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Analysis of the Genome of *Symbiobacterium toebii* by Pulsed-Field Gel Electrophoresis

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Abstract We have studied the genome of an obligately commensal thermophile, *Symbiobacterium toebii*. The chromosome was extracted from pure cultures of *S. toebii* recently established. Total DNA of *S. toebii* was resolved by pulsed-field gel electrophoresis (PFGE) into discrete numbers of fragments by digestion with the endonuclease *Ssp*I, *Spe*I, *Xba*I, and *Hpa*I. Estimated sizes of fragments produced by the four enzymes and their sum consistently yielded a total genome size of 2.8 Mb. Because restriction endonucleases *Not*I and *Swa*I, recognizing 8 bp, released too many fragments, these enzymes could not be used for the estimation of the genome size. Considering no mobility of undigested genome under PFGE, the genome of *S. toebii* appears to be circular. The presence of extrachromosomal DNA in *S. toebii* was excluded by the results of the conventional 1% agarose gel electrophoresis and the field inversion gel electrophoresis of undigested *S. toebii* DNA.

Key words: *Symbiobacterium toebii*, obligately commensal thermophile, genome

Symbiobacterium is a thermophilic bacterium showing obligately commensal interaction with a thermophilic *Bacillus* strain [8, 11, 13]. Growth of *S. toebii* essentially requires growth of a thermophilic *Bacillus* strain. Although *S. toebii* has high G+C content (65 mol %) of genomic DNA, phylogenetic analysis based on 16S rRNA sequences placed this novel bacterium among the members of the gram-positive, low G+C content anaerobic thermophilic bacteria within the *Bacillus-Clostridium* subphylum [unpublished results].

S. toebii contains thermostable biocatalysts useful in industrial applications [4, 6]. Despite the availability of cloned genes, pure cultures of *S. toebii* have been unsuccessful. However, recently, we isolated a single colony of *S. toebii* by using crude extracts and culture supernatants of the partner *Bacillus* strain [unpublished results].

The recent development of pulsed-field gel electrophoresis (PFGE) allows the separation and analysis of large restriction fragments of an entire bacterial chromosome [2, 11]. Rare cutting enzymes employed in conjunction with PFGE have allowed species identification and strain classification within the same species and have also provided useful data for estimating genome size and mapping [12].

Preparation of pure genomic DNA of *S. toebii* was unsuccessful because the genomic DNA from mixed cultures always contained *Bacillus* DNA. Here, we report the estimation of chromosome size and investigation of extrachromosomal content of *S. toebii* prepared from recently established pure cultures.

Materials and Methods

Bacterial Strain and Culture Conditions

S. toebii (KCTC 0685BP) requires essential growth factors from its partner strain thermophilic *Bacillus* sp. SK-1 (KCTC 0306BP). Therefore, *S. toebii* was cultivated with the following medium. The composition of basal medium (BM) was 0.05% (w/v) L-tyrosine, 0.5% (w/v) polypeptone, 0.1% (w/v) yeast extract, 0.1% (w/v) KH_2PO_4, 0.3% (w/v) K_2HPO_4, 0.1% (w/v) $NaNO_3$, and 0.05% (w/v) $MgSO_4 \cdot 7H_2O$. After autoclaving BM, 1.5 g of crude extract and 500 ml of culture supernatant of *Bacillus* strain were filter-sterilized and added to 500 ml of BM as growth factors. Because growth of *S. toebii* was inhibited by oxygen, we cultivated *S. toebii* under nitrate-reducing conditions for 48 h [11].

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Preparation of Intact Chromosomal DNA

Agarose plugs containing genomic DNA were prepared following the procedure of Park and Kim [9]. Cells were washed by suspending cell paste in 10ml buffer (0.01 M 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 cell suspension was warmed in an incubator at 40–45°C, then diluted with an equal volume of 1.2% (w/v) low-melting-temperature agarose (InCert™ Agarose, FMC Bio-Products, U.S.A.) made up in sterile water at 42°C. The resulting solution was then poured into a mould chamber (Bio-Rad, U.S.A.). Solidified blocks were incubated at 37°C for 12 h in lysozyme (Sigma, St. Louis, U.S.A.) solution [1 mg/ml in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)]. The blocks were treated overnight at 50°C with an equal volume of buffer containing proteinase K (1 mg/ml; Boehringer Mannheim, Germany), 0.5% *N*-laurylsarcosine (Sigma) and 1 mM EDTA, pH 8.0. Proteinase activity was inhibited by washing the blocks twice for 2 h at room temperature in phenylmethylsulphonyl fluoride (40 μg/ml; PMSF). The blocks were then stored in 0.05 M Na-EDTA (pH 8.0) at 4°C.

Restriction Endonuclease Digestion of DNA Gel Plugs and PFGE

One DNA gel plug containing 2 μg *S. toebii* DNA was equilibrated with 200 μl of restriction endonuclease buffer (supplied with the enzyme) at 4°C for 20 min. The buffer was replaced, and DNA was digested with 15 to 20 U of endonuclease for 20 h at 37°C in a 100 μl reaction volume. After digestion, the plugs were equilibrated in TE buffer, then mounted on the teeth of an electrophoresis comb. The gel was electrophoresed at 14°C in a CHEF DR II apparatus (Bio-Rad). For separation of fragment sizes between 40 and 300 kb, the gel was run for 22 h at 200 V with a ramped pulse time from 5 to 25 sec. The same time and voltage were used for separation of the size ranges 200–600 kb, but the ramped pulse time was from 25 to 75 sec. CHEF DNA size standard (8–48 kb, Bio-Rad) was used as

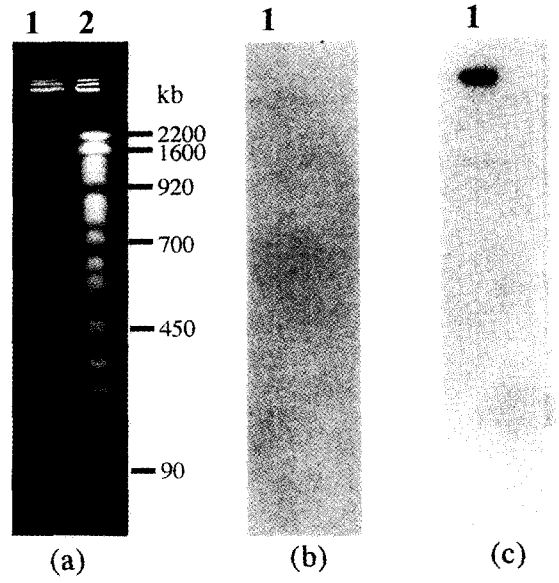

Fig. 1. Undigested DNA from a pure culture of *S. toebii* separated by PFGE. (a) Ethidium bromide-stained gel, containing undigested *S. toebii* DNA (*lane 1*) and size standard *S. cerevisiae* chromosomes (*lane 2*). Electrophoretic conditions of 200 V, 25–75 sec switch rate, and a 23 h run time were used. (b) Autoradiograph of a Southern blot hybridization of this gel probed with ^{32}P-labelled D-amino acid aminotransferase gene from *B.* sp. SK-1. (c) The same blot, stripped and then probed with ^{32}P-labelled tyrosine phenol-lyase gene.

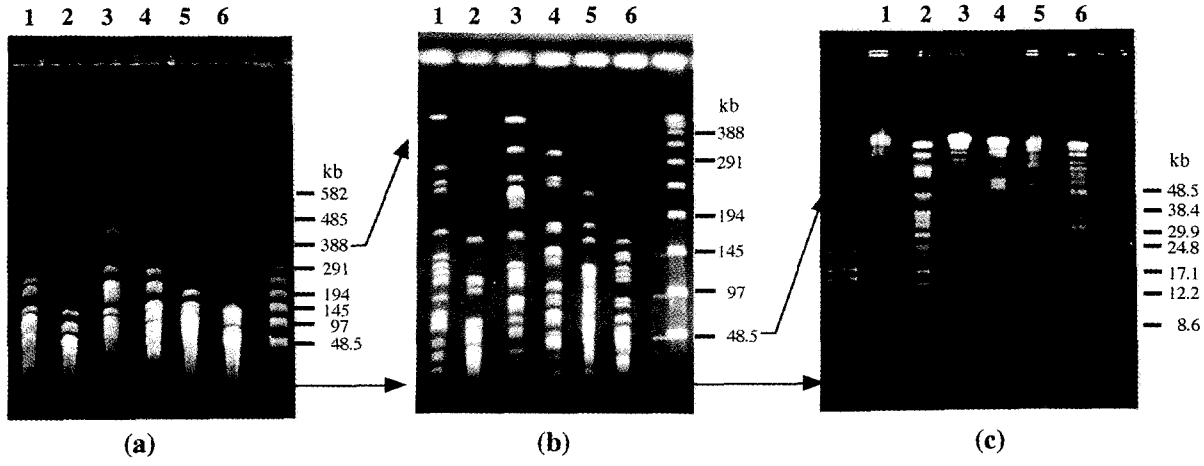

Fig. 2. Restriction fragments of *S. toebii* genomic DNA separated by PFGE. Lanes show digestion with *Ssp*I (*lane 1*), *Eco*RI (*lane 2*), *Spe*I (*lane 3*), *Xba*I (*lane 4*), *Hpa*I (*lane 5*), and *Hin*dIII (*lane 6*). (a) High-range size fragment; ramped pulsed time from 25 to 75 sec, during 22 h. (b) Mid-range size fragments; ramped pulsed time from 5 to 25 sec, for 22 h. (c) Low-range size fragments; ramped pulsed time from 0.5 to 3 sec, for 18 h. Size in kb are indicated on the left and right.

Table 1. Size analysis of PFGE-separated restriction fragments of *S. toebii* genome.

Name and size (kb) of fragments:							
*Ssp*I		*Spe*I		*Xba*I		*Hpa*I	
Fragment	Size	Fragment	Size	Fragment	Size	Fragment	Size
Ss1	490	Sp1	450	Xb1	320	Hp1	240
Ss2	280	Sp2	320	Xb2	270	Hp2	190
Ss3	270	Sp3	270	Xb3	260	Hp3	170
Ss4	250	Sp4	250	Xb4	190	Hp4	130
Ss5	180	Sp5	180	Xb5	180	Hp5	125
Ss6	150	Sp6	140	Xb6	160	Hp6	120
Ss7	140	Sp7	130	Xb7	150	Hp7	115
Ss8	130	Sp8	120	Xb8	120	Hp8	110
Ss9	120	Sp9	110	Xb9	115	Hp9	105
Ss10	100	Sp10	95	Xb10	100	Hp10	100
Ss11	80	Sp11	90	Xb11	90	Hp11	95
Ss12	70	Sp12	80	Xb12	80	Hp12	85
Ss13	60	Sp13	70	Xb13	75	Hp13	80
Ss14	55	Sp14	60	Xb14	70	Hp14	75
Ss15	54	Sp15	55	Xb15	60	Hp15	73
Ss16	50	Sp16	50	Xb16	50	Hp16	70
Ss17	48	Sp17	36	Xb17	50	Hp17	68
Ss18	45	Sp18	25	Xb18	50	Hp18	66
Ss19	40	Sp19	20	Xb19	45	Hp19	62
Ss20	34	Sp20	17	Xb20	30	Hp20	60
Ss21	32	Sp21	13	Xb21	28	Hp21	55
Ss22	31			Xb22	26	Hp22	54
Ss23	28			Xb23	24	Hp23	52
Ss24	22			Xb24	22	Hp24	51
Ss25	21			Xb25	18	Hp25	50
Ss26	20			Xb26	17	Hp26	49
Ss27	14			Xb27	15	Hp27	47
Ss28	13			Xb28	11	Hp28	40
Ss29	12			Xb29	8	Hp29	38
Ss30	10					Hp30	37
Ss31	8					Hp31	34
						Hp32	27
						Hp33	24
						Hp34	23
						Hp35	19
						Hp36	18
						Hp37	17
						Hp38	16
						Hp39	13
						Hp40	8
Total	2,857		2,801		2,634		2,811
Average size:	2,775						

[a]Each restriction fragment was named by the initial letters of the enzyme used to produce it (Ss, *Ssp*I; Sp, *Spe*I; Xb, *Xba*I; Hp, *Hpa*I). The fragments from each digest were numbered in order, from the largest to the smallest.

the size marker for DNA fragments smaller than 50 kb, and lambda DNA concatemers (Bio-Rad) was used as the size marker for high-molecular-mass DNA fragments. After electrophoresis, gels were stained with 0.5 × TBE containing ethidium bromide (0.5 μg/ml) for 30 min, then destained in distilled water.

Southern Blot Hybridization

After electrophoresis, DNA fragments in the gels were depurinated in 0.2 N HCl for 20 min and vacuum transferred onto positively charged nylon membranes (Hybond N+, Amersham, Braunschweig, Germany) by the alkaline transfer procedure [10]. Nick-translated probes were prepared from the [α-^{32}P]dCTP-labelled D-amino acid aminotransferase gene (unpublished data) from *Bacillus* sp. SK-1 or the tyrosine phenol-lyase gene [5] from *S. toebii*. Conditions for hybridization and washings were done as described by Sambrook *et al.* [10]. To reprobe the blots, hybridized probes were removed by incubating the blot in 0.4 N NaOH for 20 min at room temperature and

the hybridization procedure was repeated with different probes.

RESULTS AND DISCUSSION

Preparation of Genomic DNA from Pure Cultures of *S. toebii*

Contamination of *Bacillus* DNA was the main problem in preparing chromosomal DNA of *S. toebii* from mixed cultures. Using cells from pure cultures of *S. toebii*, we prepared agarose plugs containing sufficiently pure genomic DNA of *S. toebii* (Fig. 1). In Southern blot of undigested *S. toebii* DNA, the sample was hybridized with the ^{32}P-labelled tyrosine phenol-lyase gene probe (Fig. 1c). The lack of a hybridization signal of pure-cultured *S. toebii* DNA with the D-amino acid aminotransferase gene fragment of *B.* sp. SK-1 as a probe confirmed that this sample contains undetectable level of *Bacillus* DNA contamination (Fig. 1b).

Selection of Suitable Restriction Enzymes for PFGE Analysis of *S. toebii*

The genome of *S. toebii* has a high guanine and cytosine content (65 G+C mol%) [10]. Therefore, in the present work, the restriction enzymes *Ssp*I, *Spe*I, *Xba*I, and *Hpa*I which recognize adenosine and thymine (A+T)-rich sequences were found to give a reasonable number of DNA fragments in the *S. toebii* genome. All the fragments separated by PFGE were greater than 200 kb in size. However, because the four enzymes generated too many fragments from *S. toebii* genome, none of them were suitable for the constructing of physical mapping.

Other restriction enzymes including 8-base-recognizing enzymes were tested to select suitable restriction enzymes. *Mlu*I treatment produced no fragments, while the 8-base-recognizing restriction enzymes *Not*I (GCGGCCGC) and *Swa*I (GGCCN5GGCC) cut the genomic DNA into many fragments which were too small and numerous for genome sizing (data not shown).

Size Determination of *S. toebii* Chromosome

The total genome size was determined by adding the sizes of the restriction fragments obtained using each enzyme and resolved by PFGE (Fig. 2). The genome size determined from each enzymatic digestion is given in Table 1. The average size of the intact chromosomal DNA of *S. toebii* is approximately 2,775 kb (Table 1). As shown in Fig. 1a, undigested *S. toebii* DNA remained primarily within the wells, even under PFGE conditions normally used for separation of linear fragments in the 0.1-1.0 Mb size range. These results suggest that *S. toebii* has a circular genome, consistent with most prokaryotic organisms. PFGE experiments using a 25-75 sec pulse time for 23 h with undigested genomic DNA showed that no plasmid was present in the *S. toebii*. Additionally, the presence of extrachromosomal DNA in *S. toebii* was excluded considering that no plasmid was detected on conventional 1% agarose gel electrophoresis.

Obligately parasitic bacteria require numerous factors for their growth, and they generally live in a constant or rich environment. The relatively small size (about 1 Mb) of their chromosomes could reflect the absence of functions required for respiration and survival in fluctuating ecosystems [1, 3, 7]. However, *S. toebii* has a relatively large chromosome compared with other strict parasitic bacteria. This fact implies that the association between *S. toebii* and *Bacillus* sp. SK-1 might not be as strict as that of other parasitic bacteria.

This work constitutes, to our knowledge, the first estimation of the genome size of a *Symbiobacterium* sp. These results provide a framework for future studies on the organization of the *S. toebii* genome.

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