JOURNAL OF BACTERIOLOGY, Mar. 1981, p. 1365-1373
0021-9193/81/031365-09$02.00/0

Vol. 145, No. 3

Rapid Procedure for Detection and Isolation of Large and Small Plasmids

C. I. KADO* AND S.-T. LIU

Department of Plant Pathology, University of California, Davis, California 95616

Procedures are described for the detection and isolation of plasmids of various sizes (2.6 to 350 megadaltons) that are harbored in species of *Agrobacterium, Rhizobium, Escherichia, Salmonella, Erwinia, Pseudomonas,* and *Xanthomonas.* The method utilized the molecular characteristics of covalently closed circular deoxyribonucleic acid (DNA) that is released from cells under conditions that denature chromosomal DNA by using alkaline sodium dodecyl sulfate (pH 12.6) at elevated temperatures. Proteins and cell debris were removed by extraction with phenol-chloroform. Under these conditions chromosomal DNA concentrations were reduced or eliminated. The clarified extract was used directly for electrophoretic analysis. These procedures also permitted the selective isolation of plasmid DNA that can be used directly in nick translation, restriction endonuclease analysis, transformation, and DNA cloning experiments.

To determine whether a given bacterium harbors plasmids, it is necessary to isolate DNA by plasmid isolation procedures. Chromosomal and plasmid DNA are often obtained from cells that are treated with lysozyme and lysed with a detergent. The DNAs are freed of RNA and proteins by RNase and protease treatments that are followed by extraction with phenol. Just before phenol extraction, the plasmid DNA is released from the folded chromosomal complex by a shearing step or by RNase treatment. The plasmid DNA can be resolved as covalently closed circular molecules by isopycnic centrifugation in cesium chloride containing ethidium bromide (25).

To avoid such time-consuming steps, rapid techniques were reported for resolving plasmid and recombinant DNA in *Escherichia coli* (1, 3, 10, 14, 20, 21, 28), *Salmonella* spp. (31), *Rhizobium meliloti* (6), *Agrobacterium tumefaciens* (6), *Pseudomonas* spp. (6, 10, 24), *Bacillus subtilis* (10), and *Neisseria gonorrhoeae* (10). Such techniques still require partial purification steps that we viewed as unessential and time consuming. For example, selective precipitation of chromosomal complexes by centrifugation (2) or by adding salts or organic solvents (12) seems unnecessary and complicated. Furthermore, very large plasmids like those in *Agrobacterium* species (6, 9) were irreproducibly recovered by these precipitation steps (P. F. Lurquin and C. I. Kado, unpublished data).

To circumvent these problems, it was necessary to review the differences in plasmid DNA structure relative to linear DNA, RNA, and proteins that were the masking contaminants. The result of this review allowed us to develop an efficient reproducible method that utilized the least time and was applicable to many different bacteria. In the present report we describe a protocol for the detection of plasmid DNA within 3 to 4 h from either a small broth culture or a single colony of bacterial cells. We have also developed a procedure to circumvent the use of dye-buoyant density equilibrium cesium chloride centrifugation for the purification and isolation of plasmids.

An outline of the protocol was distributed at the annual meeting of The American Society of Biological Chemists, New Orleans, 1 to 5 June, 1980, and appeared in abstract form (Fed. Proc. **39**:2013, 1980).

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains of *A. tumefaciens* were obtained as follows: strain 1D1 (ATCC 15955) was from J. E. DeVay, strain ACH-5 was from J. Schell, strain C-58 was from R. Dickey, strains 1D135 and 1D1422 were isolated in our laboratory, strain NT1 was from M.-D. Chilton, and strain IIBNV6 was from J. Lippincott. *Agrobacterium radiobacter* 22 was obtained from A. Kerr, strains 12D18 and 12D120 were from our laboratory. *E. coli* 1830(pJB4J1) was from J. Berringer and A. Johnston, strain J53(pRK2) was sent by N. Panapoulos, and strain P678-54 was obtained from A. J. Clark. *Salmonella typhimurium* TA1534 was obtained from B. Ames. *Erwinia rubrifaciens* 6D318 was isolated in this laboratory, *Erwinia carotovora* 3D31 was obtained from R. H. Segall, *Erwinia stewartii* SW2 was sent by D. L. Coplin, and *Erwinia amylovora* 1D314 was isolated from *Cotoneaster* sp. in this laboratory. *Providencia stuartii* 164 was sent by F. Bolivar. *Serratia marcescens* 5B was obtained from C. Mulder.

Pseudomonas tomato 14D43 was provided by K. Kimble, and *Pseudomonas angulata* BF was from R. Fulton. *Pseudomonas savastanoi* 1D419 was isolated in our laboratory from infected olive, and strain 1D450 was isolated from infected oleander. *Pseudomonas eriobotryae* 4004 was provided by M. T. Lai, and *Pseudomonas glycinea* 12D43 was sent by H. Hoitink. *Xanthomonas pruni* 8D51 was isolated in this laboratory, and *Xanthomonas vitians* 068790 was from C. Wehlburg. All of the above cultures were grown in L-broth (10 g of casein hydrolysate [Calbiochem, La Jolla, Calif.], 10 g of NaCl, 5 g of yeast extract [Difco Laboratories, Detroit, Mich.], 1 liter of distilled water) or on L-agar plates at 30°C. *Rhizobium trifolii* 162P17, *Rhizobium legiminosarum* 128C53, and *Rhizobium japonicum* 3I1634 were received from D. Gross. They were grown in YEM broth (10 g of mannitol, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, 0.4 g of yeast extract, 1 liter of distilled water).

Chemicals and reagents. Sodium dodecyl sulfate (SDS) and Tris were purchased from Sigma Chemical Co., St. Louis, Mo. Sodium lauryl sarcosinate was obtained from CIBA-Geigy, Greensboro, N.C. Phenol obtained from Mallinckrodt was distilled and stored frozen until use. The frozen phenol was liquefied at 50°C and mixed with chloroform (50:50, vol/vol). The lysing solution contained 3% SDS and 50 mM Tris (pH 12.6). The solution was adjusted to pH 12.6 by adding 1.6 ml of 2 N NaOH by using a glass electrode (model GK2322C Radiometer; London Company, Copenhagen), the volume was adjusted to 100 ml with distilled water, and the solution was filtered through a membrane filter (2-μm pore size; Millipore Corp., Bedford, Mass.). All solutions may be kept at room temperature.

Gel electrophoresis. Agarose gel electrophoresis was performed as described previously (19, 21). Either 0.7 or 1% Seakem ME agarose (Marine Colloids, FMC Corp., Rockland, Maine) was used depending on whether whole plasmids or fragments of plasmid DNA were to be resolved. To avoid overheating and to resolve high-molecular-weight plasmids (21), a low-salt buffer system composed of 40 mM Tris-acetate and 2 mM sodium EDTA was used. The Tris was adjusted to pH 7.9 with glacial acetic acid (E buffer). Agarose was melted briefly (3 to 5 min) in E buffer either in an autoclave or in a microwave oven and mixed well before pouring. All electrophoresis was performed on a horizontal apparatus as described by McDonell et al. (19), except that the gel-holding base was fitted with a water-cooling plate. Electrophoresis was carried out at 12 V/cm and usually required about 2 h for the bromocresol purple tracking dye in the sample to migrate 12 cm. The gel (5 mm thick) was stained with 0.5 μg of ethidium bromide per ml for 30 min at 23°C. Photographs were taken of gels that were positioned over a shortwave UV light source (Ultraviolet Products, Inc., San Gabriel, Calif.). Polaroid type 55 film exposed through a Tiffen 15 orange filter was used.

Plasmid detection procedure. Cells were grown in 3 ml of L-broth overnight at 30°C to an optical density at 600 nm of 0.8 and pelleted by centrifugation (5,700 rpm, 4°C, for 7 min in a Sorvall SS34 rotor). The cell pellet was thoroughly suspended in 1 ml of E buffer. The cells were lysed by adding 2 ml of lysing solution (see above), which was mixed by brief agitation. The solution was heated at 50 to 65°C for 20 min in a water bath, and 2 volumes of phenol-chloroform solution (1:1, vol/vol) were added. The solution was emulsified by shaking briefly, and the emulsion was broken by centrifugation (6,000 rpm, 15 min, 4°C in a Sorvall SS34 rotor). Avoiding the precipitate at the interface, the upper aqueous phase was transferred to a screw cap tube by using a polyethylene Pasteur pipette (Ulster Scientific, Inc., Highland, N.Y.). Samples could be withdrawn directly for electrophoresis immediately. Usually 35 μl of sample was mixed on a square of parafilm "M" (American Can Company, Greenwich, Conn.) with 10 μl of 0.25% bromocresol purple in 50% glycerol–0.05 M Tris-acetate (pH 7.9). To minimize shearing of large plasmids it is important that samples be withdrawn and transferred with Drummond 50-μl micropipettes and an Adams no. 4555 suction apparatus (Becton, Dickinson & Co., Rutherford, N.J.).

Colony plasmid isolation. The procedures for screening recombinant plasmids in *E. coli* were the same as those described above, except that the cells were first streaked in a 1-cm^2 area on L-agar plates and allowed to grow at 37°C overnight. After incubation, about one-fourth of the cells on the 1-cm^2 area were scraped off from the agar surface with a wooden applicator, and the cells were suspended in 100 μl of lysing solution in a 750-μl Eppendorf microcentrifuge tube. Alternatively, a few large colonies could be picked up directly from a plate and suspended directly in 50 μl of the lysing solution. The lysate was subsequently incubated at 55°C for 15 to 60 min, followed by extraction with an equal volume of phenol-chloroform solution. After centrifugation in an Eppendorf model 5412 microcentrifuge for 5 min, 20 μl of aqueous phase was mixed with 5 μl of 0.25% bromocresol purple in 50% glycerol–0.05 M Tris-acetate (pH 7.9) on a square of parafilm and then loaded in a 25-μl sample slot in an agarose gel for electrophoresis. The heat step could be omitted when *E. coli* containing recombinant plasmids were to be screened. However, the lysate was still subjected to phenol-chloroform extraction and loaded on a gel for electrophoresis.

Rapid isolation of plasmid for restriction enzyme digestion, transformation, and nick translation. Cells were grown either on L-agar plates as mentioned above or in L-broth. For broth cultures, cells from 0.2 to 1 ml of the culture were pelleted by centrifugation in an Eppendorf microcentrifuge model 5412 with 1.5-ml Eppendorf microcentrifuge tubes. The pelleted cells were lysed with the lysing solution, incubated either at 55°C for 1 h for *E. coli* or at 65°C for 1 h for *A. tumefaciens* and extracted with phenol-chloroform as described above. The aqueous DNA solution was then freed of phenol by repeated extractions (usually two to four times) with diethyl ether and then transferred to a new centrifuge tube, at which time 100 μl of 3 M sodium acetate was added. The plasmid DNA was precipitated by adding 800 μl of cold (−20°C) 95% ethanol and incubating the mixture in a dry ice-ethanol bath for 5 min. The precipitated plasmid DNA was pelleted by centrifugation at 2°C for 15 min, dried under nitrogen, and suspended in 50

to 100 μl of autoclaved water. Usually, 20 μl of the solution was sufficient for restriction enzyme digestion, transformation, or nick translation. The entire procedure took less than 2 h.

RESULTS

Rapid lysis. Unlike *E. coli*, bacteria of the *Rhizobiaceae* and other *Enterobacteriaceae* such as *Erwinia* spp. and members of the *Pseudomonadaceae* are difficult to lyse adequately to insure reproducible recovery of intact plasmids. Lysis methods employing lysozyme in an isotonic buffer were unsuitable because good lysis seemed to depend on the stage of cell growth and on the temperature and length of incubation. Limitations were also experienced as variations in lysozyme activity depended on the commercial source of the enzyme. The enzyme had to be freshly prepared, and the incubation was time consuming. Surveys made of various described techniques generally showed reliance on lysozyme and, in some cases, proteinase and RNase followed by lysis with detergents such as Triton X-100 or SDS or by use of phenol. Heat shock or alkali treatment of the lysozyme- or detergent-treated cells seemed to aid in plasmid recovery (1, 3, 6, 13). We have investigated several of these parameters and have arrived at the following conclusions. Cells do not require washing after harvesting. They should be thoroughly suspended in a buffer of low ionic strength. The E buffer described above that was used for electrophoresis was used to suspend the cells. Generally, pelleted cells from a 3-ml broth culture were suspended in 1 ml of E buffer, and cells from a single colony scraped from an agar plate were suspended in 50 μl of the same buffer. Lysis was accomplished by the addition of two volumes of 3% SDS (in 50 mM Tris-hydroxide, pH 12.6). Usually members of the *Enterobacteriaceae* gave lysates that quickly cleared, whereas those of the *Rhizobiaceae* and *Pseudomonadaceae* remained slightly turbid. Sodium lauryl sarcosinate (3%) can be substituted for SDS when plasmid DNA is prepared for large-scale isolation by standard isopycnic centrifugation with cesium chloride-ethidium bromide density gradient procedures (25).

Clarification by heat treatment. Immediately after the addition of the alkaline SDS solution, the mixture was incubated at 50 to 65°C and then extracted with unbuffered phenol-chloroform (see below). The temperature treatment was essential for eliminating the "feathery" effect of chromosomal DNA commonly observed in agarose gels after electrophoresis and may be extended to eliminate chromosomal DNA altogether (Fig. 1). The temperature treatment also quickly eliminated RNA, which migrated faster than DNA and was usually positioned in the gel nearest the anode (compare lanes 1 and 2 in Fig. 1). RNA in a plasmid preparation of *X. vitians* was completely eliminated by heating for 5 min at 65°C. Linear DNA mainly composed of chromosomal DNA was minimized by the temperature treatment and became imperceptible after 72 min of incubation at 65°C. By contrast, plasmid DNA was concentrated for maximum visualization. Such a plasmid band could be dissected from the gel and eluted by one of several techniques (4, 29). For *E. coli*, the 55°C treatment was important. Usually 15 min of the heat treatment eliminated most of the chromosomal DNA band; however, 60 min of incubation was required to completely obliterate the chromosomal contamination (Fig. 2). For *Agrobacterium* spp. the 55°C heat treatment was not fully effective for complete chromosome elimination, but heating the DNA solution for 5 min at 95°C and then

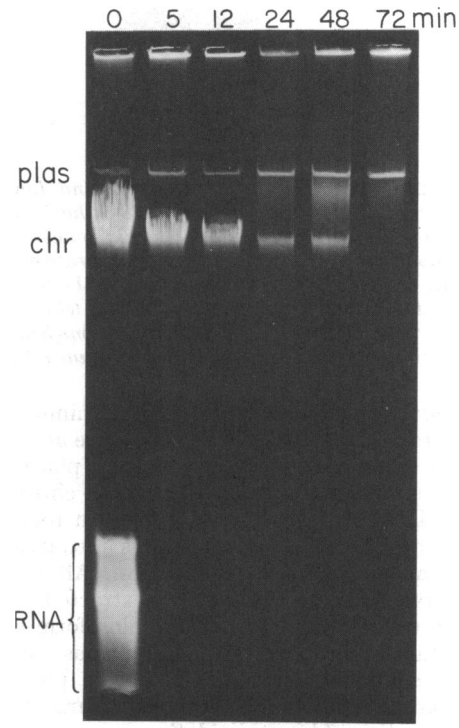


FIG. 1. *Kinetics of chromosomal DNA (chr) elimination at 65°C without loss of 43-Mdal plasmid (plas) in X. vitians. Temperature treatment as a function of time (minutes) followed lysis of cells in 1% SDS in 50 mM Tris-hydroxide (pH 12.6), after which the lysate was extracted with phenol-chloroform. DNA molecules were resolved by electrophoresis in 0.7% agarose (Seakem) prepared in E buffer.*

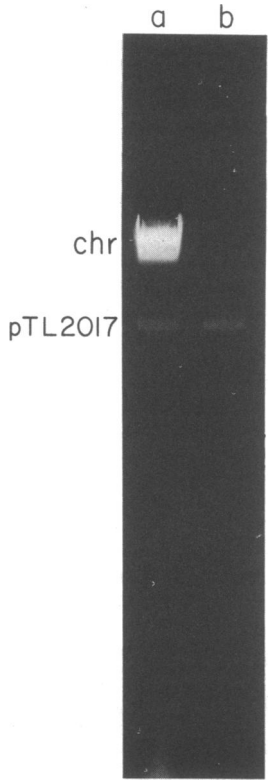


FIG. 2. *Elimination of chromosomal and linear DNAs from cell lysates of E. coli P678-54 harboring pBR325-pTi1422 recombinant plasmid molecules. Electrophoresis was performed in 0.7% agarose gel in E buffer at 12 V/cm with water cooling. (a) Unheated preparation showing chromosomal DNA (chr) and recombinant DNA pTL2017. (b) Preparations heated for 60 min at 55°C showing only recombinant DNA.*

cooling it immediately in crushed ice minimized the chromosomal DNA. The procedure at 95°C may be employed when the presence of plasmids that migrate in the same position as chromosomal DNA is suspected. However, in routine screenings the 55°C temperature is adequate.

Phenol-chloroform extraction. After the heat treatment, the lysate was extracted very briefly with unbuffered phenol-chloroform and centrifuged to obtain a clear aqueous phase which contained plasmid DNA. The phenol-chloroform mixture was designed to minimize the formation of brown oxidation pigments that usually occur with phenol solutions alone and at the same time be capable of lowering the pH of the lysate to 9.3. It also conveniently separated into hydrophobic and aqueous phases quickly and facilitated denaturation and the extraction of proteins.

Screening of plasmids. *A. tumefaciens* strains harboring large plasmids of 120 (Ti plasmid) and 260 (cryptic plasmid) megadaltons (Mdal) were screened (Fig. 3). The Ti plasmid of 28 to 29 Mdal in strain 1D1422 is unique among oncogenic *A. tumefaciens* strains (Kado et al., manuscript in preparation). A 3- to 4-kilobase deletion in this plasmid is sufficient to cause complete loss of virulence. A larger plasmid of 58 Mdal was observed in avirulent strain IIBNV6 and was similar to that reported previously (16). Furthermore, this strain and strains ACH-5, B6, 1D135, and C58 all harbored 260-Mdal cryptic plasmids. The large 260-Mdal cryptic plasmid was not detected in strains 1D1 and 1D1422. The absence of this cryptic plasmid in 1D1 (ATCC 15955) was also noted by others (S. K. Farrand, personal communication). *A. radiobacter* strains also harbor large cryptic plasmids (Fig. 3). Large cryptic plasmids of approximately 350 Mdal were also resolved in *R. leguminosarum, R. japonicum,* and *R. trifolii* (Fig. 4).

Analysis made on various members of the *Enterobacteriaceae* indicated that plasmids of this group were easily resolved by the rapid procedure. Plasmids of various sizes harbored in *E. coli, S. typhimurium* TA1534, *E. stewartii* SW2, and *E. amylovora* 1D314 are shown in Fig. 5. *E. coli* 1530, harboring the conjugally proficient plasmid pJB4J1, also harbored a larger cryptic plasmid of 102.5 Mdal. A 57.5-Mdal cryptic plasmid was observed in *S. typhimurium* TA1534 and was very similar to that observed in strain LT2 (18). *E. stewartii* SW2 harbored 12 cryptic plasmids of widely different sizes (Fig. 5). These plasmids are naturally maintained in *E. stewartii* and serve as useful molecular weight standards for covalently closed molecules (D. L. Coplin, personal communication). *E. amylovora* 1D314 harbored two cryptic plasmids with molecular masses of 20 and 47.5 Mdal.

Plasmids harbored in *Pseudomonas* species were equally well resolved by these procedures (Fig. 6). Cryptic plasmids in various randomly selected plant pathogenic *Pseudomonas* species were uncovered. Table 1 summarizes various plasmids that were resolved in different bacteria, including some species in which plasmids remained undetected.

Application to resolving recombinant plasmid DNA. The Ti plasmid of *A. tumefaciens* C-58 was digested with restriction endonuclease *Sal*I and ligated with plasmid pBR325, a cloning vector described by Bolivar (5). Figure 7 displays an array of recombinant DNA molecules of the Ti plasmid that were readily resolved by screening individual *E. coli* transformants. In this case, the heat treatment step was

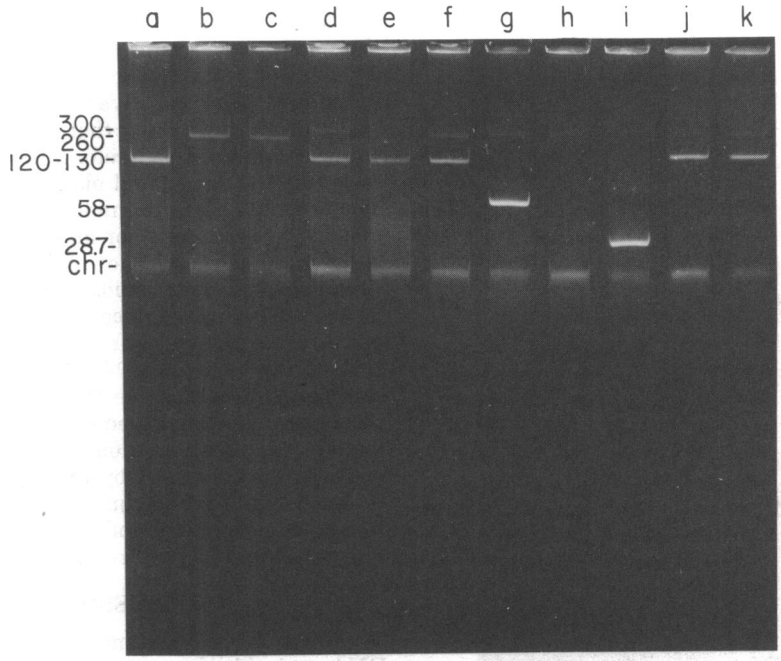
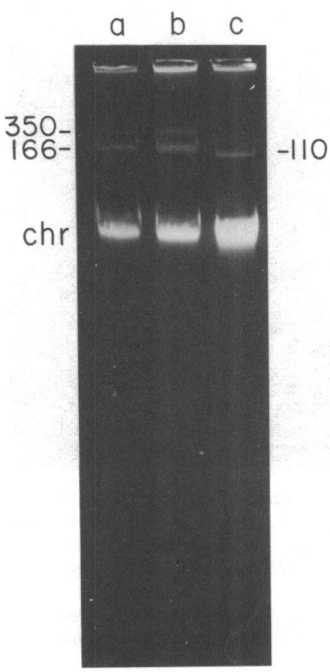


FIG. 3. *Resolution of high-molecular-weight plasmids of Agrobacterium strains. (a) A. radiobacter 22 containing a 120-Mdal cryptic plasmid. (b) A. radiobacter 12D18 and 12D120 (c) harboring 250-Mdal cryptic plasmids. (d) A. tumefaciens ACH-5 harboring the 120-Mdal Ti plasmid and a 300-Mdal cryptic plasmid, strain 1D1 (e) harboring the 120 mdal Ti plasmid, strain B6 (f) with the 120-Mdal Ti plasmid and a 300-Mdal cryptic plasmid, strain IIBNV6 (g) with 58- and 300-Mdal cryptic plasmids, avirulent strain NT1 (h) with a 260-Mdal cryptic plasmid, strain 1D1244 (i) harboring a mini-Ti plasmid of 28.7 Mdal but lacking the 260-Mdal cryptic plasmid, and strains 1D135 (j) and C58 (k) harboring a 130-Mdal Ti plasmid and the 260-Mdal cryptic plasmid. Electrophoresis was in 0.7% agarose in E buffer. Numbers indicate megadaltons; chr, chromosomal and linear DNA.*



omitted since plasmids in cloning experiments are small enough to migrate beyond the chromosomal DNA. Even without the heat treatment the chromosomal DNA was much reduced in intensity. Some lanes in the agarose electrophoretogram showed more than one band due to the presence of multimeric plasmid species that occur with pBR325, a feature not unusual for this and related plasmid vectors (17).

Stable maintenance of plasmid DNA. Plasmid preparations from various sources were stored at 4°C for 6 weeks. Samples (30 μl) were taken at weekly intervals and resolved by aga-

FIG. 4. *High-molecular-weight plasmids of Rhizobium species. (a) R. trifolii 162P17 harboring two large cryptic plasmids, (b) R. leguminosarum 128C53 harboring two large cryptic plasmids, and (c) R. japonicum 3I1634 harboring a 110-Mdal cryptic plasmid. Cells of strains 162P17 and 128C53 were lysed and heat treated for 15 min at 55°C. Strain 3I1634 was heat treated for 5 min at 55°C. Chr, Chromosomal and linear DNA. The plasmids were resolved in 0.7% agarose gel in E buffer. Numbers indicate megadaltons.*

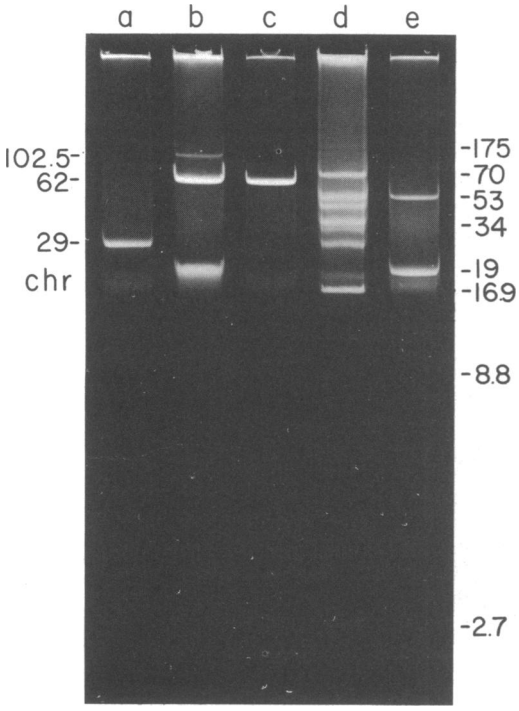


FIG. 5. *Gel electrophoretogram of some Enterobacteriaceae. (a) E. coli harboring pRK2 with a deletion in the region conferring tetracycline resistance. (b) E. coli containing pJB4J1 and a larger cryptic plasmid of 102.5 Mdal. (c) S. typhimurium TA1534 harboring a 57.5-Mdal cryptic plasmid. (d) E. stewartii SW2 harboring 12 cryptic plasmids of diverse sizes (see Table 1). (e) E. amylovora 1D314 harboring two cryptic plasmids of 20 and 47.5 Mdal. Numbers indicate megadaltons; chr, chromosomal and linear DNA. Electrophoresis was in 0.7% agarose in E buffer.*

rose gel electrophoresis. The 120-Mdal Ti plasmid of *A. tumefaciens* as well as the mini-Ti plasmid of 28.7 Mdal were stably maintained, as were the multi-cryptic plasmids harbored in *E. stewartii* SW2 (Table 2).

Plasmid isolation for cloning, nick translation, and restriction analysis. Plasmids isolated by this procedure were suitable for direct use in transformation experiments, for restriction analysis, and for cloning and could be labeled by nick translation. Illustrated in Fig. 8 is a typical agarose gel electrophoretogram of *E. coli* plasmid pTL2015, an example of a cloned restriction fragment of pTi1422 of 16.7 kilobase pairs, that was purified by the rapid procedure and was digested with restriction endonuclease *Sal*I. Note that there was no detectable smearing as would be expected if traces of chromosomal DNA were contaminating the preparation. These restriction fragments readily ligated to the cloning vector pBR325 as expected. Furthermore, recombinant plasmids yielded transformants with a frequency equal to that yielded by using plasmid from dye-cesium chloride density gradient procedure; that is, a frequency of 10^{-5} was obtained. Therefore, the rapid purification method expedited plasmid DNA cloning experiments. The plasmid prepared by this method was also used for nick translation with good results (data not shown). With large *Agrobacterium* spp. Ti plasmids, the precipitation of plasmid DNA was unnecessary. The aqeuous phase after phenol-chloroform extraction was simply dialyzed against 10 mM Tris-hydrochloride (pH 7.4) to remove traces of phenol and chloroform and then used directly for transformation. A transformation frequency of 10^{-7}, equal to that obtained by using plasmid DNA from standard dye-cesium chloride centrifugation procedure was reproducibly obtained (J. C. Kao et al., unpublished results).

DISCUSSION

Various cryptic plasmids and recombinant DNA molecules were equally resolved by the protocol described herein. Plasmids ranging from 2.6 to 350 Mdal were easily detected. Bac-

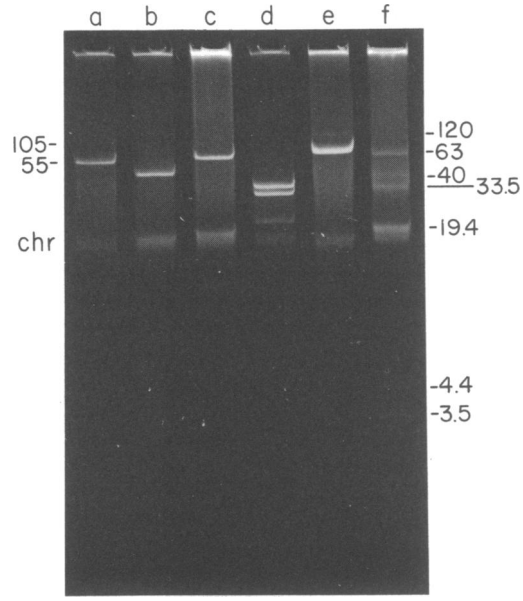


FIG. 6. *Resolution of diverse cryptic plasmids of Pseudomonas species. (a) P. tomato 14D43, (b) P. angulata BV, (c) P. savastanoi olive strain, (d) P. savastanoi 1D450 from oleander galls, (e) P. eriobotryae 4004, and (f) P. glycinea 12D43. Gel electrophoresis was in 0.7% agarose in E buffer. Numbers indicate megadaltons; chr, chromosomal and linear DNA.*

TABLE 1. *Plasmids of various bacteria resolved by the mini-screen procedure*

Bacterium	Plasmids detected	Molecular mass of plasmids (Mdal)
A. tumefaciens ACH-5, B6	Yes	120, 300
A. tumefaciens C58, 1D135	Yes	130, 260
A. tumefaciens 1D1	Yes	120
A. tumefaciens 1D1422	Yes	28.7
A. radiobacter 12D18, 12D120	Yes	250
R. leguminosarum 128C53	Yes	160, 350
R. trifolii 162P17	Yes	166, 350
R. japonicum 3I1634	Yes	110
E. amylovora 1D314	Yes	20, 47.5
E. stewartii SW2	Yes	2.8, 8.8, 16.9, 23.2, 29.5, 33.0, 34.5, 43.3, 49.2, 51.6, 69.8, 178[a]
E. carotovora 3D31	No	
E. rubrifaciens 6D318	No	
E. coli J53(RK2Δ)	Yes	29.0
E. coli 1530(pJB4J1)	Yes	19.0, 62, 102.5
S. typhimurium TA1534	Yes	57.5
S. marcescens 5B	No	
P. stuartii 164	No	
P. tomato 14D43	Yes	55, 105
P. angulata BF	Yes	41.5
P. savastanoi 1D419 (olive)	Yes	60
P. savastanoi 1D450 (oleander)	Yes	20.5, 28.5, 34.5, 60
P. eriobotyrae 4004	Yes	67, 120
P. glycinea 12D43	Yes	3.5, 4.4, 33.5, 40, 63
X. pruni 8D51	Yes	26
X. vitians 068790	Yes	43

[a] Cryptic plasmid molecular masses of this strain were obtained by electron microscopy and gel electrophoresis by D. L. Coplin.

terial cells that were tested were lysed quickly by SDS at pH 12.6, and the lysate was cleared by heat treatment. With the exception of *E. coli*, SDS at high pH conditions alone lysed the cells ineffectively. Hansen and Olson (13) described a plasmid isolation procedure which included an alkaline denaturation step. They noted low yields of plasmids of the P1 incompatibility group and postulated that the diminished yields may have resulted from either a possible covalently integrated RNA sequence, an alkali-sensitive modified base of DNA, or an alkali-sensitive relaxation complex of Inc-P1 plasmids. At least two Inc-P1 plasmids (RK2 and pJB4J1) that we tested showed no loss of yield as judged by band intensities in agarose gel electrophoretograms using the same amounts of cells as comparative standards (Fig. 5). We noted, however, that reduced band intensities occurred when disposable pipette tips with small orifices were used. We assume that there was a great deal of mechanical shearing of the plasmids during sample transfer to agarose gels. When this is avoided as outlined in the methods section, our procedure seems applicable to Inc-P1 plasmids; at least it permits the easy detection of these plasmids. Under the given alkaline conditions, plasmid DNA bands remained unaffected when heated between 50 and 95°C for 20 to 30 min. In contrast, chromosomal DNA diminished with time at these elevated temperatures, because of the highly denaturing conditions above pH 11 (7, 26, 32). DNA primary structure remains relatively stable under such alkaline conditions, and deprotonation of guanine residues is experienced with guanine- and cytosine-rich DNAs (e.g., *Micrococcus lysodeikticus* DNA) (26, 32). The heat treatment permitted complete denaturation and abolishment of the secondary structure of linear DNA, which then was unable to renature under the conditions prescribed.

Alternative alkaline treatment methods have required a neutralization step by the careful addition of buffer at low pH (6, 9, 13). We have had difficulty in obtaining reproducible results owing to irregularities in the neutralization step. We found no real need for a neutralization step, and, in fact, the extraction of the alkaline lysate with unbuffered phenol-chloroform simply lowered the pH to 9.3. This condition maintains plasmid DNA at a most desirable pH (7, 26), whereby plasmid DNA can be stored for several weeks at 4°C without appreciable deterioration (Table 2). We have found that the phenol-chloroform step is necessary, but cannot be used directly for lysis. This step must follow the alkaline SDS lysis step. It is well established that phenol readily extracts proteins and nucleases (27) and therefore serves to extract proteins from the SDS-lysed cells. It also probably helps to remove denatured DNA, since this step is essential for the isolation of plasmid DNA free of chromosomal DNA, particularly with *E. coli* containing recombinant plasmids. It has been shown (9) that denatured DNA is preferentially entrapped in the precipitate at the interface after extraction with phenol.

The procedure described above is applicable to various bacteria. Undescribed cryptic plas-

TABLE 2. *Stability of various plasmids stored at pH 9.3 at 4°C*

Storage time (wk)	Stability of plasmid		
	pTiC58	pTi1422	*E. stewartii* SW2 (cryptic)
0	+	+	+
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	±
5	+	+	−
6	±	±	−

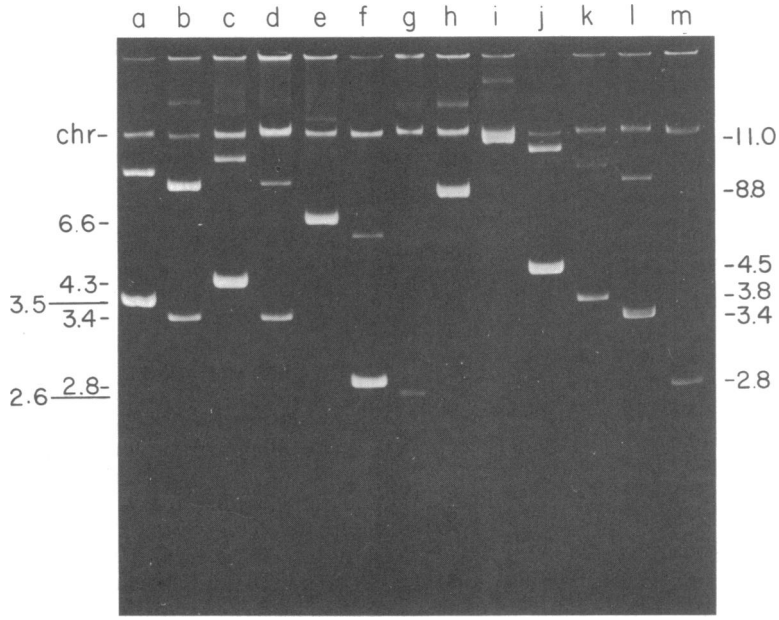


FIG. 7. *Cloned plasmid pTiC58 fragments generated by restriction endonuclease SalI. Lanes a to f and h to m represent recombinant plasmid molecules each of which were resolved from a single colony of E. coli P678-54. Lane g shows the pBR322 vector of 2.6 Mdal. Multimeric forms of each recombinant molecule were obtained in each lane. Electrophoresis was in 1% agarose gel. Numbers indicate megadaltons; chr, chromosomal and linear DNA.*

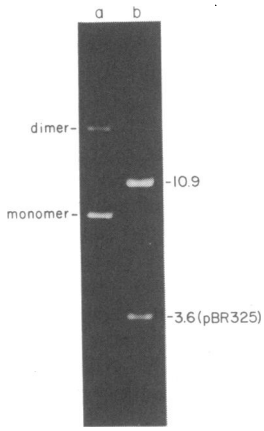


FIG. 8. *Plasmid pTL2015 subjected to agarose gel electrophoresis before and after digestion with restriction endonuclease SalI. (a) Covalently closed circular molecules as dimers and monomers of pTL2015. (b) Linear cloned fragment of pTi1422 and cloning vector pBR325 after cleavage with SalI. Chr, chromosomal and linear DNA.*

mids were uncovered in *A. tumefaciens* 1D1422, ACH-5, B6, and IIBNV6; *S. typhimurium* TA1534; *E. amylovora* 1D314; *P. tomato* 14D43; *P. angulata* BV; *P. eriobotyrae* 4004; *R. trifolii* 162P17; *R. leguminosarium* 128C53; *R. japonicum* 3I1634; *X. pruni* 8D51; and *X. vitians* 068790. We have confirmed the presence of cryptic plasmids in *A. tumefaciens* IIBNV6 (16), *S. typhimurium* LT2 (data not shown) (17), strain Ea46 of *E. amylovora* (23), *P. glycinea* (8), *P. phaseolicola* (11, 21), *P. savastanoi* (L. Comai, personal communications), *X. manihotis* (data not shown) (15), and *Rhizobium* spp. (6, 22, 30). Furthermore, plasmids in yeast cells were resolved by this procedure (A. Howarth, personal communication). This should facilitate rapid screening of genetically modified yeasts.

Our protocol requires about 3 to 4 h to obtain the results, that is, less than 60 min to obtain the sample for agarose gel electrophoresis and 120 to 180 min for the electrophoresis step. Thus, we routinely screen individual colonies or small broth cultures by this method.

Application of this protocol for the preparative isolation of plasmid DNA in *E. coli* was shown to be effective. For *E. coli* the phenol and chloroform were removed by ether extraction, and the plasmid DNA was concentrated by ethanol precipitation and rapid drying under nitrogen. Such DNA preparations are suitable for restriction analysis and are of sufficient homogeneity for use as probes and in cloning experiments. Also, large Ti plasmid DNAs, which have been tedious to prepare, can now be isolated in less than 1 h and used directly for transformation experiments. These procedures

may also be applicable for isolating cryptic plasmids from various bacteria heretofore untested.

ACKNOWLEDGMENTS

We thank Sandy Woodward, Jesse Dutra, Lisa McDaniel, and Jeff Hall for capable technical assistance.

This work was supported by Public Health Service grant CA-11526 from the National Cancer Institute and by grants from the Science and Education Administration, U.S. Department of Agriculture, and the Science Advisory Council, North Atlantic Treaty Organization.

LITERATURE CITED

1. **Barnes, W. M.** 1977. Plasmid detection and sizing in single colony lysates. Science **195**:393–394.
2. **Bazaral, M., and D. R. Helinski.** 1968. Circular DNA forms of colicinogenic factors E1, E2, and E3 from *Escherichia coli*. J. Mol. Biol. **36**:185–194.
3. **Birnboim, H. C., and J. Daly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Blin, N., A. Gabain, and H. Bujard.** 1975. Isolation of large molecular weight DNA from agarose gels for further digestion by restriction enzymes. FEBS Lett. **53**: 84–86.
5. **Bolivar, F.** 1978. Molecular cloning vectors: derivatives of plasmid pBR322, p. 59–63 *In* H. W. Boyer and S. Nicosia, (ed.), Genetic engineering. Elsevier-North Holland Publishing Co., New York.
6. **Casse, F., C. Boucher, J. S. Julliot, M. Michel, and J. Dénarié.** 1979. Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J. Gen. Microbiol. **113**:229–242.
7. **Chapman, R. E., Jr., and J. M. Sturtevant.** 1970. Volume changes accompanying thermal denaturation of deoxyribonucleic acid. II. Denaturation at alkaline pH. Biopolymers **9**:445–457.
8. **Curiale, M. S., and D. Mills.** 1977. Detection and characterization of plasmids in *Pseudomonas glycinea*. J. Bacteriol. **131**:224–228.
9. **Currier, T. C., and E. W. Nester.** 1976. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. **76**:431–441.
10. **Eckhardt, T.** 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid **1**: 584–588.
11. **Gantotti, B. V., B. S. Patil, and M. Mandel.** 1979. Apparent involvement of a plasmid in phaseotoxin production by *Pseudomonas phaseolicola*. Appl. Environ. Microbiol. **37**:511–516.
12. **Guerry, P., D. J. LeBlanc, and S. Falkow.** 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116**:1064–1066.
13. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227–238.
14. **Klein, R. D., E. Selsing, and R. D. Wells.** 1980. A rapid microscale technique for isolation of recombinant plasmid DNA suitable for restriction enzyme analysis. Plasmid **3**:88–91.
15. **Lin, B.-C., H.-J. Day, S.-J. Chen, and M. E. Chien.** 1979. Isolation and characterization of plasmids in *Xanthomonas manihotae*. Bot. Bull. Acad. Sinica **20**:157–171.
16. **Lippincott, B. B., J. B. Margot, and J. A. Lippincott.** 1977. Plasmid content and tumor initiation complementation by *Agrobacterium tumefaciens* IIBNV6 J. Bacteriol. **132**:824–831.
17. **Lurquin, P. F., and C. I. Kado.** 1977. *Escherichia coli* plasmid pBR313 insertion into plant protoplasts and into their nuclei. Mol. Gen. Genet. **154**:113–121.
18. **Manis, J. J., and H. J. Whitfield.** 1977. Physical characterization of a plasmid cointegrate containing an F1 *his gnd* element and the *Salmonella typhimurium* LT2 cryptic plasmid. J. Bacteriol. **129**:1601–1606.
19. **McDonnell, M. W., M. N. Simon, and F. W. Studier.** 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. **110**:119–146.
20. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.
21. **Mickel, S., V. Arena, Jr., and W. Bauer.** 1977. Physical properties and gel electrophoresis behavior of R12-derived plasmid DNAs. Nucleic Acids Res. **4**:1465–1482.
22. **Nuti, M. P., A. M. Ledeboer, A. A. Lepidi, and R. A. Schilperoort.** 1977. Large plasmids in different Rhizobium species. J. Gen. Micorbiol. **100**:241–248.
23. **Panopoulos, N. J., W. V. Guimaraes, S.-S. Hua, C. Sabersky-Lehman, S. Resnik, M. Lai, and S. Shaffer.** 1978. Plasmids in phytopathogenic bacteria, p. 228–241. *In* D. Schlessinger (ed.), Microbiology—1978. American Society for Microbiology, Washington, D.C.
24. **Pemberton, J. M., and A. J. Clark.** 1973. Detection and characterization of plasmids in *Pseudomonas aeruginosa* PAO. J. Bacteriol. **114**:424–433.
25. **Radloff, R., W. Bauer, and J. Vinograd.** 1967. A dye-bouyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. **57**:1514–1521.
26. **Record, M. T., Jr.** 1967. Electrostatic effects on polynucleotide transitions. II. Behavior of titrated systems. Biopolymers **5**:993–1008.
27. **Rushizky, G. W., A. E. Greco, R. W. Hartley, Jr., and H. A. Sober.** 1963. Concentration and desalting of ribonucleases. Biochem. Biophys. Res. Commun. **10**: 311–314.
28. **Telford, J., P. Boseley, W. Schaffner, and M. Birstiel.** 1977. Novel screening procedure for recombinant plasmids. Science **195**:391–393.
29. **Thurnig, R. W. J., J. P. M. Sanders, and D. Borst.** 1975. A freeze-squeeze method for recovering long DNA from agarose gels. Anal. Biochem. **66**:213–220.
30. **Tshitsenge, G., N. Luyindula, P. F. Lurquin, and L. Ledoux.** 1975. Plasmid deoxyribonucleic acid in *Rhizobium vigna* and *Rhizobium trifolii*. Biochim. Biophys. Acta **414**:357–361.
31. **Willshaw, G. A., H. R. Smith, and E. S. Anderson.** 1979. Application of agrose gel electrophoresis to the characterization of plasmid DNA in drug-resistant enterobacteria. J. Gen. Microbiol. **114**:15–25.
32. **Zimmer, C.** 1968. Alkaline denaturation of DNA's from various sources. Biochim. Biophys. Acta **161**:584–586.