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Restriction Fragment Fingerprint of an Alkaliphilic *Micrococcus* sp. Y-1 Genome by Pulsed-field Gel Electrophoresis

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A genomic DNA of alkaliphilic bacterium, *Micrococcus* sp. Y-1, was analysed using the physical mapping method of pulsed-field gel electrophoresis (PFGE). Five restriction enzymes of *Ssp*I, *Hpa*I, *Xba*I, *Nde*I or *Eco*RI, which recognize the Adenine-Thymine-rich sequences of genomic DNA, were used for the generation of few (7 to 20) distinctly separate fragments, with average sizes in the range of 200~500 kb. However, the sites for *Not*I and *Sfi*I, 8 base-recognizing enzymes, were highly frequent. The genome size of this strain was determined to be 4 mega base pairs (Mb) from restriction fragments separated by PFGE. This is the first case of restriction mapping in alkaliphilic bacterium.

In previous papers (6, 7), a producer of alkaline pullulanase, *Micrococcus* sp. Y-1, was isolated. *Micrococcus* sp. Y-1 is a gram-negative, extremely aerobic and alkaliphilic bacterium. It can grow at 60°C and its bacterial growth and bacterium occurs at pH values between 6.0 and 12.0. The strain secretes enzymes that possess amylolytic and pullulannolytic activities (6). The discovery of the genus Micrococcus sp. Y-1 provided a detailed study on the alkaline bacterium (6). However, genetic information concerning this strain is limited, and only two enzymes, pullulanase and amylase, have been reported, even though it was shown that the genomic mapping results on the bacterial chromosome revealed the order of the six *Ssp*I fragments on the chromosome.

Most bacterial genomes contain one circular chromosome as determined by genetic mapping and confirmed by physical mapping (14). Two exceptions have been reported in *Rhodobacter sphaeroiedes* (15) and *Spirochetes* (2, 3). Genomic mapping can either be performed at the level of chromosomal DNA itself (physical mapping) or by following the pattern in which portions of the genomic DNA are passed to the progeny (genetic linkage mapping). The method of pulsed-field gel electrophoresis (PFGE) of large fragments of bacterial chromosomal DNA (15), generated by using rarely cutting restriction endonucleases, has made it possible to map megabase (Mb) regions of whole genomes of prokaryotes (1, 12, 13). Up to now, approximately 40 bacterial genomic maps (8) have been constructed by this way. These maps provided low physical resolution, so their utility has been primarily to demonstrate the mapping strategy and to confirm existing genetic maps (14).

The aim of this work was to continue our previous investigation (9) on the fingerprints produced by restriction enzymes that cleave infrequently, and to establish a fine fingerprint of the alkaline *Micrococcus* sp. Y-1 genome by PFGE.

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Key words: Mapping, pulsed-field gel electrophoresis, *Micrococcus* sp. Y-1 genome

MATERIALS AND METHODS

Bacterial Strain and Culture

Alkaliphilic *Micrococcus* sp. Y-1 cells (6) were grown at 50°C for 16 hours in a medium (pH 7.2) consisting of 0.8% polypeptone, 0.2% yeast extract and a basal salt mixture as decribed previously (7) and then chloramphenicol (made up in 95% ethanol, 100 mg/ml) was added to a final concentration of 180 μg/ml. The culture was maintained for 4 hours.

Preparation of Bacterial Chromosome in Agarose Plug

Agarose plugs containing genomic DNA were prepared following procedure (1). In brief, cells grown till the late log or stationary phase were pelleted by centrifugation at 3500 rpm for 10 min at 4°C in a clinical centrifuge. The pellets were then resuspended in 10 ml of the suspension buffer (10 mM Tris-HCl and 1 M NaCl, pH 7.6) and again spinned at 3500 rpm for 10 min

at 4°C. After resuspending the cell thoroughly in 2 ml of the suspension buffer, the cells were warmed in an incubator at 30~40°C and then diluted with an equal volume of 1% low melting temperature agarose (FMC Bio-Products, Rockland, Maine) in sterile water at 42°C. The agarose blocks were cast in the size of 1 by 10 by 20 mm. The blocks were transferred to the solution of 0.1 M EDTA (pH 8.0)-0.01 M Tris-0.02 M NaCl containing proteinase K (mg/ml), 0.5% sarkosyl, 1 mg/ml of lysozyme, 20 μg/ml of RNase and 40 μg/ml of phenylmethylsulfonyl fluoride (PMSF) and the blocks were incubated for 1 h at 50°C. After incubation, the blocks were stored in 0.05 M EDTA (pH 8.0) at 4°C for the next experiment.

Restriction Enzyme Digestion of DNA in Agarose Blocks and Pulsed-field Gel Electrophoresis (PFGE)

Agarose blocks (1 by 5 by 5 mm) containing 1 μg of Micrococcal DNA were subjected to single or double digestion with restriction endonucleases in 0.1 ml of the respective restriction endonuclease buffer containing 0.01% bovine serum albumin for 20 h at 37°C. For a total digestion of DNA, 10 units (U) of restriction endonuclease *Ssp*I (Boeringer), and 10 U of *Eco*RI (Promega) were used. For a partial digestion with *Ssp*I, 5 U was used. After the digestion of restriction endonuclease, the blocks were dialyzed in a buffer solution containing 10 mM Tris (pH 8.0), 1 mM EDTA and mounted on the agarose gel for the electrophoresis. The gel was casted with 1.0% Sea-Kem agarose at 55°C using 0.5 ×TBE buffer (45 mM Tris base, 45 mM borate, 1 mM EDTA, pH 8.0). The gel was electrophoresed at 14°C with a CHEF II apparatus (Bio-Rad Laboratories, Richmond, CA). For the separation of fragments between 4 and 200 kb sized, the gel was run for 20 h at 200 V with a ramp of the switch time from 5 to 25 s. For the separation of the range of 6 to 600 kb, the same electrophoresis time and voltage were used but the ramp of the switch time was from 15 to 75 s. As size markers, a lambda ladder (Promega Corp., Madison, WI) and *Saccharomyces cerevisiae* chromosomes (Bio-Rad) were used. After electrophoresis, the gels were stained in 0.5×TBE containing ethidium bromide (0.5 μg/ml) for 30 min and destained in distilled water.

Preparation of λ-DNA Marker for PFGE

100 mg of low-melting-temperature agarose was dissolved in 10 ml of 20 mM $MgCl_2$, 100 mM Tris-HCl (pH 7.6) and the solution was chilled to 37°C, and then 10 μg of λ-DNA in TE (pH 7.6) was added. After heating for 5 min at 56°C, the solution was maintained at 37°C and rapidly the solution of the following ingredients (1% PEG 8000, 1 mM ATP, 1 mM DTT, T4 DNA ligase, 0.75% agarose solution) was added. After chilling the solution with ice for 15 min, at least 3 volumes of 1×ligation buffer containing polyethylene glycol (8%) was added to blocks. Then the blocks were incubated for 24 hours at room temperature and transferred to a tube containing 10 volumes of 20 mM EDTA (pH 8.0). The blocks were stored at 4°C for usage.

RESULTS AND DISCUSSION

High GC-content-recognizing Restriction Enzymes Are Suitable for Analyzing and Genome Sizing *Micrococcus* sp. Y-1

In the previous paper, it was suggested that *Micrococcus* sp. Y-1 has a high Guanine-Cytocine content of about 70% (6). Thus, the sites of restriction enzymes that cleave at Adenine-Thymine sequences are expected to be rare in the genome of *Micrococcus* sp. Y-1. This allowed us to predict theoretically the existence of a set of rare-cutting restriction enzymes. Seven such enzymes, *Spe*I (ACTAGT), *Hpa*I (GTTAAC), *Nde*I (CATATG), *Xba*I (TCTAGA), *Dra*I (TTTAAA) or *Eco*RI (GAATTC), *Ssp*I (AATATT) were experimentally identified as being suitable for the generation of relatively few distinct fragments in *Micrococcus* sp. Y-1 genome (data not shown).

Genome size can be determined by adding the sizes of macrorestriction fragments obtained using several enzymes resolved by PFGE. The intact size of the genome of the *Micrococcus* sp. Y-1 is about 4.0 Mb (Fig. 1).

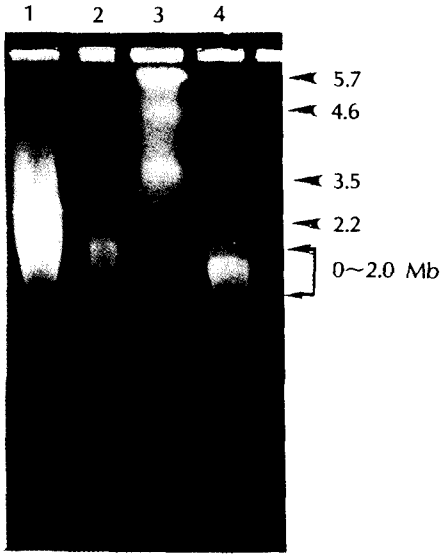

Fig. 1. PFGE of *Micrococcus* sp. Y-1 intact genomic DNA. Lanes: 1, *Micrococcus* sp. Y-1 intact genomic DNA; 2, *Thermus caldophilus* GK24 intact genomic DNA (2.4 Mb); 3, DNA size standard of yeast *Saccharomyces cerevisiae* chromosomal DNA; 4, *Micrococcus* sp. Y-1 genomic DNA digested with *Ssp*I. The numbers on the right show the positions for the DNA size standard markers of the sizes indicated. The pulse times were 30 min for 72 hours at 14°C, with ramped time, 50 Volts. The gels was 0.5% Sea Plaque agarose in 0.5 X TBE.

Compared with the genome size of other procaryotes, which range between 600 and 6,000 kb, the genome of the *Micrococcus* sp. Y-1 has average value. Probably, *Micrococcus* sp. Y-1 carries one circular chromosome. The total size of the undigested *Micrococcus* sp. Y-1 genomic DNA is about 4,000 Kb.

A chromosomal DNA of *Micrococcus* sp. Y-1 was digested as described above and it was electrophoresed under migration conditions convenient for resolving each restriction fragment: the pulse times were ramped 5 to 25 sec for 20 h at 200 volt to resolve the shorter fragments, as high molecular mass fragments (up to 1000 kb) were resolved using 15 to 75 sec ramped pulse times for 20 h at 200 volt. Fig. 2 shows the pattern of several restriction fragments separated by PFGE for *Micrococcus* sp. Y-1 genomic DNA. The molecular size of PFGE-separated fragments for each enzyme was determined with respect to the λ concatamer and *S. cerevisiae* molecular weight standards (Table I). The molecular size distribution of fragments, being specific for a particular enzyme, provides a genome sizing fingerprint. A ramp of the pulse time from 15 sec to 75 sec over 20 h was found to be optimal for a successful separation and size determination of the fragments in that size range. The separation of restriction fragments by PFGE allowed the estimation of molecular size of the genome for *Micrococcus* sp. Y-1. The genome size determined by each enzymatic digestion is given in Table I. The variation in the estimated genome size for each restriction endonuclease enzyme was within 50 to 200 kb. A double digestion with both enzymes of *Ssp*I and *Spe*I revealed 15 fragments, three of which contained comigrating bands. For separating of these comigrating bands, a ramp of the pulse time from 5 sec to 25 sec over 20 h was optimal (Fig. 2, lane 2). *Micrococcus* sp. Y-1 genomic DNA digested with *Spe*I contained 12 fragments (760, 560×2, 520, 470, 345, 320, 260, 180, 120,

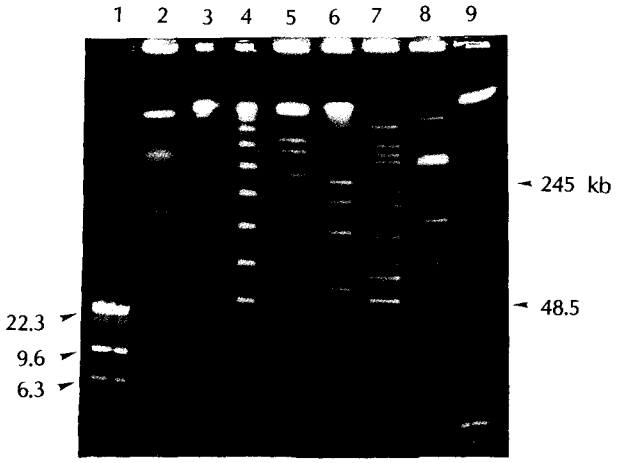

Fig. 2. PFGE of restriction endonuclease digests (*Spe*I, *Hpa*I, *Nde*I, *Xba*I, *Dra*I) of *Micrococcus* sp. Y-1 genomic DNA.
Lanes: 1, λ-DNA digested with *Hin*dIII; 2, *Micrococcus* sp. Y-1 genomic DNA digested with *Ssp*I and *Spe*I; 3, DNA size standard of yeast *Saccharomyces cerevisiae* chromosomal DNA; 4, λ-DNA concatamer; 5, *Micrococcus* sp. Y-1 genomic DNA digested with *Spe*I; 6, *Micrococcus* sp. Y-1 genomic DNA digested with *Hpa*I; 7, *Micrococcus* sp. Y-1 genomic DNA digested with *Nde*I; 8, *Micrococcus* sp. Y-1 genomic DNA digested with *Xba*I; 9, *Micrococcus* sp. Y-1 genomic DNA digested with *Dra*I. The numbers on the right show the positions for the DNA size standard markers of the sizes indicated. The pulse times were 5~25 sec. for 20 hours at 14°C, with ramped time, 200 Volts. The gels was 1.0% Sea Plaque agarose in 0.5 X TBE.

Table 1. Size of the chromosomal *Spe*I, *Hpa*I, *Xba* and *Eco*RI restriction fragments and partial *Ssp*I restriction fragments from *Micrococcus* sp. Y-1 in kilo base pairs

*Spe*I	length (kb)	*Hpa*I	length (kb)	*Xba*I	length (kb)	*Eco*RI	length (kb)	*Ssp*I-*Spe*I	length (kb)	*Ssp*I	length (kb)	Possible combination
Sp-1	760	Hp-1	800	Xb-1	960	Ec-1	730	Sd-1	592×2	Ss-1p	2910	(Ss-1,2)
Sp-2	560×2	Hp-2	720	Xb-2	470	Ec-2	710			Ss-1	2280	
Sp-3	520	Hp-3	660	Xb-3	370	Ec-3	620	Sd-2	580	Ss-2p	920	(Ss-2,4)
Sp-4	470	Hp-4	580	Xb-4	348×3	Ec-4	372	Sd-3	520	Ss-3p	820	(Ss-3,5,6)
Sp-5	345	Hp-5	540	Xb-5	302	Ec-5	360	Sd-4	330×2	Ss-4p	680	(Ss-4,5,6)
Sp-6	320	Hp-6	240	Xb-6	240	Ec-6	280	Sd-5	320	Ss-2	580	
Sp-7	260	Hp-7	220	Xb-7	150	Ec-7	180	Sd-6	185	Ss-5p	530	(Ss-3,6)
Sp-8	180	Hp-8	170	Xb-8	109	Ec-8	260	Sd-7	120	Ss-3	460	
Sp-9	120	Hp-9	50	Xb-9	97	Ec-9	127	Sd-8	45	Ss-6p	400	(Ss-5,6)
Sp-10	45	Hp-10	40	Xb-10	68	Ec-10	100	Sd-9	34	Ss-4	330	
Sp-11	30	Hp-11	31	Xb-11	48	Ec-11	80			Ss-5	280	
		Hp-12	30	Xb-12	43	Ec-12	60			Ss-6	108	
				Xb-13	29	Ec-13	48					
						Ec-14	43					
						Ec-15	40					
						Ec-16	35					
						Ec-17	30					
	4,170		4,081		3,930		4,075				4038	

* Average size: 4000 kb.

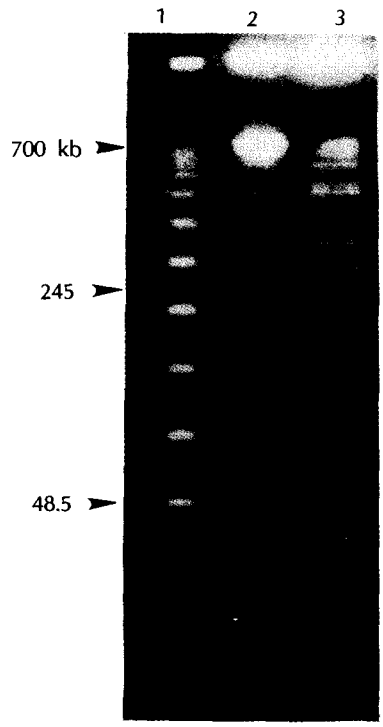

Fig. 3. PFGE of restriction endonuclease *Eco*RI digestion of *Micrococcus* sp. Y-1 genomic DNA.
Lane 1, λ-DNA concatamer; lane 2, DNA size standard of yeast *Saccharomyces cerevisiae* chromosomal DNA; lane 3, *Micrococcus* sp. Y-1 genomic DNA digested with *Eco*RI. The numbers on the right show the positions for the DNA size standard markers of the sizes indicated. The pulse times were 15~75 sec. for 20 hours at 14°C, with ramped time, 200 Volts. The gels was 1.0% Sea Plaque agarose in 0.5 X TBE.

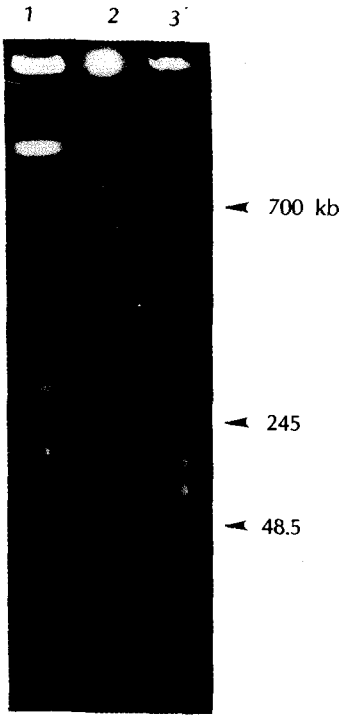

Fig. 4. PFGE of restriction endonuclease *Ssp*I partial digestion of *Micrococcus* sp. Y-1 genomic DNA.
Lanes: 1, *Micrococcus* sp. Y-1 genomic DNA partially digested with *Ssp*I; 2, DNA size standard of yeast *Saccharomyces cerevisiae* chromosomal DNA; 3, λ-DNA concatamer. The numbers on the right show the positions for the DNA size standard markers of the sizes indicated. The pulse times were 15~75 sec. for 20 hours at 14°C, with ramped time, 200 Volts. The gels was 1.0% Sea Plaque agarose in 0.5 X TBE.

45, 30) and the total size of the genomic DNA was 4,170 bp (Table 1). *Micrococcus* sp. Y-1 genomic DNA digested with *Hpa*I contained 12 fragments (800, 720, 660, 580, 540, 240, 220, 170, 50, 40, 31, 30) and the total size of the genomic DNA was 4,081 bp (Table 1). *Micrococcus* sp. Y-1 genomic DNA digested with *Xba*I had 14 fragments (960, 470, 370, 348×3, 302, 240, 150, 109, 97, 68, 48, 43, 29) and the total size of the genomic DNA was 3,930 bp (Table 1). The chromosomal restriction pattern for *Eco*RI involved seventeen restriction fragments ranging between 730 and 30 kb (Fig. 3, lane 3). The restriction endonuclease *Ssp*I cleaved the *Micrococcus* sp. Y-1 genomic DNA into 6 fragments ranging in size from 108 to 2,280 kb (2,280, 580, 460, 330, 280, 108) and the total size of the genomic DNA is 4,038 bp (Table 1). The genome size can be determined by adding the sizes of the macrorestriction fragments obtained with several enzymes resolved by PFGE. This value is similar to the size of the intact chromosome. On the other hand, *Dra*I (TTTAAA) digestion produced only two fragments, while *Not*I (GCGGCCGC) and *Sfi*I (GGCCN5CCGG), 8 base-recognizing restriction enzymes cut the genome DNA into many fragments were too small and numerous for genome sizing (data not shown).

The analysis of chromosomal restriction fragment length polymorphisms of bacterial strains has been reported (12) using frequent-cutting restriction enzymes. However, a large number of chromosomal fragments generated by these enzymes produce complicated banding patterns that are difficult to resolve and compare. Even though high-resolution restriction fragment fingerprints of bacterial chromosomes can be produced with frequent-cutting restriction enzymes using two-dimensional electrophoresis, comparison with multiple enzyme fragmentation can not be done in one such experiment (10). The present PFGE study shows a direct comparison of large restriction fragment band patterns generated by each of the seven infrequently cutting enzymes. The molecular size of several bacterial genomes such as *E. coli* (14), *Mycoplasma mycoides* (11), *Haemophilus influenza* (5) and *Pseudomonas aeruginosa* (4) has been reported based on PFGE. There is hardly any PFGE data available on the genome sizes of Micrococcus species. According to the present PFGE study, the genome size of Micrococcus sp. Y-1 was estimated. Although any

contribution from the plasmid in the estimation of genome size would be insignificant, PFGE experiments using 15 sec pulse time, for 14 h with undigested genomic DNA, showed no evidence of any plasmid in the strain (data not shown).

On the other hand, the chromosomal restriction pattern for *Sspl* was completely established and the following step was done to identify the DNA fragments linkage in order to determine the physical map. The chromosomal restriction patterns for *Sspl* involved six restriction fragments ranging from 2,280 to 108 kb (Table 1). A partially digested fragment of 2910 kb is Ss-1 and Ss-2 (Ss-1p); another fragment of 920 kb is Ss-2 and Ss-4 (Ss-2p); another fragment of 820 kb is Ss-3, Ss-5 and Ss-6 (Ss-3p); another fragment of 680 kb is Ss-4, Ss-5 and Ss-6 (Ss-4p); another fragment of 530 kb is Ss-3 and Ss-6 (Ss-5p); another fragment of 400 kb is Ss-5 and Ss-6 (Ss-6p) (Table 1 and Fig. 4). And then we detected Ss-2 and Sp-2 bands in the double digestion but not Ss-1 and Sp-1 fragments. This result is very important because with this we can determine the existence of Ss-1 fragment. An Ss-1 band is not an intact chromosome but a fragment digested with *Spel*. We can obtain more information from the results of partial digestion. An Ss-4 fragment has a Spel site but the Ss-2 fragment have not. Now we are in the process of constructing the high-density map of an industrially useful bacterium.

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