of TI-plasmid isolated with these procedures from the different strains. The mean value varies from 0,4 - 1,7%.

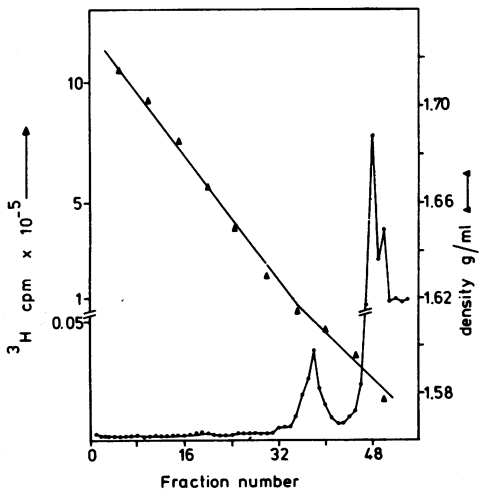

Fig.2 : The separation of the TI-plasmid from strain B6S3 on a CsCl-Propidium-di-iodide buoyant density gradient.

TABLE III : The detection and the percentage of TI-plasmid in different *Agrobacterium* strains

Strain	Isolation procedure	plasmid detected	% with respect to chromosomal DNA (means)
A6	alkaline sucrose	yes	0.61% (4 exp.)
B6S3	alkaline sucrose	yes	1.71% (21 exp.)
	neutral sucrose	yes	1.21% (3 exp.)
	CsCl-dye	yes	0.8% (2 exp.)
C58	alkaline sucrose	yes	1.0% (4 exp.)
C58-C9	alkaline sucrose	no	(2 exp.)
LBA 601	alkaline sucrose	yes + RP4	0.91%, RP4 0.31% (6 exp.)
ID135(AO)	alkaline sucrose	yes	0.4% (2 exp.)
ID135-10a	alkaline sucrose	no	(2 exp.)

Because the molecular weights of the chromosomal DNA and the TI-plasmid DNA are respectively 3.7×10^9(22) and 1.12×10^8 daltons (1) 3.1% plasmid DNA ought to be present if each bacterium does contain one copy per genome, as was shown to be true for strain C58 by Watson *et al.*(23) using renaturation kinetics. We investigated if this loss of TI-plasmid could be prevented by changing the lysozyme concentration , the incubation time and the shear time after lysis. As shown in Table IV, the conditions described in materials and methods

TABLE IV : Determination of the optimal lysozyme incubation time, the optimal lysozyme concentration and the optimal shear time for the isolation of the TI-plasmid from strain B6S3 following the alkaline procedure.

	Lysozyme conc. in µg/ml	Incubation time in min.	Shear time in sec.	% plasmid	Remarks
1	200	30	15	1.0	bottom component
2	200	30	60	0.8	
3	200	30	120	1.1	
4	200	30	180	0.8	
5	200	30	300	0.7	
6	200	10	120	1.5	
7	200	30	120	1.9	
8	no lysozyme	--	120	1.3	bad separation of plasmid from chrom. DNA.
9	600	30	120	1.9	Flocky material in gradients.

Note: As experiment 1 - 5 and 6 - 9 were done at different times, it is only possible to compare the results within one group.

gave the best results. Change in conditions only resulted in minor differences.

c. Large scale isolation of the TI-plasmid. Using one of the lysis procedures, described in materials and methods, attempts were made to isolate the TI-plasmid on a large scale. It was impossible to make use of cleared lysates, neither by the method of Clewell and Helinski (5) using Sarkosyl instead of Brij 58 and Triton X100 - the two latter nonionic detergents did not give any lysis at all - nor by the SDS-1 M NaCl procedure described by Guerry *et al.* (24). Contrary to the TI-plasmid, RP4 could be cleared from chromosomal DNA by the last mentioned method. The plasmid band, obtained after CsCl-ethidium bromide density centrifugation, contained 30 times more RP4 than TI-plasmid, as shown by electron microscope measurement. Since the TI-plasmid might precipitate because of its attachment to the membrane, or by association to the chromosome in which RNA is involved, as shown for the F' plasmid by Kline (25), the following attempts have been made to avoid its loss.

A - The neutral lysate was treated with pronase B.

B - The carbenicillin treated bacteria were grown for 15 min.with resp. 10 and 50 µg/ml rifampicin (Sigma U.S.A.) before harvest.

C - The lysate was treated with 100 µg/ml pancreatic RNase(NBC A grade U.S.A.) and 30 U/ml RNase T1 (Merck,Germany), during incubation with lysozyme.

All these attempts did not result in the enrichment of TI-plasmid by clearing.

Sharp *et al.*(26) have described a procedure for the isolation of large quantities of F'plasmid. The bulk of the chromosomal DNA was removed after controlled denaturation under alkaline conditions (pH 12.5), followed by a quick neutralisation and binding to nitrocellulose. The plasmid ought to remain native. However, in our hands this method did not work for the mass isolation of the TI-plasmid from *A.tumefaciens*. Neither could the plasmid be isolated from alkaline denatured DNA, by hydroxylapatite chromatography (27). We also tried the selective retention of plasmid to nitrocellulose filters by rapid filtration(5 ml/min.) (28) using DNA purified by CsCl density centrifugation. This method too was not successful.

We found that the best way of isolating the TI-plasmid on a large scale is making use of its sedimentation properties, as described in materials and methods. Fig.3 shows the function of the sucrose cushion in the case of the alkaline procedure. The sheared lysate was put into a SW41 centrifuge tube containing a 20% sucrose layer on an alkaline CsCl cushion (1.8g/ml), and centrifuged for 75 min. at 4°C and 40.000 rpm. The chromosomal DNA is separated from the TI-plasmid by the sucrose cushion. Usually no alkaline CsCl cushion is used because it does not allow further purification of the enriched plasmid fraction

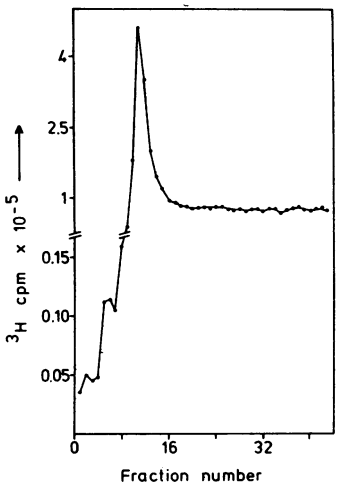

Fig.3 : Sedimentation of linear DNA on a sucrose cushion. 8 ml alkaline B6S3 lysate is layered in a SW41 tube on a 2 ml 20% sucrose cushion whereunder a 1 ml 1.8 g/ml alkaline CsCl cushion.

on alkaline sucrose gradients. With both the alkaline and the neutral procedure it is possible to isolate 100 µg of pure TI-plasmid from 1 l culture with a cell density of 2 x 10^9 cells/ml as tested by renaturation kinetics (section d). This is about 25% of the theoretically obtainable amount if each cell does contain one plasmid.

d. Renaturation kinetics. With the use of the method of DNA renaturation kinetics (17), the degree of purification of the plasmid preparation was determined. The chromosomal DNA will renature 33 times slower as the plasmid DNA, because of difference in molecular weight, resulting in a $C_0t_{\frac{1}{2}}$ that is 33 times lower (Fig.4). According to Britten and Kohne

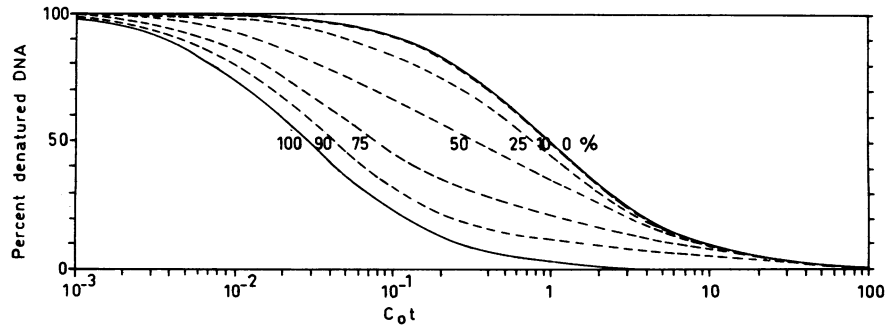

Fig.4 : Theoretical C_0t curves of mixtures of chromosomal and plasmid DNA, with increasing amounts of plasmid DNA. The $C_0t_{\frac{1}{2}}$ value of chromosomal DNA is taken to be 1.

$$\frac{C}{C_0} = \frac{1}{1 + KC_0t} \quad \text{(I)}$$

C_0 = Concentration total DNA
C = Concentration single stranded DNA
K = Rate constant

Mixtures of TI-plasmid and chromosomal DNA will result in a C_0t curve, deviating from the normal second order kinetics, because in this case two DNA's with different complexity are renaturating at the same time. If K of chromosomal DNA is taken to be 1, than K of TI-plasmid has to be 33. Now it is possible to construct theoretical curves of mixtures of chromosomal and TI-plasmid DNA, by making use of the following formula :

$$\frac{C_{plasmid} + C_{chromosomal}}{C_0} = \frac{C}{C_0} \quad \text{(II)}$$

If the plasmid fraction is p, then the chromosomal fraction will be 1 - p. Combination of (I) and (II) gives :

$$\frac{C}{C_0} = \frac{C_{plasmid} + C_{chromosomal}}{C_0} = \frac{p}{1 + 33pC_0t} + \frac{1 - p}{1 + (1-p)C_0t} \quad \text{(III)}$$

Plotting the amount single stranded DNA versus the log C_0t and filling up for several C_0t values, the curve for a special TI-plasmid - chromosomal mixture can be found (fig.4).

The theoretical curves were verified by the renaturation of artificial mixtures of TI-plasmid and chromosomal DNA. All our TI-plasmid preparations proved to be more than 90% pure, as shown for the neutral large scale isolation (fig.5).

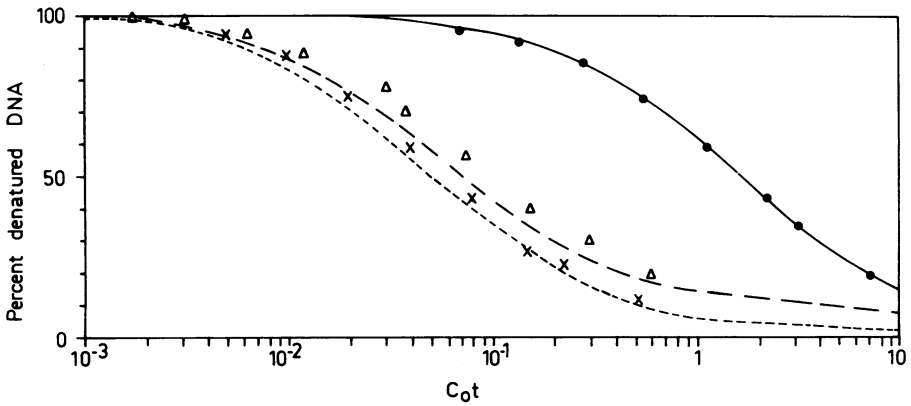

Fig.5 : Control of the purity of the TI-plasmid from strain B6S3, isolated with the neutral mass isolation procedure (Δ) compared to the TI-plasmid(✱) and chromosomal DNA of this strain (●), isolated from an alkaline sucrose gradient. Theoretical C_0t curves of 100% plasmid (---). 90% plasmid (— —) and chromosomal DNA (——) were drawn on basis of chromosomal DNA (●).

DISCUSSION

It is not always possible to isolate pure TI-plasmid from *A.tumefaciens* with the procedure described by Zaenen *et al.*,(1). Frequently no clear lysates are obtained and generally the lysates show flocky material in sucrose and CsCl-dye buoyant density gradients. By this,uncontrolled losses of TI-plasmid occur and large scale isolation of the plasmid is not possible. One of the serious drawbacks of the method described is the need for a quite long proteolytic enzyme treatment before lysis is completed. This allows endogeneous nucleases to be active before becoming inactivated by penetrating proteolytic enzymes. Moreover, a 2 hrs pretreatment at 37°C of the pronase B used for lysis, may not be sufficient to inactivate nuclease activity, giving the risk of exogeneous nuclease activity also during lysis (29).

Agrobacterium usually is not sensitive enough to lysozyme treatment under the conditions described for lysis (8). Sometimes clearance upon addition of detergent is improved by increasing the lysozyme concentration, but this invariably leads to an increase in the mentioned flocky material due to still incomplete digestion of the cell wall constituents which form aggregates in the presence of large amounts of lysozyme (21). Considerable amounts of DNA are present in these aggregates. So, the first requirement was to obtain a more complete cellwall digestion to avoid aggregates and secondly, lysis should be quick using conditions to avoid nuclease activity. None of the known methods for lysis (4, 11, 24) we tried, were successful for *A.tumefaciens*. We found that cells growing in the presence of carbenicillin upon lysozyme treatment on ice, lyse immediately by the addition of detergent. Lysates obtained in this way did not show flocky material in gradients. Nuclease activity in the detergent - lysis step was repressed by working either under alkaline conditions or by the use of DEP (12).

TI-plasmid recoveries varied between 25 - 80%, the higher values generally found for the alkaline procedure. The remainder is supposed to be under the chromosomal peak. Because variations of lysozyme concentration , lysozyme incubation time and shear time were of little influence on the TI-plasmid yields and as the recoveries depend strongly upon the bacterial strain used, it is supposed that the variable recoveries are a result of the growth conditions. Especially during carbenicillin incubation, nucleases activated by damaging the cells, can nick the TI-plasmid. Therefore, incubation must not last very long and the culture must not be shaken vigerously. However, the carbenicillin incubation period cannot be shortened very much, because it has to be continued somewhat more than one generation time.

The effectiveness of the procedure is demonstrated by the isolation of

the TI-plasmid from strains A6 and ID135(A0), which strains gave non reproducible results using other isolation procedures. Even with this effective lysis procedure we were not able to make use of cleared lysates for the large scale isolation of the TI-plasmid (5, 24). It was shown that this was not a result of RNA mediated chromosome attachment (25) or attachment to the membrane. Contrary to the TI-plasmid, RP4 in strain C58-RP4 could be cleared from chromosomal DNA. So we suppose that the TI-plasmid, because of its size is too much intertwisted in chromosomal DNA aggregates to become freed on centrifugation. Also other methods frequently used for large scale plasmid isolation from crude lysates were not successful (26, 27, 28). The problem to isolate large plasmids on a large scale does not seem to be unique (30, 31). We developed two procedures, namely a neutral and an alkaline, for a large scale plasmid isolation, based on the sedimentation properties of the plasmid. In both cases 100 µg TI-plasmid DNA could easily be isolated. The efficiency is about 25%. Taking into consideration the sedimentation properties of the RP4 plasmid from strain LBA 601, the alkaline mass isolationprocedure is applicable to plasmids larger than 40-50 x 10^6 daltons, while, for the neutral method the plasmid ought to be at least about 90 x 10^6 daltons.

The TI-plasmid isolated with the lysis method presented, was pure for more than 90%, as indicated by renaturation kinetics. This test is accurate, easy to perform and has several advantages over the determination of purity by electron microscope. First the plasmid need not to be in the supercoiled or even open circular form to be identified. Secondly one needs no expensive equipment and results are obtained in a shorter time.

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