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Isolation of Large Bacterial Plasmids and Characterization of the P2 Incompatibility Group Plasmids pMG1 and pMG5

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Large plasmids from *Agrobacterium tumefaciens*, *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas putida*, and *Pseudomonas aeruginosa* were routinely and consistently isolated using a procedure which does not require ultracentrifugation but includes steps designed to separate large-plasmid DNA from the bacterial folded chromosome. It also selectively removes fragments of broken chromosome. A variety of large plasmids was readily visualized with agarose gel electrophoresis, including five between 70 and 85 megadaltons (Mdal) in size, six between 90 and 143 Mdal, one that was larger than 200 Mdal, and one that was larger than 300 Mdal. This isolation procedure allowed initial estimation of the molecular sizes of the two IncP2 plasmids, pMG1 and pMG5, which were 312 and 280 Mdal, respectively. A standard curve for size determination by gel electrophoresis including plasmids between 23 and 143 Mdal in size did not extrapolate linearly for plasmids of the 300-Mdal size range. Unique response of different plasmids to the isolation procedure included sensitivity of IncP1 plasmids to high pH and the co-isolation of a 20-Mdal "cryptic" plasmid in conjunction with pMG1.

The isolation and description of "large" plasmids having molecular sizes of 100 megadaltons (Mdal) or more have included such plasmids as the tumor-inducing plasmids of *Agrobacterium tumefaciens* (43), the H incompatibility group antibiotic resistance plasmids (12), and the CAM degradative plasmid of *Pseudomonas putida* (3, 34), as well as various plasmid-chromosome recombinants made with F (25, 39).

Procedures that have been developed which will isolate large plasmids include those of Humphreys et al. (16), Sharp et al. (39), Palchaudhuri and Chakrabarty (35), Currier and Nester (6), and Fennewald et al. (11). Aside from the strict problem of size, many plasmids from the genera *P. putida* and *Pseudomonas aeruginosa* are often quite difficult to isolate, prompting the development of protocols specifically adapted to that purpose of Palchaudhuri and Chakrabarty (35), Johnston and Gunsalus (20), and Fennewald et al. (11).

Our interest in the P2 incompatibility group plasmids, a group including both antibiotic resistance and degradative plasmids, has been tempered by the difficulty of obtaining sufficient amounts of purified plasmid DNA. Employing various of the above procedures (6, 16, 20), we have still found the yield of the P2 incompatibility group resistance plasmids pMG1 and pMG5 to be poor or negligible (unpublished data).

There appear to be two problems with the extant isolation procedures in regard to large plasmids. With methods that depend on the selective precipitation of chromosomal complexes by either high-gravity centrifugation (4) or high-salt precipitation (14), large plasmids do not remain in the supernatant (D. Lorenz, unpublished data). The protocol of Humphreys et al. attempts to counter this loss by increasing the input volume and concentrating plasmid DNA after the chromosome has been removed (16). Other methods employ a shearing step to break chromosomal DNA, which may act to help release plasmid. This is followed by cesium chloride-ethidium bromide density equilibrium centrifugation to separate the unbroken plasmid DNA in a satellite band (2). However, shearing appears also to break much of the large-plasmid DNA (6), which necessitates an increase of input cells to provide sufficient plasmid yield. Increasing the input, however, requires a further elaboration: excess linear DNA is removed before equilibrium density centrifugation. This is done in several protocols by denaturing the linear DNA and selectively removing the single-stranded DNA pieces (6, 35, 39).

One protocol attempts to avoid the two prob-

lems of plasmid loss, caused by shearing or by co-precipitation with the chromosomal DNA, by defining strict lysis conditions that appear to improve release of plasmid DNA from the chromosome (20).

For reasons of yield and efficiency, these approaches were not satisfactory for our purposes. We thus sought to develop a method of large-plasmid isolation that would be rapid, would not require large input volumes of cells, and would allow agarose gel electrophoresis of a crude lysate to visualize plasmid bands without any prior need of equilibrium density centrifugation in the manner suggested by Meyers et al. for smaller plasmids (26).

To meet those criteria, we evolved a protocol which is in part a synthesis of portions of three extant plasmid isolation procedures (6, 14, 16) and which also incorporates changes designed to help release plasmid DNA from the folded chromosome and to reduce contamination of plasmid DNA with pieces of broken DNA.

(This report is part of an investigation being conducted by J.B.H. in partial fulfillment of the requirements for the Ph.D. degree at the University of Michigan, Ann Arbor.)

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used are listed in Tables 1 and 2.

TABLE 1. *Bacterial strains*

Bacterial strains	Parent bacterial strain relevant characteristics	Strain source and reference	Plasmid source and reference[a]
P. aeruginosa			
PAO2(pRO12)	Ser^-	[b]	[c]
PAO2(FP2)	Ser^-		10[b]
PAO2(pRO271)	Ser^-		30[c]
PAO2(pRO161)	Ser^-		40[c]
PAO38(RP1)	Leu^-	[b]	31[c]
PAO38(pRO12)	Leu^-		[c]
PAO38(pMG1)	Leu^-		17[d]
PAO38(pMG5)	Leu^-		18[d]
P. putida			
AC10	Met^-, plasmid free	[e]	
AC541	Met^-, contains K plasmid	[e]	
S. typhimurium			
LT2	Wild-type; contains cryptic plasmid	25, 29[f]	
AA2103(F'*80his-gnd*)	Chromosomal *his* deletion, Arg^- Ser^-; cured of LT2 cryptic plasmid.	25, 29[f]	
AA0019(pAS19)	Chromosomal *his* deletion, Arg^- Ser^-; cured of LT2 cryptic plasmid	25, 29[f]	
E. coli			
ROE531(pRO11)	Met^-	33[c]	
AB1133(pRO11)	Leu^- Pro^- Met^- His^- Thi^- SmR^g	[c]	
DT41(R27)	Lac^- Arg^- Leu^- Thi^-	42[h]	
DT78(TP116)	Lac^- Arg^- Leu^- Thi^-	12[h]	
CSH24	Pro^- Thi^- SuII; contains F'*lac*(Ts)	27[i]	
A. tumefaciens			
C58(RP4)	Also contains tumor-inducing (TI) plasmid	6[j]	

[a] Entry in this column signifies that host strain designated was not the original strain in which the plasmid listed was received, isolated, or constructed; transfer of plasmids to host strains listed was performed in this laboratory using procedures previously published (30).

[b] Bruce Holloway, Monash University, Clayton, Victoria, Australia.

[c] This laboratory.

[d] George Jacoby, Massachusetts General Hospital, Boston, Mass.

[e] A. M. Chakrabarty, General Electric Research and Development Center, Schenectady, N.Y.

[f] Harvey Whitfield, Department of Biochemistry, University of Michigan Medical School.

[g] SmR, Chromosomal resistance to streptomycin.

[h] Diane Taylor, The Hospital for Sick Children, Toronto, Ontario, Canada.

[i] Cold Spring Harbor Laboratory strain.

[j] Thomas Currier, Michigan State University, East Lansing, Mich.

TABLE 2. *Plasmids*

Plasmids	Relevant characteristics[a]	Molecular size in Mdal (reference)
IncP1[b] R-plasmids[c] from *P. aeruginosa*		
RP1	Wide host range	40 (13)[d]
RP4[e]	Wide host range	36 (26)
pRO161	Transductionally shortened RP1 derivative	23 (40)
IncP2 R-plasmids from *P. aeruginosa*		
pMG1		312[f]
pMG5		280[f]
IncA-C R-plasmids from *Serratia marcescens*[g]		
pRO11		97[f]
pRO12	Derivative of pRO11 formed by spontaneous deletion in *P. aeruginosa*	73[f]
IncH R-plasmids		
R27[h]	IncH1, from *S. typhimurium*	112 (12)
TP116	IncH2, from *Salmonella typhi*	143 (12)
Sex factors		
FP2	IncP8, from *P. aeruginosa*	59 (37) 58 (35) 71[i]
pRO271	FP2 with CbR[j] added from RP1	73[i] 76[f]
K	From *P. putida*	80 (3) 64 (34) 85[i]
F'*lac*(Ts)	IncFI, temperature sensitive for replication, carries *E. coli lac* genes	95[i]
F'*80his-gnd*	IncFI, carries *E. coli his-gnd* genes	79 (25)
Other plasmids		
LT2 cryptic	From *S. typhimurium*	60 (25)
pAS19	Recombinant of LT2 cryptic and F'*80his-gnd*	125 (25)
TI	Tumor inducing for plants	120 (43)

[a] Antibiotic resistance markers of plasmids conformed with previously published phenotypes and were variously used to select for maintenance or transfer.
[b] Plasmid incompatibility group P1; for review of P incompatibility groups, see Jacoby (19).
[c] "R-plasmid" connotes antibiotic resistance plasmid of nosocomial origin.
[d] Contour length of 19.3 μm; Mark Richmond, personal communication.
[e] Similar to RP1.
[f] By contour length measurement, this paper; see Table 3.
[g] Manuscript in preparation.
[h] Also called TP117 (see 7, 12).
[i] By agarose gel electrophoresis, this paper. See Results.
[j] CbR, Resistance to carbenicillin.

Buffers. TE buffer contained 0.05 M tris-(hydroxymethyl)aminomethane (Tris) and 0.02 M disodium ethylenediaminetetraacetate (Na_2EDTA) (pH 8.0). TES buffer consisted of 0.05 M Tris, 0.05 M NaCl, and 5 mM Na_2EDTA (pH 8.0).

Cell growth. Bacteria were grown to approximately 2×10^8 cells per ml in broth medium as described previously (32), except that a 30°C incubation temperature was used for *P. putida* and *A. tumefaciens* strains.

Cell lysis and plasmid isolation. Except where otherwise noted, plasmid isolation was performed by the following standard protocol (see Table 3 for summary of steps). The following steps show amounts used for a 40-ml culture input; for 80 ml, the volumes were doubled unless otherwise noted. The cells were washed by centrifugation in a polypropylene centrifuge tube in a Sorvall SS34 head at ambient temperature, 10,000 rpm, 30 min, followed by suspension of the pellet in 10 ml of 0.01 M sodium phosphate buffer (pH 7.0) and recentrifugation for 20 min.

(i) Cell lysis. The washed pellet was resuspended

TABLE 3. *Steps of plasmid isolation*[a]

Step	Operation	Conditions
1	Cell growth	40 or 80 ml of cells in complex broth medium, to about 2 × 10^8 cells per ml.
2	Cell lysis	Washed cells resuspended at high osmolarity, cold; addition of lysozyme, Na_2EDTA, SDS to 4%; intermittent 55°C pulses.
3	Alkaline denaturation	pH 12.1 to 12.3, 3 min.
4	Neutralization	Addition of 2 M Tris (pH 7.0) to lower pH to 8.5 to 9.0.
5	Removal of membrane-chromosome complexes	Addition of SDS to 4%, NaCl to 1.0 M; refrigeration for 6 h; centrifugation, 30 min at 12,000 rpm.
6	Concentration of plasmid DNA from supernatant	Addition of PEG 6000 to 10%, refrigeration for 6 h; centrifugation at 2,500 rpm, 5 min; resuspension in about one-fortieth volume.
7	Agarose gel electrophoresis	

[a] Conditions of treatment for each step are summarized. For details, see the text.

by mixing in a Vortex mixer at maximum speed in 1.35 ml of 25% sucrose-0.05 M Tris (pH 8.0) at ambient temperature. All mixing steps from this point through polyethylene glycol 6000 (PEG 6000) addition were by gentle inversion of the centrifuge tube at a frequency no greater than 20 times per min. The numbers of inversions used pertaining to various steps in the procedure are specified below. First, 0.1 ml of lysozyme (10 mg/ml in 0.25 M Tris, pH 8.0) was added and mixed in by four inversions, and the tubes were put in an ice-water bath for 5 min. Then, 0.5 ml of Na_2EDTA (0.25 M, pH 8.0) was added and mixed in by five inversions, and the tubes were chilled for 5 min more in ice water. The addition of 0.5 ml of sodium dodecyl sulfate (SDS; Pierce Chemical Co.) (20%, wt/vol, in TE), followed by eight cycles of a heat pulse and mixing (one cycle was 15 s in a 55°C water bath, and then five inversions during 15 s after being removed from the water bath), produced a clear viscous solution of lysed cells.

(ii) Alkaline denaturation and neutralization. At ambient temperature, 0.5 ml of NaOH (a freshly prepared 3 N solution) was added, immediately followed by 3 min of inversion (at 20 inversions per min). For our cultures, this raised the pH to between 12.1 and 12.3. Then 1.0 ml of Tris (2 M, pH 7.0) was added in two 0.5-ml aliquots each followed by 30 s of inversion (at 20 inversions per min), to lower the pH to between 8.5 and 9.0.

(iii) Removal of membrane-chromosome complexes. The addition of 0.65 ml of SDS (20%, wt/vol, in TE), immediately followed by addition of 1.25 ml of NaCl (5 M) and 20 inversions (at 20 per min) caused the appearance of white floc-like material. Failure to add and mix quickly at ambient temperature gave incomplete removal of the chromosomal DNA. After inversion, the tubes were chilled in an ice-water bath and refrigerated (4°C) for 6 h or overnight as convenient. Centrifugation in a Sorvall SS34 head at 12,000 rpm (4°C, 30 min) caused the salt-precipitated chromosome-membrane complexes to form a large white pellet.

(iv) Concentration of plasmid DNA. The supernatants were poured into chilled plastic conical tubes and put in an ice-water bath. Some pieces of white floc might float at the top of the solution; if so, these were removed with a Pasteur pipette. The conical tubes had two effects: small particles of floc that could not be pipetted off stuck to the sides when the solution was decanted, and markings on the sides of the tubes allowed estimation of volumes without pipetting. The volumes were measured (usually about 4.8 ml for 40 ml of culture input) and decanted into chilled polypropylene centrifuge tubes set in ice-water baths, and 0.313 volume of PEG 6000 ("Carbowax 6000," Union Carbide, Schwarz/Mann) (42%, wt/vol, in 0.01 M sodium phosphate buffer, pH 7.0) was added, giving a concentration of 10%. Mixing was done by stirring with a plastic pipette, the solution becoming slightly cloudy. The tubes were refrigerated for 6 h or overnight as convenient. Centrifugation in a Sorvall SS34 head for 5 min at 2,500 rpm (4°C) yielded pellets; these were resuspended in 0.15 ml of cold TES (for 80-ml culture inputs, 0.20 ml of TES) and kept in an ice-water bath. For a 40-ml culture input, the volume of the plasmid solution after addition of the 0.15 ml of TES was usually about 0.25 ml. This solution constituted a highly enriched crude plasmid preparation that could be subjected to electrophoresis without further purification.

Lysis without alkaline denaturation. To perform the above lysis and plasmid enrichment while omitting the pH excursion caused by NaOH addition, the 0.5 ml of NaOH (3 N) and 1.0 ml of Tris (2 M, pH 7.0) were mixed together before their addition to the lysate. The same number of inversions were done as above to control for mechanical effects. Thus the same ionic environment and volume were obtained, but the pH went no higher than 8.5 to 9.0.

Agarose gel electrophoresis. Samples of 10 to 40 μl of crude plasmid preparation were subjected to electrophoresis and visualized by the method of Meyers et al. (26), except that the 0.7% agarose gels were run at 100 V for 3 h on a Savant model SGE 1310 slab gel electrophoresis assembly with an E-C model 458 constant voltage power source. Our plastic comb for forming slots in the gel had 10 teeth (3 by 6.5 mm). The Tris-borate running buffer of Meyers et al. is 89 mM Tris, 2.5 mM Na_2EDTA, and 89 mM boric acid, and the tracking dye solution consists of bromophenol blue (0.07%), SDS (7%), and glycerol (33%) in water (26). After staining overnight with 0.4 μg of ethidium

bromide per ml of water, gels were visualized on a Ultra-Violet Products transilluminator model 0-62. Plasmid DNA appears as a band that fluoresces when excited with UV light. Commonly, we used 25 μl of DNA solution and 10 μl of tracking dye solution. The crude plasmid solution often appeared as a particulate suspension which was difficult to dissolve, but mixing with the dye solution at ambient temperature gave a solution. Photographs of gels presented in Results show migration from top to bottom. Photographs were taken with Plus-X pan film (4 by 5 inches, ca. 10.2 by 12.7 cm), using a no. 9 Wratten gelatin filter (Kodak) and a J-344 contrast filter (Ultra-Violet Products, Inc.) to remove blue and UV light. Photography enhanced the visualization of faint bands.

Standard curve construction. Since there was variation in the mobility observed between different electrophoresis runs, the mobility data of plasmids needed to be normalized to construct the standard curve seen in Fig. 3. Data analysis and plotting were done using a Hewlett-Packard 9825A calculator and 9872A plotter.

One representative electrophoresis gel was chosen as the standard, and the mobility data from the other electrophoresis gels were normalized to the standard data by the following algorithm. If A was the gel whose data were being normalized and B was the standard gel, and if A and B both had in common n different plasmid DNA bands, designated $1,2 \ldots, n$, and if the relative mobilities of the plasmids (distance migrated from the origin divided by length of the gel) were designated as $A_1, A_2, \ldots, A_n$; $B_1, B_2, \ldots, B_n$, then the constant coefficient, K_a, for which the expression $\sum|K_aA_i - B_i|/(K_aA_i + B_i)$ (which $i = 1$) was at a minimum, could be calculated. Then all the mobility data in A were normalized by multiplying by K_a.

A different constant was calculated for each gel whose data were to be normalized to the standard gel's data. Inspection of the algorithm will show that its effect was to minimize, for all plasmids common to two gels, the sum of the proportional differences between the standard mobilities and the normalized mobilities. This provided a closest-fit normalization with regard to all plasmids in common between two gels. For all the normalizations, the average number of plasmids in common was 4, with a minimum of 2 and a maximum of 7.

To give a positive logarithm, 1.0 was added to each normalized relative mobility value. The normalized data were then plotted as log of relative mobility versus log of molecular weight. A least-squares regression line with log of relative mobility as the independent variable and log of molecular weight as the dependent variable was also calculated and plotted (see Fig. 3).

Electron microscopy. To prepare DNA for electron microscopy, the crude plasmid preparation resulting from 240 to 320 ml of input cells was centrifuged, in a Beckman type 65 rotor at 40,000 rpm and 15°C for 65 h, to equilibrium in a cesium chloride-ethidium bromide gradient (6). The satellite band was collected from above by syringe with a 17-gauge needle, extracted with cold isopropanol to remove ethidium bromide, and dialyzed against two changes of cold TES buffer. Analysis of DNA contour length using the basic protein film technique (9) was performed with a Zeiss EM10 electron microscope. Tracings of open circular molecules of plasmid DNA and of λcI857 DNA (gift from the laboratory of D. Jackson) were measured with a Numonics Corp. Electronic Graphics Calculator. Plasmid molecular size was determined by the ratio of contour lengths, using the point mutant λcI857 as a 30.8-Mdal (8) standard.

The size of the λcI857 standard itself was verified. The actual lengths of 21 open circular λcI857 molecules were measured through use of a carbon replica grating containing 2,160 lines per mm (no. 1002, Ernest F. Fullam, Inc.). The 21 molecules measured 14.96 ± 0.20 μm in length. Using the conversion factor of 2.07 Mdal/μm (23), the molecular size of the λcI857 standard was calculated at 30.97 ± 0.41 Mdal.

RESULTS

Effect of SDS concentration. In the standard protocol in Materials and Methods, the SDS was added at 20% concentration (wt/vol) to give a final concentration in the lysate mixture of approximately 4%. The input concentration of SDS could be adjusted to give various desired final concentrations in the lysate. Varying the concentration of SDS used in our protocol was found to have a marked effect on plasmid DNA yield. This is seen in Fig. 1, where we prepared pMG5 plasmid DNA with either 2% (wells A and B), 1% (wells C and D), or 4% (wells E and F) final SDS concentration. The yield of pMG5 DNA, as indicated by the intensity of the upper plasmid bands, increased as the SDS concentration was increased. The lower diffuse bands are regions of linear chromosomal DNA. We also tried a final concentration of 8% SDS, but this did not improve the yield of pMG5 DNA as compared with 4% (unpublished data); the 4% final concentration was incorporated into standard lysis protocol (see Materials and Methods).

Using the standard lysis protocol, 17 different plasmids (see Table 2) were isolated and visualized on agarose gels (wells A, C, E, and G in Fig. 2; unpublished data).

Effect of alkaline denaturation. Comparison of plasmid yield from typical lysates in which the alkaline denaturation step has either been included, i.e., the standard protocol (see Materials and Methods), or omitted is shown in Fig. 2. Files in the agarose gel on which samples were run that had not been through the alkaline denaturation step were distinctive for their large, thick region of broken chromosomal DNA (wells B, D, F, and H in Fig. 2). Inclusion of the denaturation step greatly diminished the amount of this broken chromosomal DNA. Furthermore, the denaturation step improved the DNA yield for several plasmids, as seen for pMG5, pAS19, F′*80his-gnd*, and the LT2 cryptic plasmid in Fig. 2, and pMG1, TP116, FP2, and

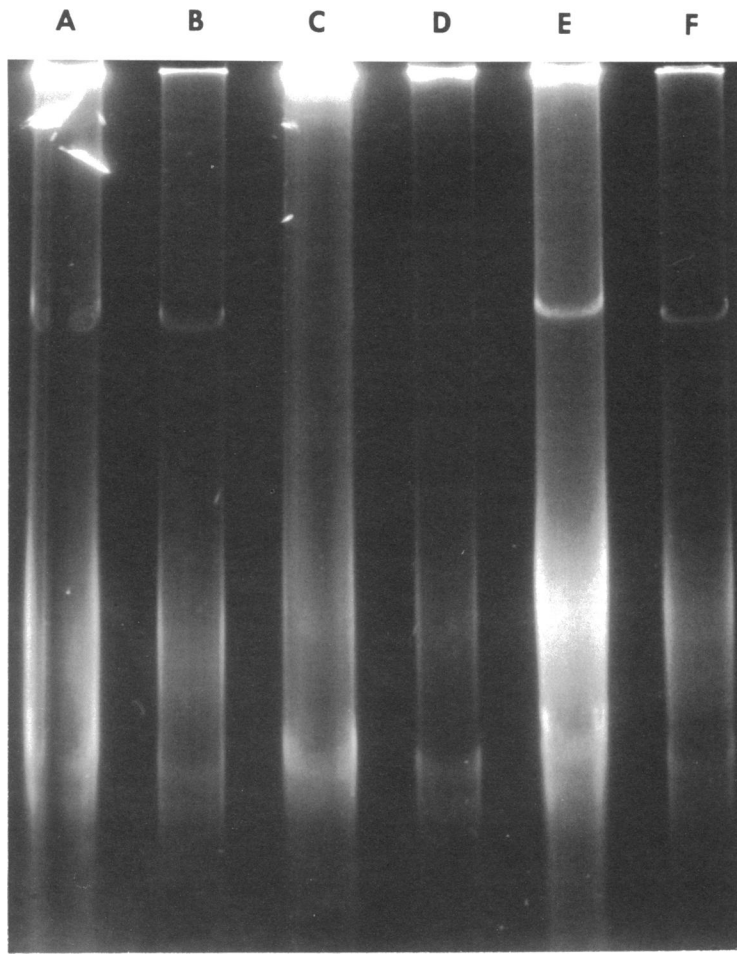

FIG. 1. *Agarose gel electrophoresis of pMG5 DNA showing effect of different SDS concentrations during lysis. Three 80-ml cultures of PAO38 (pMG5) were lysed by the standard protocol described in the text (which includes an alkaline denaturation step), but with these changes: SDS was added at either 5, 10, or 20% to give final SDS concentrations in the lysates of 1, 2, or 4%. For each lysate, the resuspension was mixed with dye at a ratio of 4.5 to 1, and samples were subjected to electrophoresis as follows: (A and B) 40- and 10-μl samples from the lysate using 2% final SDS concentration; (C and D) 40- and 10-μl samples from the lysate using 1% final SDS; (E and F) 40- and 10-μl samples from the lysate using 4% final SDS.*

pRO271 (unpublished data). Indeed, the P8 incompatibility group plasmids, FP2 and pRO271, could not be seen at all when denaturation was omitted (unpublished data).

However, the denaturation step did not appear to improve the yield of certain other plasmids, including pRO12, R27, the tumor-inducing plasmid TI. For the P1 incompatibility group plasmids RP4, RP1, and pRO161, the denaturation step acted to diminish plasmid yield (unpublished data).

Standard curve and estimating plasmid size. Figure 3 shows the standard curve that we constructed from agarose gel electrophoresis data for 11 plasmids whose range of molecular size varied from 23 Mdal for pRO161 to 143 Mdal for TP116 (see Table 2). The molecular sizes of pRO11 and pRO12 were obtained by electron microscopic contour length measurement (see Materials and Methods and Table 4); the sizes of the other plasmids were obtained from other sources as detailed in Table 2. Use of the standard curve did not yield a precise size determination, but did allow reproducible relative and approximate size determinations.

We used the standard curve to estimate the molecular sizes of six other plasmids whose mobility data were normalized to the standard curve. On the basis of one band each, FP2, K, and F'*lac*(Ts) were estimated at 71, 85, and 95

Mdal, respectively. On the basis of three bands, pRO271 was estimated at 73 ± 1 Mdal. The P2 incompatibility group plasmids, pMG1 and pMG5, were evidently larger than the largest plasmid of the standard curve, as evidenced by their slower migration during electrophoresis. If we assumed a linear extrapolation of the standard curve, the molecular size of pMG1, on the basis of four bands, was estimated at 229 ± 7 Mdal, and, on the basis of eight bands, pMG5 was estimated at 194 ± 15 Mdal. However, these values for the large plasmids pMG1 and pMG5 were inaccurate, as will be discussed subsequently.

The estimated size for K, 85 Mdal, is in fair agreement with the preliminary 80-Mdal size estimated by Chakrabarty (3). Also, the size difference between FP2 and pRO271 is close to that expected if pRO271 constitutes the addition Tn1 (3.2 Mdal) to FP2 (see Table 2) (30). However, there were two problems with the other estimations. First, the estimation for FP2 was not in agreement with the sizes of 59 and 58 Mdal previously published (34, 37). Secondly, the validity of a linear extrapolation of the standard curve was untested.

To address these two problems, preliminary contour length measurements were performed for pRO271, pMG5, and pMG1 (see Table 4). λcI857 was included as an internal standard for

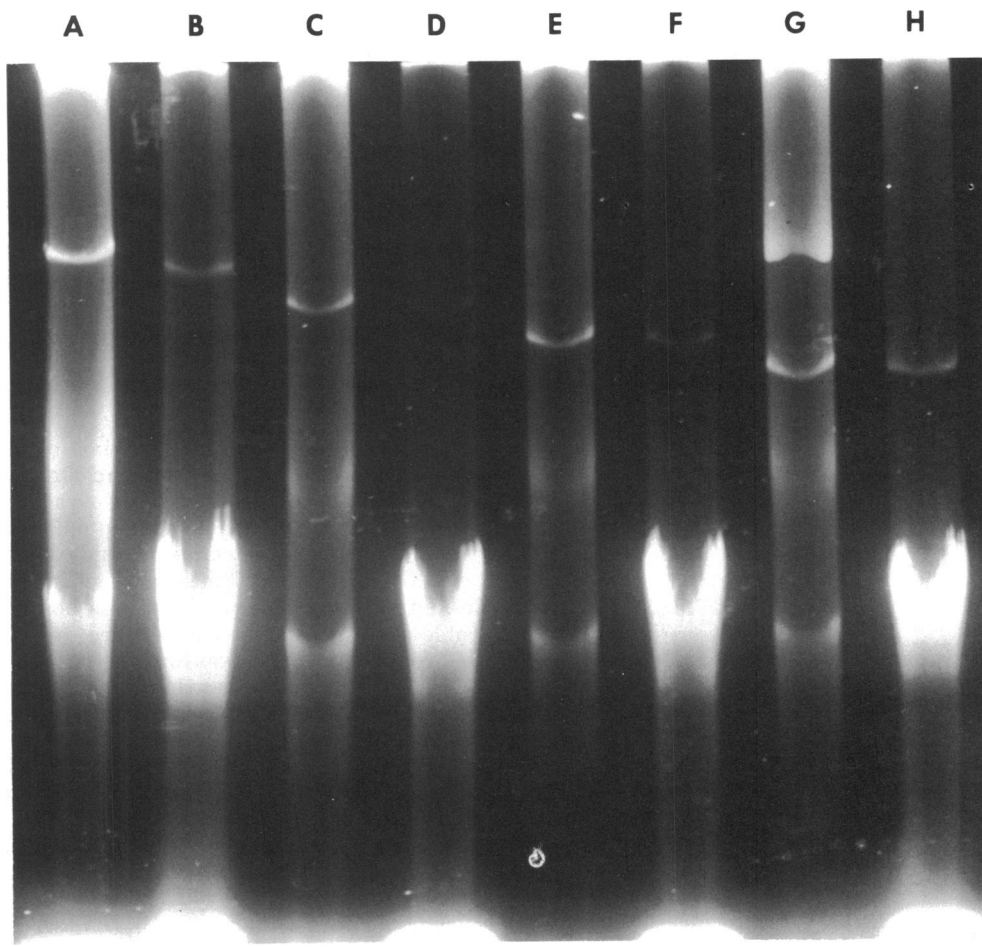

FIG. 2. *Agarose gel electrophoresis showing effect of alkaline denaturation on plasmid yield for pMG5, pAS19, F'80his-gnd, and LT2 cryptic. Four different cultures in 40-ml duplicates were prepared by either the standard protocol (includes denaturation) or with omission of denaturation. A 25-μl sample of each DNA preparation was mixed with 10 μl of dye and subjected to electrophoresis. Samples prepared by the standard protocol were run as follows: (A) PAO38 (pMG5); (C) AA0019(pAS19); (E) AA2103(F'80his-gnd); (G) LT2. Samples of lysates prepared with omission of denaturation were run in wells as follows: (B) PA038(pMG5); (D) AA0019(pAS19); (F) AA2103(F'80his-gnd); (H) LT2.*

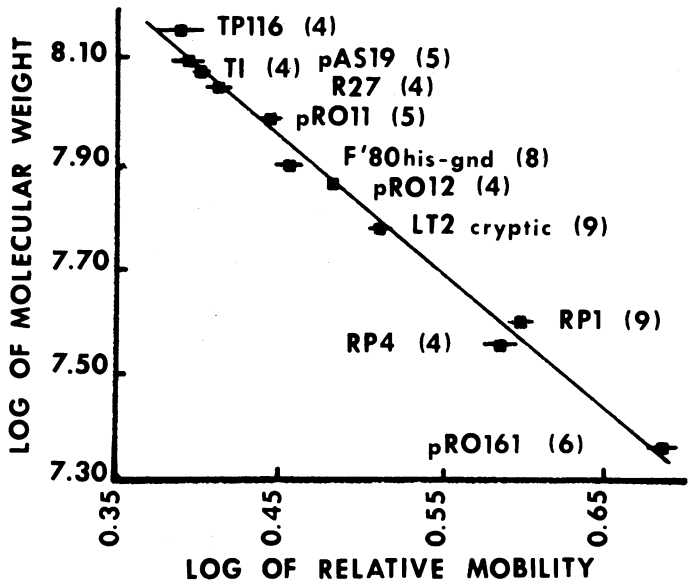

FIG. 3. *Standard curve of 11 plasmids. The data of 62 plasmid bands from nine gels were normalized, with relative mobility calculated on a scale of 0 to 10 (see the text). For each plasmid's normalized data, the filled square is the mean of the log of the relative mobility, and the line indicates one standard deviation to either side of the mean. In parentheses after each label is the number of observations for that particular plasmid. For preparations of samples and electrophoresis conditions, see the text. For plasmid sources and molecular sizes, see Tables 1 and 2. All data were generated with the lysate procedure of this paper except for two pRO11 data observations, for which DNA came from Currier and Nester (6) lysates, and three RP1, three pRO161, and one pRO11 observations of plasmid DNA derived from Guerry et al. (14) lysates. The top four plasmids in order of descending size are TP116, pAS19, TI, and R27.*

TABLE 4. *Contour length measurements of plasmids by electron microscopy*[a]

Plasmid	No. of molecules measured	Estimated molecular size (Mdal) ± SD[b]
pRO11	18	96.8 ± 2.3
pRO12	22	72.5 ± 1.7
pRO271	10	75.6 ± 2.7
pMG5	8	280.2 ± 14.8
pMG1	3	311.8 ± 17.9
Small plasmid with pMG1	13	19.8 ± 0.3

[a] All microscopy grids prepared for contour length measurement of plasmids also contained, on the same grid, λcI857 DNA as an internal standard; 10 to 40 of these were measured, depending on the sample.

[b] SD, Standard deviation.

all grids. The 75.6 ± 2.7-Mdal measurement for pRO271 was consistent with our estimation of the sizes of FP2 and pRO271 obtained by electrophoresis; however, it suggested that FP2 was approximately 10 Mdal larger than previous reports of Pemberton and Clark (37) or Palchaudhuri and Chakrabarty (35).

The measurements of pMG5 at 280.2 ± 14.8 Mdal and of pMG1 at 311.8 ± 17.9 Mdal indicated that, for the particular conditions of electrophoresis that we used, the standard curve was not linear beyond 140 Mdal, and that a linear extrapolation would, in fact, yield an underestimation of the size of very large plasmids. During the pMG1 contour length measurement, another plasmid of size 19.8 ± 0.3 Mdal was observed (Table 4). This plasmid was believed to be related to pMG1, since it was absent from preparations of other plasmids made from the same host strain, PAO38, and it was much larger than the cryptic *P. aeruginosa* plasmids reported by Pemberton and Clark (37). This 20-Mdal plasmid was also seen in some electrophoresis gels of pMG1 (data not shown).

Plasmid is isolated in the CCC form. The DNA bands containing plasmid seen in Fig. 1 and 2 and other gels (data not shown) contained covalently closed circular (CCC) DNA. Three observations support this. First, when the standard curve was constructed, the points all fitted a linear curve (see Fig. 3). This suggested that all the plasmids were in the same configuration, namely, CCC; if any were not CCC, their mobilities would have been greatly altered, and they would not have fitted onto the standard curve. Secondly, when satellite DNA from ethidium

bromide-cesium chloride equilibrium density gradients for several plasmids (pRO11, pRO12, pRO271, pMG1, pMG5) was subjected to electrophoresis on agarose gels, the plasmid bands showed the same mobility as bands given by the crude plasmid preparations (unpublished data). Thirdly, when contour length measurements were done for several plasmids, the results agreed with the standard curve size estimations (see Table 2).

Thus, CCC DNA of large plasmids is not necessarily precipitated by the high-salt method as has been previously suggested (6). Furthermore, large size per se does not appear inimical to plasmid DNA entry into the 0.7% agarose gel matrix.

Yield calculations. Although we have not performed precise quantitative yield experiments, we can estimate the amount of DNA expected in a plasmid band after agarose gel electrophoresis. The samples in each well of Fig. 2 were one-tenth of the total crude plasmid preparation obtained from a 40-ml lyate; thus they represent DNA from 4 ml of input culture. With an input cell density of 2×10^8/ml, and assuming 1.8 copies of a 100-Mdal plasmid in each cell, 4 ml of cells would contain 240 ng of plasmid DNA, some portion of which would be in CCC form. A qualitative estimate of actual yield of CCC plasmid DNA is made by observing the plasmid bands in Fig. 2; they certainly contained far more DNA than the 50 ng (26) needed for the threshold of visualization. Quantitative determination of yield is in progress.

Suitability of the protocol for isolation of various plasmids. The plasmids that are on the standard curve (Fig. 3), as well as the plasmids pMG1, pMG5, K, FP2, pRO271, and F'*lac*(Ts), which were compared against standards for size determination, were chosen using two criteria. The first was molecular size: eight of the plasmids were larger than 90 Mdal, and six were larger than 110 Mdal (see Table 2). Yet for this variety of very large plasmids, the gels showed clear and reproducible bands (see Fig. 2; unpublished data). The second criterion was the choice of several plasmids that have heretofore been very difficult to isolate (a criterion not necessarily mutually exclusive of large size): for us, these included the P2 incompatibility group plasmids pMG1 and pMG5, and the P8 incompatibility group plasmids FP2 and pRO271 (see Introduction). Of the 17 plasmids used, the only ones for which the staining intensity of bands on agarose gels was variably faint were pRO12, FP2, and pRO271. Their relative faintness, when compared with bands of the other plasmids, suggested that a poorer yield occasionally obtains for those plasmids.

For lysates of some plasmids, including RP4/TI, RP1, pRO161, LT2 cryptic, pMG1, and K, we saw after electrophoresis a broad region of brightness whose front was concave in relation to the bottom of the gel, rather than convex like the plasmid band (see G in Fig. 2; unpublished data). Notably, these aberrant fluorescent regions (aurorae) of the gels were seen only if the alkaline denaturation step was included in the plasmid isolation protocol (compare G and H in Fig. 2 for LT2 cryptic). Related plasmids did not always share this particular trait. For example, we noted that while lysates made from strains containing one IncP2 plasmid, pMG1, showed an aurora, those containing the other IncP2 plasmid, pMG5, did not (data not shown); furthermore, while the LT2 cryptic plasmid lysates showed an aurora, lysates of strains containing the recombinant of LT2 cryptic plasmid with F'*80his-gnd*, pAS19, did not (compare C and G in Fig. 2). The auroral fluorescence observed may be due to a skewed size distribution of linear DNA pieces, having a distinct size minimum (hence the sharp lower bound of the aurorae). This sharp boundary we observed may suggest the occurrence of a specific mechanism of DNA degradation that leads to the aurorae.

DISCUSSION

Variables affecting CCC plasmid isolation. The following factors seemed to contribute to the success of this plasmid isolation protocol. First, the manipulations were all very gentle, consisting only of decanting, inversion, or centrifugation; there was no pipetting. The absence of shearing, in contrast to some other isolation methods for large plasmids (6, 35, 39), should have reduced plasmid breakage through mechanical means, a loss Currier and Nester found to be considerable (6).

During lysis, the lysates were either kept cold or were in the presence of high SDS concentration or high pH. The protein denaturation effect of both of these latter conditions, as well as the effect of low temperature on endonucleolytic activity, may have served to reduce the degree of enzymatic degradation the plasmids would have otherwise incurred.

SDS, alkali, and the heat-pulse step may all have helped increase plasmid yield in another way. The folded-chromosome model for the bacterial genome (38, 44, 45) suggests that the continuous chromosomal DNA molecule comprises 12 to 80 separate loops or domains, and RNA is involved in maintaining the domains separate from each other. If DNA in one domain undergoes a single-strand break, that domain alone will lose its supercoils. Ribonuclease digestion of folded chromosome will decrease the number of

loops, but will not alter the supercoiling (38, 44, 45). Kline and Miller (21) and Kline et al. (22) postulate that in vivo a supercoiled plasmid makes a nonintegrative association with the folded chromosome because the physicochemical structure of the plasmid resembles one of the supercoiled domains of the bacterial genome, and that such a nonintegrated association is important for both replication and segregation of the plasmid. Evidence for such association derives mainly from observation of co-sedimentation of the plasmid with isolated folded-chromosome complexes. Included among the plasmids that exhibit such an association are the *Escherichia coli* sex factor F, derivatives of F (21, 22), and the cryptic plasmid of the *Salmonella typhimurium* LT2 strain (24; see Tables 1 and 2). For plasmids whose replication is stringently controlled, the association with the folded-chromosome complex is nearly complete (22). Since the large plasmids tend to be stringently controlled for replication (5), their complete association with the folded chromosome may be interfering with plasmid isolation. However, both SDS and heat (50°C for 5 min) are observed to cause the complete unfolding of the condensed chromosome similar to that caused by ribonuclease (though probably through different mechanisms; SDS acts to dissociate bound proteins and is not known to have any direct effect on nucleic acid-nucleic acid interactions [41]). This suggested to us that in our lysis procedure one contribution of the SDS, the 55°C heat pulses, and perhaps the alkali treatment as well, was to help unfold the bacterial genome, allowing release of the supercoiled plasmid into solution before precipitation of the chromosome-membrane complex by high salt. If this interpretation is correct, the successful application of our lysis procedure to recovery of large plasmids from a variety of bacterial genera may imply a widespread occurrence of the nonintegrative association between plasmid and folded genome.

It was not clear why the SDS effect was titratable as seen with pMG5 yield in Fig. 1, since at all SDS concentrations there was complete lysis of cells. We considered that the effect was related to plasmid-folded chromosome association, but we only studied the effect of varying the SDS concentration with *P. aeruginosa*.

The effect of the alkali treatment on reducing the chromosomal debris (see Fig. 2) probably resulted from an inability of the PEG 6000 step to concentrate single-stranded DNA. DNA concentration by PEG 6000-NaCl is believed to be a phase partition phenomenon rather than a precipitation (46), and may operate analogously to the PEG-dextran two-phase partition system (1) which separates single-stranded from double-stranded DNA. With a pMG1 lysate, it was seen that reduction of chromosomal DNA allowed visualization of the 20-Mdal cryptic plasmid.

Deleterious effect of alkali on P1 incompatibility group plasmids. A reduction in yield of IncP1 plasmids caused by the alkali denaturation step (see Results) contrasted with the neutral or positive effect of the alkali step on recovery of all other plasmids examined. It suggested the possibility that the IncP1 plasmids shared either a covalently integrated RNA sequence, alkali-sensitive modified bases of DNA (28), or an alkali-sensitive relaxation complex (15). Some experience in this laboratory with the effect of a ribonuclease treatment during the isolation of RP1 plasmid DNA (unpublished data) supported the first possibility. Another wide-host range plasmid, the IncW plasmid, R388, also has demonstrated ribonuclease sensitivity during isolation (unpublished data).

P2 incompatibility group plasmids pMG1 and pMG5. The two IncP2 plasmids that we examined were large, with a molecular size of 280 Mdal for pMG5 and 312 Mdal for pMG1 (see Table 4). What genetic information besides the known transfer functions, antibiotic or metallic ion resistances, and alteration of phage typing patterns (17, 18) are present on these plasmids that are 13 and 15% the size of the *P. aeruginosa* chromosome (estimated at 2.1×10^9 daltons by Pemberton [36])? If the plasmid DNA is unique, without any repeated regions, the plasmids might contain more than 300 separate cistrons each. We don't know at this point whether the plasmids pMG1 and pMG5 have a unit length that is less than the size we have measured (implying that they exist as stable multimers) and also whether any of their genetic content is shared with the chromosomal genome or with each other. The relation of the "cryptic" 20-Mdal plasmid to pMG1 is also unknown at this time. It is hoped that the isolation method employed in this study will help answer some of these questions, as well as facilitate examination of natural and clinical isolates for large plasmids. By their large size, pMG1 and pMG5 represent archetypes of very large plasmids.

The isolation of six other large IncP2 plasmids, not including pMG1 or pMG5, has been recently reported. Fennewald et al. obtained DNA of P2 incompatibility group plasmids from *P. aeruginosa*, using a quantitative isolation procedure that employs an adenine-thymine-specific dye to accentuate density differences between plasmid and chromosomal DNA. A molecular size of greater than 200 Mdal for all six

plasmids was suggested by the examination of endonuclease restriction fragments. However, as is the case with some other large plasmid isolation procedures (see Introduction), breakage appears to be a problem with their procedure; plasmid DNA was only recoverable in the form of linear fragments, from 15 to 20 Mdal in size. Furthermore, because the mole percent of guanine plus cytosine of IncP2 plasmid DNA is close to that of *P. putida* chromosomal DNA, the procedure is not applicable for isolation of IncP2 plasmids from *P. putida* (11). The minimum size of 200 Mdal for the six plasmids isolated by Fennewald et al. corresponds with the large sizes for pMG1 and pMG5 reported herein. Taken together, they suggest that the P2 incompatibility group may generally represent an archetypal class of large plasmids. This is supported by our finding that 12 different IncP2 plasmids of diverse geographic origin all had sizes between 280 and 310 Mdal (manuscript in preparation).

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