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RESTRICTION ENDONUCLEASE SITES AND AROMATIC METABOLIC PLASMID STRUCTURE

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SUMMARY: Plasmid DNA isolated from Pseudomonas putida strains which metabolize naphthalene (Nah^+), salicylate (Sal^+), or toluate (Tol^+) contain multiple restriction endonuclease sites. Eco R1, Bam H1, and Hind III produce 10, 11 and 12 fragments respectively from plasmids NAH (∿48 Md)§ and SAL (∿49 Md). The restriction fragments from NAH and SAL are identical in molecular weight for each enzyme except for 1 fragment which is ∿2 Md larger in SAL than its corresponding fragment in NAH. When the ∿80 Md TOL plasmid was treated with the same endonucleases, the yield was 17, 18 and 14 fragments, respectively; correspondence of the latter to the NAH-SAL fragments is suggested.

Self transmissible plasmids carry genes for growth of Pseudomonas on many natural and synthetic organic compounds (1-4). Several were isolated from cesium chloride-ethidium bromide equilibrium gradients as supercoiled DNA and the size was estimated by sedimentation (5) or by electron microscopy (6). Genetic analyses of the fluorescent genospecies, especially of P. putida and P. aeruginosa, by enzyme complementation (9), conjugal crosses, transduction and transformation (10,11,1-3) have been summarized recently (12-14).

To compare the restriction endonuclease hydrolytic patterns of three possibly related aro plasmids (plasmids which code for dissimilation of an aromatic molecule) each plasmid was transferred from the wild type isolate to a strain used previously for genetic studies (1,2,5) which does not yield plasmid DNA in the plasmid isolation procedure. The current unexplained variability in reports on aro plasmid genetic (4,11) and physical (5-8) properties and inferred host effects (5,7,11) prompted the use of the isogenic carrier cell. The aro plasmids are associated with self and chromosome fertility (1,2,12), and code

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§Md = megadalton.

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for gene products initiating aromatic ring opening and oxidation of catechol by the "meta" pathway (15). The plasmid coded physiological functions are lost on mitomycin c curing (1,2,11,12). Dunn and Gunsalus (2) provided the genetic data for NAH on one of two strains studied; Chakrabarty (3) did similar studies for SAL. Williams with Murray (4) reported the plasmid control of the TOL properties in the much studied mt-2 strain and initiated, with Duggleby, Bayley, Worsey and Broda (8), the analysis of the molecular genetics of TOL plasmids for multiple strains.

This paper reports the DNA fragments formed from three of the known aromatic plasmids from an isogenic cell environment by three type II restriction endonucleases, each with a different 6 base pair palindromic sequence.

MATERIALS AND METHODS

The recipient PpG 1360, a CAM deleted $Trp^-Ade^-Cam^-$ strain, was crossed with an auxotrophic mutant of each of the wild type aro^+ strains to provide an isogenic cell background for isolation of the plasmid DNA according to Johnston and Gunsalus (5). The PpG strain numbers are /NAH 2121, /SAL 2115, and /TOL 2114.

Transformation of the Trp^-Cam^- recipient PpG 277 (trpB615/CAM^d) with supercoiled DNA from equilibrium density gradients identified the plasmid phenotypes. PpG 277 failed to show plasmid DNA by two isolation procedures, an alkaline denaturation procedure by Duggleby et al. (8) and the sarkosyl lysing procedure by Johnston and Gunsalus (5).

All reagents and glassware used in preparation and analysis were autoclaved prior to use. Three units of restriction endonucleases, provided by Boehringer-Mannheim, were used per ∿1 µg plasmid DNA. The DNA was added in not more than 30 µl of TES buffer (50 mM Tris-Cl pH 8, 50 mM NaCl, 5 mM EDTA), diluted to 100 µl with the suppliers recommended reaction buffer for each enzyme and incubated 3 hr at 37°C. The reactions were stopped by adding 100 µl of 25 mM EDTA, 0.01% bromophenol blue in 20% glycerol and heating immediately to 60°C for one minute, then cooling quickly to room temperature. Electrophoresis of the digests was carried out according to Ito et al. (16) in 0.8% agarose (Calbiochem) with a 36 mM Tris-Cl buffer, pH 7.8, 30 mM NaH_2PO_4, 1 mM EDTA, employing a vertical gel apparatus similar to the Roberts et al. apparatus (17). The linear DNA fragments were separated after about five hours with 50 mA constant current across 3 mm x 15 cm^2 gel at room temperature. The gels were transferred to 250 ml of electrophoresis buffer with 0.5 µg/ml ethidium bromide added. The fluorescence of the DNA bands in the gel, emitted on excitation by short wavelength UV from a low pressure mercury arc, was photographed after 15 minutes or more with Polaroid No. 57 film through a Kodak No. 23A red filter.

RESULTS AND DISCUSSION

The data in Table 1 indicate the properties of the DNA from the three isolated aromatic plasmids NAH, SAL, and TOL when subjected to digestion with

Table 1. Metabolic Plasmid DNA Restriction Endonuclease Digest

Endonuclease			Eco Rl			Bam Hl			Hind III		
DNA fragment	λ	φ29	/NAH	/SAL	/TOL	/NAH	/SAL	/TOL	/NAH	/SAL	/TOL
					(Mass in megadaltons)						
A	13.7	6.1	14	15	10.8	15.8	15.8	14.3	10.7	12.5	13
B	4.5	3.9	14	14	7.4	15.8	15.8	12	10.5	10.5	12.5
C	3.5	1.1	4.8	4.5	6.6	4.1	5.5	10	6.4	6.4	11.0
D	3.5	0.54	3.7	3.6	4.8	3.1	3.1	9.4	4.2	4.2	6.7
E	3.0	0.36	3.0	2.9	4.2	3.1	3.1	8.5	3.6	3.6	5.6
F	2.4		2.2	2.2	3.5	2.5	2.5	5.6	2.5	2.6	4.6
G			2.0	1.8	3.5	1.5	1.5	4.4	2.1	2.1	4.6
H			1.6	1.7	3.2	1.3	1.3	3.9	2.0	2.0	4.0
I			1.4	1.5	2.8	1.3	1.3	3.4	1.4	1.4	3.6
J			1.0	1.3	2.5	0.8	0.8	2.7	1.3	1.3	3.5
K					2.2	0.5	0.5	2.1	1.1	1.1	3.1
L					2.0			1.9	0.8	0.8	2.7
M					1.8			1.9			1.9
N					1.7			1.4			1.4
O					1.5			1.2			
P					0.9			1.1			
Q					0.6			0.9			
R								0.7			
Number of Fragments	6	5	10	10	17	11	11	18	12	12	14
Sum $M_R \cdot 10^6$	30	12	47	48	76	50	51	85	46	48	79

Estimated Molecular Weight:	/NAH	/SAL	/TOL
EM	--	54*	78.1†
Sedimentation	37-41§	39§	55§

*Palchaudhuri and Chakrabarty (6). †Duggleby et al. (8).
§Johnston and Gunsalus (5).

Type II restriction endonucleases. The products were separated by electrophoresis in agarose gel and their sizes estimated from plots of log molecular weight vs. migration of λ and φ29 DNA and their Eco RI digests as internal standards (18). Two points are immediately apparent: a) within the precision of the methods, the sum of DNA in the fragments produced from each plasmid by single endonuclease hydrolysis approximates the molecular weight estimated by

electron microscopy (6-8); and b) the number and sizes of the NAH and SAL products are strikingly similar. With Bam Hl, the slightly high sums of DNA products may arise from limited accuracy in determining molecular weight in agarose gels of plasmid fragments above 10 Md. The 10 to 20 restriction sites per plasmid are within the anticipated range for procaryote DNA of 50-80 Md with endonucleases which cleave 6 base pair palindromes. Separation of fragments is well within the analytical range of current procedures (18).

Plasmid phenotypes were identified by transformation (5) into a plasmid free strain, PpG 277. Plasmid DNAs were then reisolated from Nah^+, Sal^+, and Tol^+ transformed clones. Their restriction endonuclease digestion patterns were indistinguishable from the respective patterns of the original plasmid DNAs used in the transformations.

The recurrent analogies between the NAH and SAL plasmid DNA digests with three restriction enzymes and their common control of salicylate oxidation, meta cleavage of catechol, and self fertility, lead us to the working hypothesis of a common plasmid origin. The insertion of a 1-2 Md fragment of DNA coding for the gene products which control the dissimilation of naphthalene to salicylic acid into an essential region in the NAH plasmid could result in the SAL plasmid, which initiates its coded metabolic function at the dissimilation of salicylic acid. This insertion on SAL would then correspond to a piece on fragments A, from Eco RI and Hind III, and C from Bam HI action and is verifiable by the current work of ordering the restriction fragment within the plasmids. Genetic crosses already in progress via transduction and transformation of mutant strains containing the two plasmids, and physical evidence from heteroduplex observation of NAH and SAL, clearly can further resolve these questions. The TOL plasmid treated with Hind III yielded 14 DNA segments compared to 12 each from NAH and SAL. With Eco RI and Bam HI the number of fragments is proportional to the plasmid mass, i.e. 17-18 vs. 10-11 / $\sim$80 Md vs. 46-48 Md plasmid--ratio $\sim$1.6. A second working hypothesis can serve to examine the eight TOL·Hind III products which are similar in size to the largest of the NAH·Hind III and SAL·Hind III

fragments. This comparison leaves unpaired four NAH•Hind III - SAL•Hind III fragments: G, J, K, L--2.1, 1.3, 1.1, 0.8 (sum 5.3 Md); and six TOL•Hind III fragments: A--13; E, F, G--5.6, 4.6, 4.6; J, K--3.5, 3.1 (sum 34.4 Md). The discrepancy in the comparison of the TOL•Hind III E or F fragments to the small remainder from the NAH• or SAL•Hind III pattern is perhaps due to the mass difference between the two plasmid sets, TOL vs. NAH-SAL. Again the fragment sequences in the plasmids will afford valuable clues.

Duggleby, et al., (8) have made electron microscope (EM) measurements of TOL DNA and have also prepared Eco R1 digests and run agarose gel electrophoretic separations (8). Their EM data indicate a genome size of 78.1±1 Md for TOL mt-2 from the wild type cell. Of seven other Tol^+ isolates yielding plasmid DNA of 74-80 Md, three were larger, 103-170 Md, and one smaller, ∿52 Md; one strain contained circular DNA of 6 sizes in the range of 25-202 Md. These workers photographed the TOL mt-2•Eco RI digest by ethidium bromide fluorescence in agarose gel on Ilford FP4 film and scanned it on a MK III double beam recording micro densitometer. A reproduction of the scan, depicted in their Figure 3, shows 19 to 22 peaks. By estimating double bonds from intensity they report about 29 Eco RI fragments. Our fluorescent intensities were not quantitated and the fragment number is thus subject to upward revision should multiples be shown. Conversely, in our work the sum of digest fragments from 3 restriction endonuclease hydrolysates approximates 80 Md, which we tentatively accept as a correction on our earlier estimate of TOL mt-2 DNA size in the PpG 1 cell line (5). The Williams group did not present size estimates of the Eco RI fragments.

As indicated earlier (5) several reports on P. putida plasmids are still divergent. For the SAL plasmid in strain AC 36, Palchaudhuri and Chakrabarty (6) report from EM measurements circular DNA of contour lengths 18 and 27 μm (approximately 36 and 54 Md) and more recently Palchaudhuri (7), from the same strain, reports again the EM contour lengths of 18 and 26.5 μm and adds 31.4 and 48.8 μm. The 31.4 μm molecule is a fertility factor and it is suggested that the 48.8 μm is an 18 + 31.4 μm cointegrate. The AC 36 strain is a CAM^d Ade^-

auxotroph derived from the PpGl cell line. The DNA from the Sal^+ strain PpG 2100 used here would seem to correspond to the 26.5-27 μm molecule of Palchaudhuri and Chakrabarty (6) and Palchaudhuri (7).

In summary, three aromatic metabolic plasmids carried by an isogenic Pseudomonas putida strain of the fluorescent pseudomonad genospecies exhibit common genetic and molecular properties. The similarity in number and size of fragments produced with each restriction endonuclease implies the plasmids are related and suggests the potential for further studies on the molecular level.

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