Determination of genome size of *Pseudomonas aeruginosa* by PFGE: analysis of restriction fragments

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Received March 20, 1990; Revised and Accepted April 9, 1990

ABSTRACT

Genomic DNA size was measured in three strains of *Pseudomonas aeruginosa*, ATCC 29260 (exotoxin A), ATCC 33467 (type I smooth) and ATCC 33468 (type 2 mucoid) by transverse alternating field electrophoresis of restriction fragments. Because of the high (67%) G + C content of *Pseudomonas aeruginosa*, restriction enzymes that recognize sequences with at least 4 AT base pairs were expected to be rare cutters. Eight enzymes produced fragments greater than 200 kb in size: *Dra*I (TTT/AAA), *Asn*I (ATT/AAT), *Hpa*I (GTT/AAC), *Afl*III (C/TTAAG), *Xba*I (T/CTAGA), *Spe*I (A/CTAGT), *Ssp*I (AAT/ATT) and *Nde*I (CA/TATG). All eight enzymes recognized one of three rare tetranucleotide sequences, TTAA, CTAG or ATAT. *Pseudomonas aeruginosa* strain 29260 has a genomic DNA size of 5573 kb. Strains 33467 and 33468 have identical restriction patterns and a possible deletion with a genomic size of 5407 kb.

INTRODUCTION

The recent development of pulsed field gel electrophoresis (PFGE) (1–4) provides the means to separate large DNA fragments and whole chromosomes up to 10,000 kb in size (5–8). Whole chromosomes from *Saccharomyces cerevisiae* and *S. pombe* are readily separated by transverse alternating field electrophoresis (TAFE). Chromosome sizes vary from 200–1140 kb for *S. cerevisiae* (9) and 3000–9000 kb for *S. pombe* (10). Human chromosomes are significantly larger, ranging from 50,000 kb (chromosome 21) to 250,000 kb (chromosome 1), and have not yet been separated successfully by alternating current electrophoresis systems (11). However, this technique, combined with restriction analysis (12), has been applied successfully to estimate sizes of circular bacterial chromosomes from *E. coli* (13), *Mycoplasma mycoides* (14) and *Haemophilus influenzae* (15–17).

We report here the genomic DNA size of three *Pseudomonas aeruginosa* strains determined with PFGE and report significant differences in restriction fragment sizes in one of the strains.

METHODS

Pulsed field gel electrophoresis

Bacterial DNA was separated in a GeneLine TAFE system (Beckman) developed by Gardiner (18). Gels were made of 1%

low endosmosis (LE) agarose (Beckman) and run at 12°C, 150 mA, constant current, in TAE buffer (0.01M Tris, 0.0001M EDTA, 0.00435M acetic acid). Pulses from 1 to 60 seconds were applied over 15–18 hr to separate 2–1600 kb DNA fragments. The gels were stained with ethidium bromide and photographed using an ultraviolet transilluminator (Spectroline, 302 nm).

DNA standards

Four DNA standards were used. *S. cerevisiae* plugs (9) and lambda concatemers (43.7 and 48.5 kb monomer) were purchased from Beckman. High molecular weight DNA standards from 50 µg lambda restriction digests (BRL) were incubated at 65°C for 10 minutes to minimize spontaneous annealing at cos sites, then mixed with 1.2 ml 1% LMP agarose and molded into plugs. Lambda HindIII digests (500 µg Sigma) were suspended in a mixture of 1.5 ml TE buffer and 1.5 ml 1% LMP agarose and molded into plugs. All plugs were stored at 4°C in TE buffer.

Pseudomonas strains

Pseudomonas aeruginosa strains ATCC 29260, 33467 and 33468 were chosen for restriction fragment analysis. Strain 33467 is smooth and strain 33468 is mucoid, but both lack indophenol oxidase usually found in *Pseudomonas* (19). Strain 29260 (PA-103) is a poor producer of proteases, but produces large amounts of exotoxin A (20). The bacteria were grown overnight at 37°C in 10 ml trypticase soy (TCS) broth with shaking, suspended in 2.0 ml TE buffer, mixed with an equal volume of 1% LMP agarose in lysis buffer and poured into plug molds. Plugs were shaken overnight at 37°C in 5 ml TE buffer containing 1M NaCl and 1 mg/ml lysozyme to lyse the cells. A second incubation was performed at 50°C for 48 hours in 5 ml TE buffer containing 1% SDS and 1 mg/ml proteinase K. Plugs were then incubated overnight at room temperature, in TE buffer with 10 mg/ml PMSF. The plugs were washed four times in TE buffer for 12 hours and stored at 4°C until used.

Restriction enzymes

Restriction enzymes, selected for their specific cleavage sites, were purchased from commercial sources and used according to the recommendations of the manufacturer. *Pseudomonas* plugs were incubated overnight in 60 µl of restriction buffer with 1–2 µl of the selected enzyme (21). All materials and utensils were autoclaved or washed in alcohol to eliminate contaminating

Table 1. Restriction enzymes, restriction sequence and largest restriction fragment resulting after *Pseudomonas* 33468 digestion. (Key: A = adenosine, C = cytosine, G = guanine, T = thymidine, U = purine, Y = pyrimidine, N = nucleotide)

All Fragments <2 kb in size >3000 fragments	All Fragments <60 kb in size >300 fragments	One or more fragment >200 kb 30–200 fragments
AluI 2 AG/CT	AatII 40 GACGT/C	AflII 340 C/TTAAG
BssHI 2 G/CGCGC	AccI 40	AsnI 370 ATT/AAT
HaeIII 2 GG/CC	GT/(AC)(GT)AC	DraI 500 TTT/AAA
HhaI 2 GCG/C	ApaI 50 GGGCC/C	HpaI 410 GTT/AAC
HinfI 2 G/ANTC	AvaI 25 C/YCGUG	NdeI 300 CA/TATG
HpaII 2 C/CGG	AvaII 3 G/G(AT)CC	SpeI 695 A/CTAGT
MboI 2 /GATC	BamHI 50 G/GATCC	SspI 200 AAT/ATT
MboII 2 GAAGAN ₈ /	BanI 25 G/GYUCC	XbaI 270 T/CTAGA
NaeI 2 GCC/GGC	BanII 7 GUGCY/C	
NspII 2 GNGCN/C	BclI 10 T/GATCA	
NspIII 2 C/YCGUG	BglI 25 GCCN ₄ /NGGC	
NspIV 2 G/GNCC	BglII 50 A/GATCT	
Pall 2 GG/CC	BstI 40 G/GATCC	
TaqI 2 T/CGA	BstEII 20 G/GTNACC	
	ClaI 50 AT/CGAT	
	DdeI 6 C/TNAG	
	EcoRI 25 G/AATCC	
	EcoRV 45 GAT/ATC	
	HaeII 25 UGCGC/Y	
	HincII 2 GTY/UAC	
	HindIII 60 A/AGCTT	
	KpnI 25 GGTAC/C	
	MluI 60 A/CGCGT	
	MspI 30 C/CGG	
	NcoI 10 C/CATGG	
	NheI 60 G/CTAGC	
	NotI 40 GC/GGCCGC	
	NruI 50 TCG/CGA	
	PstI 5 CTGCA/G	
	PvuI 15 CGAT/CG	
	PvuII 30 CAG/CTG	
	SacI 40 GAGCT/C	
	SacII 6 CCGC/GG	
	Sall 25 G/TCGAC	
	SauI 60 CC/TNAGG	
	Sau3A1 25 /GATC	
	ScaI 40 AGT/ACT	
	SfiI 50	
	GGCCN ₄ /NGGCC	
	SmaI 20 CCC/GGG	
	StuI 10 AGG/CCT	
	StyI 4 C/C(AT)(TA)GG	
	TthIII 10 GACN/N ₂ GTC	
	XhoI 20 C/TCGAG	
	XmnI 40	
	GAAN ₂ /N ₂ TTC	

DNases, and all manipulations were performed under a laminar flow hood.

The presence of plasmids was investigated in samples subjected to pulsed field electrophoresis for 18 hr (60 and 120 second pulses) without restriction cleavage (22).

RESULTS

A total of 65 restriction enzymes were used to screen for fragments from the *Pseudomonas* 33468 genome. All 65 restriction enzymes cut the genome into multiple fragments. Only 8 of the 65 enzymes (AflII, AsnI, DraI, HpaI, NdeI, SpeI, SspI and XbaI) produced fragments greater than 200 kb in size. SpeI cut the *Pseudomonas* genome into less than 35 fragments, while

Figure 1. Digestion of *Pseudomonas* strains with SpeI. Lane A lambda concatemers (48.5 kb monomer), lane B strain 29260 SpeI digest, lane C strain 33467 SpeI digest, lane D strain 33468 SpeI digest, lane E yeast.

the other seven rare cutting enzymes cut it into over 80 fragments each. Table 1 lists the restriction enzymes screened for this study, and the largest fragment detected for each enzyme.

Three tetranucleotide sequences, ATAT, TTAA and CTAG, were extremely rare in *Pseudomonas* DNA, and each of the 8 rare cutting restriction enzymes contained one of these tetranucleotides in their recognition sequences.

Strains 33467 and 33468 had identical restriction patterns with all restriction enzymes tested (AflII, AsnI, DraI, HpaI and SpeI). The fragments produced by digesting strain 29260 with the same enzymes were markedly different (figures 1–2).

To determine the size of the *Pseudomonas* genome, restriction fragments resulting from SpeI digestion were compared with standards. Strain 29260 yielded 29 fragments ranging in size from 20 to 720 kb, and strains 33467 and 33468 had 31 identical fragments ranging from 11 to 695 kb. The total size of the *Pseudomonas* genome was 5573 kb (29260) and 5407 kb (33467 and 33468) (table 2).

No plasmids were detected in either strain. Since all the

Figure 2. Digestion of *Pseudomonas* strains with AflIII and HpaI. Lane A lambda concatemers (48.5 kb monomer), lane B strain 29260 AflIII digest, lane C strain 33467 AflIII digest, lane D strain 33468 AflIII digest, lane E strain 29260 HpaI digest, lane F strain 33467 HpaI digest, lane G strain 33468 HpaI digest, lane H yeast.

bacterial DNA remained in the well during electrophoresis, these results also indicate that the *Pseudomonas* genome is intact, and that no shearing or degradation of DNA occurred during preparation, storage or electrophoresis.

DISCUSSION

Estimations of the *Pseudomonas* genome sizes vary widely. Pemberton estimated the *P. aeruginosa* genome to be 3624 kb (23), while Bak *et al.* (24), using renaturation kinetics, obtained a value of 10,440 kb. For other organisms studied by both PFGE and renaturation, the PFGE values tend to be higher than the renaturation values.

Using PFGE electrophoresis we found the *Pseudomonas aeruginosa* genomes to be 5407 kb (strains 33467 and 33468) and 5573 kb (strain 29260). Since strains 33467 and 33468 lack the enzyme indophenol oxidase, the gene for this enzyme may be deleted. Both strains were isolated from sputum of a single patient and have identical fragment patterns. They may represent one strain with a difference in expression of mucoid protein, or one strain may have produced a mutant which coexisted in the sputum and subsequent culture. Strain 29260 is very similar to strains 33467 and 33468 in genome size and number of fragments

Table 2. Restriction fragments from *P. aeruginosa* 33468 and 29260 genomes

<i>Spe I</i> 29260	<i>Spe I</i> 33468
720	695
540	500
410	430
330	385
325	325
305	312
285	310
265	265
260	260
210	230
180	220
170	180
160	170
150	160
140	150
135	145
130	105
120	100
110	99
95	70
90	50
85	37
75	36
70	34
65	29
63	24
40	23
25	21
20	16
	15
	11
Total number of fragments	
29	31
Total kilobase size of genome	
5573	5407

and is described as a fairly typical *Pseudomonas*. We found no plasmids to account for the size difference between strains. The similarity in size and number of fragments of the three genomes, however, supports the ATCC listing that all strains are of the same species.

McClelland *et al.* (21) calculated trinucleotide frequencies from sequenced Genbank DNA and found the rarest sequences to be CTA/TAG (1.44%), TAA/TTA (1.70%) and ATA/TAT (1.89%). Our own analysis of the 29 *Pseudomonas* sequences currently available in Genbank showed the three same rare trinucleotides in the order TAA/TTA (0.88%), ATA/TAT (1.11%) and CTA/TAG (1.29%). Our experimental data confirms those results, with all 8 rare cutting enzymes recognizing one of these three sequences.

We conclude that the best approach to genomic size studies is to choose restriction endonucleases with recognition sites that contain trinucleotides occurring infrequently in sequenced genomic DNA from the organism. If genomic DNA has not been sequenced, then the restriction enzymes can be selected based on G+C content of the organism.

We also conclude that PFGE provides a highly sensitive method for distinguishing different strains of bacteria. The size of the *Pseudomonas aeruginosa* genome has now been determined, and the *Spe I* restriction fragments have been identified. The next step in clarifying the gene map of *Pseudomonas aeruginosa* will be to construct a physical map of the restriction fragments.

ACKNOWLEDGEMENTS

This work was supported by the National Institute of Health grants HL-39943 and HL-36538. We thank Dr Ali Azghani for assisting with *Pseudomonas* culture methods, Dr Gokul Das for technical advice, Dr Lynn Gray for photographs, and Mrs Pat Genoway and Mrs Sara Shepherd for computer programming.

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