Determination of genome size and a preliminary physical map of an extreme alkaliphile, *Micrococcus* sp. Y-1, by pulsed-field gel electrophoresis

Jong Hoon Park,¹ Jae-Chan Song,² Myoung Hee Kim¹, Dae-Sil Lee² and Cheorl-Ho Kim²

Author for correspondence: Cheorl-Ho Kim. Tel: +82 42 860 4133. Fax: +82 42 860 4593.

Genome Program¹ and Laboratory of Molecular and Cellular Biology,² Genetic Engineering Research Institute, KIST, Taejeon, Korea

Large restriction fragments of genomic DNA from *Micrococcus* sp. Y-1 were separated by pulsed-field gel electrophoresis (PFGE). Since *Micrococcus* sp. Y-1 has a G + C content of approximately 70%, restriction fragments were obtained by digesting chromosomal DNA with endonucleases which recognize A + T-rich sequences. Five enzymes, *SspI*, *SpeI*, *XbaI*, *HpaI* and *EcoRI*, were used for generation of distinctly separated fragments in the size range 100–500 kb. No site for *DraI* was detected. In contrast, sites for 8-base-recognizing enzymes, but not for *NotI* and *SfiI*, were frequent. The genome size of *Micrococcus* sp. Y-1 was determined from restriction fragments separated by PFGE, and was estimated to be approximately 4061 kb. Partial digestion experiments revealed the order of the six *SspI* fragments on the chromosome.

Keywords: *Micrococcus* sp., alkaliphile, genome size

INTRODUCTION

Micrococcus sp. Y-1, a producer of alkaline pullulanase, is an extreme alkaliphile, growing at pH values from 6.0 to 12.0 and secreting amylolytic and pullulanolytic enzymes (Kim *et al.*, 1993a). The discovery of *Micrococcus* sp. Y-1 has permitted a detailed study of the enzymes of an alkaliphilic bacterium (Kim *et al.*, 1993a).

The alkaliphilic bacteria have been classified into three categories: (1) hyper-alkaliphiles, (2) extreme alkaliphiles and (3) moderate alkaliphiles. Extremely alkaliphilic bacteria of the genus *Micrococcus* grow over the pH range 8.0–12.0 (Kim *et al.*, 1993a).

Most bacterial genomes comprise one circular chromosome (Smith *et al.*, 1987). Two exceptions have been reported. One is *Rhodobacter sphaeroides*, which has two unique circular chromosomes of different sizes (Suwanto & Kaplan, 1989). The other exceptions are members of the genus *Borrelia*, such as *B. burgdorferi*, which appear to have a linear chromosome (Baril *et al.*, 1989; Ferdows & Barbour, 1989).

Genomic mapping can be performed by physical mapping or by genetic linkage mapping. Physical mapping by

PFGE of large fragments of DNA generated using infrequently cutting restriction endonucleases has made it possible to map megabase regions of eukaryotes and whole genomes of prokaryotes (Schwartz *et al.*, 1983; Renaud *et al.*, 1988; Bancroft *et al.*, 1989). Approximately 40 bacterial genome maps (Krawiec & Riley, 1990) have been constructed by this method. These maps provide poor physical resolution, so their utility has been primarily to demonstrate mapping strategy and to confirm existing genetic maps (Smith *et al.*, 1987). The aim of this work was to produce a preliminary physical map and to estimate the genome size of the extremely alkaliphilic bacterium *Micrococcus* sp. Y-1 using PFGE.

METHODS

Bacterial strain and culture. *Micrococcus* sp. Y-1 cells (Kim *et al.*, 1993a) were grown at 50 °C for 16 h in medium (pH 7.2) consisting of 0.8% polypeptone, 0.2% yeast extract and a basal salts mixture, as described by Kim *et al.* (1993b). Chloramphenicol (100 mg ml⁻¹ in 95%, v/v, ethanol) was added to a final concentration of 180 µg ml⁻¹ and cells grown for a further 4 h.

Preparation of bacterial plugs. Agarose plugs containing genomic DNA were prepared following the procedure of Bancroft *et al.* (1989). In brief, cells grown to late-exponential or stationary phase were pelleted by centrifugation at 3500 r.p.m.

Abbreviation: PFGE, pulsed-field gel electrophoresis.

for 10 min at 4 °C in a clinical centrifuge. Cells were then washed by resuspension in 10 ml buffer (10 mM Tris/HCl, 1 M NaCl, pH 7.6), followed by centrifugation. After resuspension of the cells in 2 ml suspension buffer (0.01 M Tris/HCl, pH 8.0, 0.1 M Na-EDTA, 0.02 M NaCl), the suspension was warmed in an incubator at 30–40 °C, then diluted with an equal volume of 1% (w/v) low-melting-temperature agarose (FMC Bio-Products) made up in sterile water at 42 °C. The resulting solution was then poured into a mould chamber (Bio-Rad). Solidified blocks were incubated at 37 °C for 12 h in lysozyme (Sigma) solution [1 mg ml⁻¹ in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0)] then treated overnight at 50 °C with an equal volume of buffer containing proteinase K (1 mg ml⁻¹; Boehringer Mannheim), 0.5% N-lauroylsarcosine (Sigma) and 1 mM EDTA, pH 8.0. Proteinase activity was inhibited by washing the blocks twice for 1 h at room temperature in phenylmethylsulphonyl fluoride (40 µg ml⁻¹; PMSF). The blocks were then stored in 0.05 M Na-EDTA (pH 8.0) at 4 °C.

Restriction enzyme digestion of DNA using agarose blocks and PFGE. Agarose blocks containing 1 µg micrococcal DNA were subjected to single or double digestion with restriction endonucleases. DNA was digested for 20 h at 37 °C in 0.1 ml restriction endonuclease buffer containing 0.01% bovine serum albumin. For total digestion of DNA, 10 U restriction endonuclease *SspI* (Boehringer Mannheim) and 10 U *EcoRI* (Promega) were used. For partial digestion with *SspI*, 5 U were used. After digestion, blocks were equilibrated in TE buffer, then mounted on the teeth of an electrophoresis comb. The gel was cast with 1.0% (w/v) SeaPlaque agarose (FMC) at 55 °C in 0.5 × TBE buffer (10 mM Tris/borate, 1 mM EDTA). The gel was electrophoresed at 14 °C in a CHEF DR II apparatus (Bio-Rad). For separation of fragment sizes between 4 and 200 kb, the gel was run for 20 h at 200 V with a ramped pulse time from 1 to 15 s. The same time and voltage were used for separation of the size range 6–600 kb, but the ramped pulse time was from 15 to 75 s. Bacteriophage λ DNA digested with *HindIII* (Promega) was used as size markers for DNA fragments smaller than 50 kb, and *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomes (Bio-Rad) were used as size markers for high-molecular-mass DNA fragments. After electrophoresis, gels were stained with 0.5 × TBE containing ethidium bromide (0.5 µg ml⁻¹) for 30 min, then destained in distilled water.

RESULTS AND DISCUSSION

Selection of suitable enzymes for PFGE analysis of *Micrococcus* sp. Y-1

Micrococcus sp. Y-1 has a G + C content of approximately 70% (Kim *et al.*, 1993a). Sites for restriction enzymes that cleave at A + T-rich sequences are expected to be rare in the genome of *Micrococcus* sp. Y-1. Seven such enzymes, *SspI* (AATATT), *SpeI* (ACTAGT), *HpaI* (GTTAAC), *XbaI* (TCTAGA), *NdeI* (CATATG), *DraI* (TTTAAA) and *EcoRI* (GAATTC) were identified as suitable for generation of relatively few numbers of distinct fragments from the *Micrococcus* sp. Y-1 genome.

Micrococcus sp. Y-1 genome size

Chromosomal DNA of *Micrococcus* sp. Y-1 was digested and electrophoresed under migration conditions appropriate for resolving each restriction fragment. Pulse times were ramped from 5 to 25 s for 20 h at 200 V in order to

resolve shorter fragments. High-molecular-mass fragments (up to 1000 kb) were resolved using 15–75 s ramped pulse times for 20 h at 200 V. Fig. 1 shows several patterns of restriction fragments separated by PFGE. The molecular size of PFGE-separated fragments for each enzyme was determined with respect to λ concatamers and *S. cerevisiae* molecular mass standards (Table 1). To obtain good separation and size determination in the size range of the molecular mass standards, a ramped pulse time from 15 s to 75 s over 20 h was optimum. Separation of restriction fragments by PFGE allowed estimation of the molecular size of the *Micrococcus* sp. Y-1 genome.

Genome size was determined by adding the sizes of the restriction fragments obtained using several enzymes and resolved by PFGE. The genome size determined from each enzymic digestion is given in Table 1. The average size of the intact chromosome of *Micrococcus* sp. Y-1 was approximately 4061 kb (Table 1).

The chromosomal restriction pattern for *EcoRI* involved 17 restriction fragments ranging in size from 730 to 30 kb (Table 1). Double digestion with *SspI* and *SpeI* resulted in 14 large fragments which were resolved by electrophoresis

Fig. 1. PFGE of restriction endonuclease digests (*SspI*, *SpeI*, *XbaI* and *HpaI*) of *Micrococcus* sp. Y-1 genomic DNA. Lanes: 1, *Micrococcus* sp. Y-1 genomic DNA digested with *SspI* and *SpeI*; 2, λ-DNA concatamer; 3, DNA size standard of *S. cerevisiae* chromosomal DNA; 4, *Micrococcus* sp. Y-1 genomic DNA digested with *SspI*; 5, *Micrococcus* sp. Y-1 genomic DNA digested with *SpeI*; 6, *Micrococcus* sp. Y-1 genomic DNA digested with *XbaI*; 7, *Micrococcus* sp. Y-1 genomic DNA digested with *HpaI*. The numbers on the right show the positions for the DNA size standard markers. The ramped pulse times were 15–75 s for 20 h at 14 °C and 200 V. The gels were 1.0% SeaPlaque agarose in 0.1 × TBE.

Table 1. Sizes of the chromosomal *SspI*, *SpeI*, *XbaI*, *HpaI* and *EcoRI* restriction fragments from *Micrococcus* sp. Y-1

<i>SspI</i>	Length (kb)	<i>SpeI</i>	Length (kb)	<i>XbaI</i>	Length (kb)	<i>HpaI</i>	Length (kb)	<i>EcoRI</i>	Length (kb)
Ss-1	2280	Sp-1	760	Xb-1	960	Hp-1	800	Ec-1	730
Ss-2	580	Sp-2a	560	Xb-2	470	Hp-2	720	Ec-2	710
Ss-3	460	2b	560	Xb-3	370	Hp-3	660	Ec-3	620
Ss-4	330	Sp-3	520	Xb-4a	348	Hp-4	580	Ec-4	372
Ss-5	280	Sp-4	470	4b	348	Hp-5	540	Ec-5	360
Ss-6	108	Sp-5	345	4c	348	Hp-6	240	Ec-6	280
		Sp-6	320	Xb-5	302	Hp-7	220	Ec-7	260
		Sp-7	260	Xb-6	240	Hp-8	170	Ec-8	180
		Sp-8	180	Xb-7	150	Hp-9	53	Ec-9	127
		Sp-9	120	Xb-8	109	Hp-10	42	Ec-10	100
		Sp-10	45	Xb-9	97	Hp-11	34	Ec-11	80
		Sp-11	34	Xb-10	68	Hp-12	30	Ec-12	60
				Xb-11	48			Ec-13	48
				Xb-12	43			Ec-14	43
				Xb-13	26			Ec-15	40
								Ec-16	35
								Ec-17	30
Total	4038		4174		3927		4089		4075
Average size: 4061 kb									

Table 2. Size of the chromosomal partial *SspI* restriction fragments from *Micrococcus* sp. Y-1

Size (kb)	Possible combination	Total size of fragments (kb)
2910	Ss-1 + Ss-2	2860
920	Ss-2 + Ss-4	910
820	Ss-3 + Ss-5 + Ss-6	848
680	Ss-4 + Ss-5 + Ss-6	718
530	Ss-3 + Ss-6	568
400	Ss-5 + Ss-6	388

(Fig. 1, lane 1) and four small fragments not shown. For further separation of small size bands, a pulsed ramp time from 5 to 25 s over 20 h was optimum (data not shown). *DraI* treatment produced no fragments, while the 8-base-recognizing restriction enzymes *NotI* (GCGGCCGC) and *SfiI* (GGCCN5GGCC) cut the genomic DNA into many fragments which were too small and numerous for genome sizing (data not shown).

Analysis of bacterial chromosomes using frequent-cutting restriction enzymes has been reported (Krawiec & Riley, 1990). However, the large number of chromosomal fragments generated by these enzymes produce complicated banding patterns which 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 cannot be done in one experiment (Poddar & Maniloff, 1989). The PFGE-determined molecular sizes of several bacterial genomes, such as *Escherichia coli* (Smith *et al.*, 1987), *Mycoplasma mycoides* (Pyle & Finch, 1988), *Haemophilus influenzae* (Kauc *et al.*, 1989) and *Pseudomonas aeruginosa* (Hector & Johnson, 1990) have been reported. In this study, the genome size of *Micrococcus* sp. Y-1 was estimated using PFGE. Although the contribution of any single plasmid to the estimation of genome size is insignificant, PFGE experiments using a 15 s pulse time for 14 h with undigested genomic DNA showed no evidence of any plasmid in the strain (data not shown).

Physical map of the *Micrococcus* sp. Y-1 genome

Digestion of chromosomal DNA with *SspI* resulted in six restriction fragments ranging in size from 2280 to 108 kb (Table 1). Partial digestion experiments suggested that the partial fragments of 2910 kb, 920 kb, 820 kb, 680 kb, 530 kb and 400 kb were combinations of the fragments from completely digested DNA (Table 2). The Ss-2 fragment (580 kb; Table 1) was also detected after double digestion with *SspI* and *SpeI*, while the Ss-1 (2280 kb) and Sp-1 (760 kb) fragments were not (Fig. 1, lane 1).

A complete *SspI* restriction map of the *Micrococcus* sp. Y-1 chromosome was obtained. The six *SspI* bands formed a circle, demonstrating the circular topology of the chromosome (Fig. 2). Use of *SpeI*, *XbaI*, *HpaI* and *EcoRI* will allow development of a high density map of the

Fig. 2. Physical map of the *Micrococcus* sp. Y-1 genome. The six *SspI* bands are aligned in the inner circle and are listed in Table 1.

circular chromosome. If, in the future, *Micrococcus* sp. Y-1 genes are cloned, hybridization methods can be used to confirm the physical map of this genome. This work constitutes, to our knowledge, the first estimation of the genome size of an alkaliphilic *Micrococcus* sp.

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