ISOLATION OF METABOLIC PLASMID DNA FROM PSEUDOMONAS PUTIDA

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SUMMARY: Metabolic plasmids conferring on Pseudomonas putida the aromatic growth phenotypes naphthalene, Nah^+, salicylate, Sal^+, or toluate, Tol^+, have been isolated as covalently closed circular DNA in 100 µg amounts. Plasmid DNA was banded in CsCl-ethidium bromide density gradients and sedimentation rates measured in sucrose gradients and by analytical centrifuge. The plasmid sizes found, in millions, were /NAH 42, /SAL 43, /TOL 55, 42. Transformation of metabolic plasmid free P. putida with the isolated DNA confirmed the respective aromatic pathway gene contents.

Plasmids have been found by genetic analysis to contain the information essential to the growth of Pseudomonas strains on selected terpene (1), aliphatic (2), and aromatic (3-6) compounds, and associated fertility factors to initiate both plasmid and chromosomal gene transfer (1,7,8). More recently the isolation of metabolic plasmid DNA from Pseudomonas strains has been reported (9,10) and correlated with peripheral metabolic phenotypes.

We report here a method for the isolation of three aromatic metabolic plasmids from an isogenic cell line, estimates from sedimentation rates of their size, and transformation with the isolated DNA's of a plasmid "cured" strain to the respective Nah^+, Sal^+ and Tol^+ phenotypes.

The estimates of plasmid size from transduction (3,8,11), electron microscope measurements (9) and sedimentation rates (10,this ms.) though convergent are not identical; see also summaries (11,12). Thus, strain derivations, Table 1, and Methods are provided in greater than the usual detail.

MATERIALS AND METHODS

Reagents, defined as underlined, are: Tris-sucrose, 50 mM Tris-Chloride buffer, pH 8, made to 25 percent w/v sucrose; Lysozyme, 250 mM Tris-Cl pH 8 with 5 mg/ml egg white lysozyme (Sigma, grade 1); Sarkosyl-DOC, suspend 50 g Sarkosyl 97 (ICN) and 10 g deoxycholic acid in 150 ml of distilled water, neutralize with NaOH, dissolve by heating to 70°, cool to 25°, adjust to pH 8

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Table 1. Strain Origin and Preparation

PpG No.	Genotype* Chromosome	Plasmid	Derivation	Ref.
CAM				
1	wild type	(CAM)	camphor en.	1
273	1 *trpB615*	(CAM)	PC	1
277	273	(CAM^-)	MC	
1273	273 *hft-601*	(CAM)	UV	7
1274	1273	(CAM^-)	MC	
572	1	(CAM^-)	PC	1
1334	572 *his-621*	(CAM^-)	NG	
1812	1334 *str-650*	(CAM^-)	Spon.	
1343	572 *met-616*	(CAM^-)	NG	
1348	1343 *ade-625*	(CAM^-)	NG	
1360	277 *ade-631*	(CAM^-)	NG	
NAH				
7	wild type	(NAH)	naphth. en.	3
378	7 *leu-801*	(NAH)	NG	3
379	378 *hft-650*	(NAH)	NG	3
1064	277	(CAM^-)(NAH)	379 x 277	
2118	1348	(CAM^-)(NAH)	1064 x 1348	
2121‡	1360	(CAM^-)(NAH)	2118 x 1360	
SAL				
2100	277	(CAM^-)(SAL)	R1 x 277	4
2111	1343	(CAM^-)(SAL)	2100 x 1343	
2115‡	1360	(CAM^-)(SAL)	2111 x 1360	
TOL				
9	wild type	(TOL)	benzoate en.	14
1813	1812	(CAM^-)(TOL)	9 x 1812	
2114‡	1360	(CAM^-)(TOL)	1813 x 1360	
RP1				
2101‡	1274	(CAM^-)(RP1)	PL1(RP1) x 1274	
P. aeruginosa PL1		$Met^-Leu^-Rif^r$(RP1)		15
E. coli W3110		K12 $Str^r(F^-)(\lambda^-)$(col IbP9)		16

*Designations as recommended by Novick *et al;* (13)
PC = penicillin-cycloserine; MC = mitomycin C; UV = ultraviolet
NG = nitrosoguanidine (8); hft = high frequency donor
‡ Plasmids isolated, see Table 2.

and dilute to 200 ml; TES buffer, 50 mM Tris-Cl, 50 mM NaCl, 5 mM EDTA pH 8. Ethidium bromide, (EB), 4 mg/ml in TES.

Bacterial strains prepared as indicated in Table 1 were in the isogenic cell line, PpG 1360 Trp^- Ade^- (Cam^-) derived from PpG 1 (1). Plasmids were kindly supplied by A. M. Chakrabarty /SAL in strain AC5 (PpG 2100) (4);

M. Nozaki /TOL strain mt-2 (PpG 9) (14); P. aeruginosa strain PL1 (RP1) by Dr. J. C. Loper (15); E. coli W3110 (Col IbP9) by Dr. J. Konisky (16). The remaining strains were from the G (Gunsalus) collection. Media, growth, and conjugation conditions were as described earlier (8).

Transformation used a modification of Cohen's procedure (17) with Pseudomonas putida strain 277, Trp^- (Cam^-) as recipient. Cultures grown in Vogel-Bonner medium E (VB) containing 1% glucose and 30 µg/ml tryptophan at ca. 10^9 bacteria/ml in late exponential phase are chilled, harvested, and kept at 4° for all subsequent steps. The cells are washed by suspension in 0.5 growth volume of 15 mM NaCl, then 30 mM $CaCl_2$, and after 20 min resuspended in 0.1 growth volume of 30 mM $CaCl_2$; for transformation 10^9 viable cells and 1 to 5 µg of supercoiled plasmid DNA, also in 30 mM $CaCl_2$ and previously sterilized by incubation with $CHCl_3$, are mixed, incubated one hour, the mixture heated, 2 min in a 41° water bath, recooled to 0°, diluted in 5 ml VB-glucose-tryptophan broth and plated immediately and, after 18 hours, at 30°. Viable cells and transformants are enumerated respectively on VB-glucose-tryptophan agar and PAS-tryptophan agar with the selective carbon source (naphthalene as vapor, sodium salicylate 0.05% or sodium p-toluate 0.2%) after 3 to 5 days at 30° (7 days for NAH). After purification by streaking on L-agar, single clones are tested for Trp^- and $plasmid^+$ phenotypes by replication.

Plasmids are prepared from bacterial stocks stored at -70° in L broth-5% DMSO, grown overnight in L broth tubes on a shaker at 30° and used at 0.25% to inoculate L broth at about 1/5 the rated capacity in erlenmeyer flasks. Incubation for 12 hrs (rotary shaker 30°) yields ca. $2 \cdot 10^9$ cells/ml ($A_{660nm} \simeq 2$). (For radiolabelled cells 250 ml amounts of L broth are supplemented with 25 µCi methyl [^{14}C]thymine (61 mCi/mmole) for Col IbP9 or 30 µg/ml cold adenine plus 500 µCi 8[^{3}H]adenine (21 Ci/mmole) for metabolic plasmids.) To isolate plasmids the 12 hr cultures are chilled to 4° for harvest and subsequent steps. Three g ($3 \cdot 10^{12}$) cells are suspended in 20 ml of Tris-sucrose with the aid of a motor-driven Potter-Elvejhem Teflon homogenizer, and diluted to 100 ml in Tris-sucrose. Spheroplasts are prepared by adding 20 ml of lysozyme, and after 20 min 20 ml of 0.25M EDTA, pH 8. Lysis is induced by adding a mixture of 50 ml of 5M NaCl and 40 ml Sarkosyl-DOC at 10° with gentle agitation. After one minute, recool in an ice bath, allow to stand 2 hrs, and clear by centrifugation (27,000 rpm for 20 min, Beckman SW 27 rotor). The supernatant is decanted into a chilled graduated cylinder without dislodging the loosely-adhering gelatinous pellet and 0.25 volume of 50% CARBOWAX 6000 (18) added to precipitate the plasmid DNA. After standing overnight, 4° and centrifugation, (16,000 xg 5 min), two liquid phases appear; they are decanted, adhering residue removed with Kimwipes and the pellet suspended in about 30 ml TES. The supercoiled (cc) and open-circular plus linear (oc,l) DNA are separated by equilibrium banding in a CsCl-EB gradient (CsCl 8.4 g, EB 1 ml added to 8 ml of the DNA in TES, 40,000 rpm, 44 hrs, Beckman Ti 50 rotor). The cc and oc,l DNA bands, visible on excitation with 545 nm light, are collected separately through a hole in the bottom of the tube with the aid of a syringe-driven fractionator (19). Each fraction is twice extracted by gentle mixing with an equal volume of CsCl-saturated isopropanol, dialyzed against two changes, 200 volumes each, of TES and the DNA stored in glass vials (previously heated 1 hour at 120° to inactivate DNAses).

Sedimentation velocities, $S_{20,w}$, are measured in sucrose gradients or in a Spinco Model E analytical centrifuge. For sedimentation in sucrose the [^{3}H]labelled metabolic, and [^{14}C]Col Ib plasmid DNAs, in 0.1 ml, are layered on 9 ml 10-30% sucrose gradient and after centrifugation (35,000 rpm, 100 min, 20°, Beckman SW 36 rotor) 0.1 ml fractions collected on 24 mm dia. Whatman No. 1 filter paper. After air drying and sequential washes with

Table II. Metabolic Plasmid Molecular Weight Estimates

Strain	Plasmid		$S_{20,w}$*	M. Wt. $\cdot 10^{-6}$*
			S/E†	S/E†
2121	NAH	cc†	70/67	41/37
		oc	46/47	42/43
2115	SAL	cc	69/69	39/39
		oc	46/48	42/46
2114	TOL	cc	77/82	/55
		cc	/71	/42
		?	48/55	
		cc	40/40	13/13
		oc?	24/23	
2101	RP1	cc	62/55¶	32/25
		oc	43/43¶	36/36
W3110	col IbP9	cc	73/74‡	44/45
		oc	45/48‡	40/47

*Calculated according to Hudson and Vinograd (20).

†S = sucrose gradient; E = Spinco Model E;
cc = covalently closed; oc = open circular

$S_{20,w}$, ‡Clewell and Helinski (24); ¶Grinsted et al. (25).

several portions each of 5% TCA, 95% ethanol and ethyl ether, ^{3}H and ^{14}C are counted in a Packard scintillation spectrometer in vials containing 5 ml of toluene with 4 g/l PPO.

For analytical Model E sedimentation the samples (DNA 4 µg/ml, 50 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA 1.32 g/ml CsCl) are pipeted directly into 30 mm double sector cells with a large bore 5 ml pipet and boundary sedimentation followed with high intensity UV source and photoelectric scanner at 12,000 rpm, 20°. $S_{20,w}$ values, calculated from A_{265} scanner readings according to Hudson and Vinograd (20) employed the density and relative viscosity indicated from the n_d^{25} and 63% GC, CsCl DNA density = 1.723 g/ml, corrected to Na DNA.

Double stranded DNA was determined as the EB-DNA complex (21) in a Hitachi-Perkin Elmer UPF-2A spectrofluorometer using calf thymus DNA (Sigma, type 1) as standard.

RESULTS AND DISCUSSION

Table 2 indicates the size of the aromatic metabolic plasmids, /NAH, /SAL, and /TOL derived from sedimentation rates in sucrose gradients, with thymine labelled Col Ib as marker, and in the analytical model E centrifuge. As indicated in the table, the agreement between the two methods is within acceptable limits for both the supercoiled and open circular-linear DNA.

The /NAH and /SAL plasmids sedimented as single cc and oc components of nearly equal size; respectively 42 and 43 million molecular weight, taken from the oc values. Two contour lengths, 18 and 27 μ, respectively 5 and 95% of the total open circles, were previously reported for the /SAL plasmid profiles under the electron microscope (9). Assuming 2.07 daltons/μ (26), these correspond to 37 and 56 million daltons. The sedimentation data coincide more nearly with the smaller species observed in the EM; the larger was not observed in our preparations. The /TOL plasmid DNA sedimented as two species corresponding to 55 and 42 million, and occasionally a smaller one of 13 million. It is tempting to speculate that the larger may be a cointegrate or catane of the smaller fragments.

Resistance plasmids of the P1 group (RP1, RP4, etc.) are in the 40 million dalton range (10,25). RP1, transferred to P. putida by conjugation (Table 1) has a sedimentation rate in agreement with the values reported by Grinsted et al. (25) for this plasmid from P. aeruginosa; Chakrabarty (22) has also transferred RP1 to P. putida by transformation. Parenthetically, the P. aeruginosa fertility factors FP2 and FP39, respectively 59 and 52 million molecular weight (27), resemble the /TOL species in size. The report (28) that treatment of RP1 with the Eco R1 restriction enzyme produces a single fragment, whereas the aromatic metabolic plasmids /NAH and /SAL yield 10 fragments and /TOL a proportionately larger number (Crawford, Farrell, Gunsalus, Ito, personal

communication) suggests that these restriction and metabolic plasmids may be quite unrelated.

Transformation with the super-coiled metabolic plasmid DNA according to Cohen (17) and Chakrabarty (22), see Methods, with strain 277 as recipient, yields between 10^2 and 10^3 transformants per microgram of DNA. Several other PpGl mutants gave similar frequencies. Transformants were not observed if the calcium treatment or the DNA addition was omitted. Although optimum conditions may increase the yields of transformants, these data clearly identify the metabolic plasmid traits with the isolated DNA's.

Several procedures have now been used to isolate resistance, metabolic, and fertility plasmids from fluorescent pseudomonads. Three conditions appear to increase plasmid yields considerably: one, a higher ionic strength, in the range of 1.0 to 1.5 molar NaCl; two, the use of gentle detergents, e.g. Sarkosyl in concentrations between 1 and 4%; and, three, a lysis temperature in the 10 to 15° C range, as suggested by Worcel (23). Although data are not available for plasmids in excess of 100 million molecular weight, preliminary experiments indicate the /CAM and possibly the /OCT plasmid cointegrate (2) may be isolated and characterized by these procedures.

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REFERENCES

1. Rheinwald, J.G., Chakrabarty, A.M. and Gunsalus, I.C. (1973) Proc. Nat. Acad. Sci. USA 70, 885-889.
2. Chakrabarty, A.M., Chou, G. and Gunsalus, I.C. (1973) Proc. Nat. Acad. Sci. USA, 70, 1137-1140.
3. Dunn, N.W. and Gunsalus, I.C. (1973) J. Bacteriol. 114, 974-979.
4. Chakrabarty, A.M. (1972) J. Bacteriol. 112, 815-823.
5. Wong, C.L. and Dunn, N.W. (1974) Gen. Res., Camb., 23, 227-232.
6. Williams, P.A. and Murray, K. (1974) J. Bacteriol. 120, 416-423.

7. Shaham, M., Chakrabarty, A.M. and Gunsalus, I.C. (1973) J. Bacteriol. 116, 944-949.
8. Chou, G.I.N., Katz, D., and Gunsalus, I.C. (1974) Proc. Nat. Acad. Sci. USA 71, 2675-2678.
9. Palchaudhuri, S., and Chakrabarty, A. (1976) J. Bacteriol. 126, 410-416.
10. Jacoby, G.A., Jacob, A.E. and Hedges, R.W. (1976) J. Bacteriol. 127, 1278-1285.
11. Clarke, P.H. and Richmond, M.H. Eds. (1975) Genetics and Biochemistry of Pseudomonas. John Wiley and Sons, N.Y. pp. 168-190.
12. Chakrabarty, A.M. (1976) Ann. Rev. Genetics 10, 7-30.
13. Novick, R.P., Clowes, R.C., Cohen, S.N., Curtiss III, R., Datta, N. and Falkow, S. (1976) Bacteriol. Rev. 40, 168-189.
14. Nozaki, M., Kagamiyama, H. and Hayaishi, O. (1963) Biochem. Z. 338, 582-590.
15. Korfhagen, T.R., Loper, J.C. and Ferrel, J.A. (1975) Antimicrobial Agents and Chemotherapy, 7, 64-68.
16. Isaacson, R.E. and Konisky, J. (1974) Molec. Gen. Genet. 132, 215-221.
17. Cohen, S.N., Chang, A.C.Y. and Hsu, L. (1972) Proc. Nat. Acad. Sci. USA 69, 2110-2114.
18. Humphreys, G.O. Willshaw, G.A. and Anderson, E.S. (1975) Biochim. Biophys. Acta, 383, 457-463.
19. Tan, K.B. (1972) Anal. Biochem. 45, 306-308.
20. Hudson, B. and Vinograd, J. (1969) Nature, 221, 332-337.
21. LePecq, J. and Paoletti, C. (1966) Anal. Biochem. 17, 100-107.
22. Chakrabarty, A.M., Mylroie, J.R., Friello, D.A. and Vacca, J.G. (1975) Proc. Nat. Acad. Sci. USA 72, 3647-3651.
23. Worcel, A. and Burgi, E. (1974) J. Mol. Biol. 82, 91-105.
24. Clewell, D.B. and Helinski, D.R. (1970) Biochem. Biophys. Res. Commun. 41, 150-156.
25. Grinsted, J., Saunders, J.R., Ingram, L.C., Sykes, R.B. and Richmond, M.H. (1972) J. Bacteriol. 110, 529-537.
26. Lang, D. (1970) J. Mol. Biol. 54, 557-565.
27. Pemberton, J.M. and Clark, A.J. (1973) J. Bacteriol. 114, 424-433.
28. Jacob, A.E. and Grinter, N.J. (1975) Nature (London) 255, 504-506.