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Analysis of the genome of the gram-negative moderate halophiles *Halomonas* and *Chromohalobacter* by using pulsed-field gel electrophoresis

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Abstract The genomes of 11 moderately halophilic bacteria belonging to the genera *Halomonas* and *Chromohalobacter* have been analyzed by restriction endonuclease digestion and pulsed-field gel electrophoresis (PFGE). By using the infrequently cutting restriction endonucleases *Spe*I and *Swa*I, highly characteristic fingerprintings were obtained for each of the isolates studied. On the basis of the lengths of the *Spe*I and *Swa*I fragments, separated by PFGE, the genome size of the 11 strains studied was estimated. The genome size for 8 *Halomonas* strains ranged from 1450 to 2830 kb, whereas for the 3 *Chromohalobacter* strains studied it ranged from 1770 to 2295 kb. Finally, we show that macrorestriction fingerprints could be a useful tool to elucidate the taxonomic position of bacteria belonging to the *Halomonas–Deleya* complex.

Key words Moderately halophilic bacteria · *Halomonas* · *Chromohalobacter* · Genome analysis · Pulsed-field gel electrophoresis

Introduction

Moderately halophilic bacteria are a group of extremophilic microorganisms defined as those that grow optimally in media containing 3%–15% (w/v) NaCl (Kushner and Kamekura 1988). They are represented by a limited number of species, including some methanogenic members of the Archaea, as well as a variety of Bacteria (Ventosa 1994). This group of microorganisms is very heterogeneous, and several gram-negative species belonging to the genera *Halomonas*, *Deleya*, *Volcaniella*, *Halovibrio*, and *Chromohalobacter* constitute a branch within the gamma subclass of the Proteobacteria, not well defined from a taxonomic point of view (Dobson and Franzmann 1996; Dobson et al. 1993; Mellado et al. 1995). Recently, we proposed that *Volcaniella eurihalina* should be transferred to the genus *Halomonas* (Mellado et al. 1995), and latterly Dobson and Franzmann (1996) proposed that the members of the genera *Halomonas*, *Deleya*, and *Halovibrio* should be placed in a single genus, *Halomonas*, and emended the description of this genus.

During recent years, these extremophiles have been considered of great interest because of their biotechnological potential (Galinski and Tindall 1992; Ventosa and Nieto 1995), notably for producing enzymes (amylases, proteases, lipases, nucleases) of industrial interest (Kamekura 1986; Ventosa and Nieto 1995) and accumulating a variety of organic compounds, called compatible solutes, useful as enzymes or cell-stabilizing agents (Galinski 1993). Selective methods for detecting and identifying these microorganisms are needed to gain insights into the population and genome dynamics of moderate halophiles in natural environments, for detecting novel strains of biotechnological importance, and for assessing the fate of genetically engineered microorganisms. On the other hand, there is little information available about the structure and organization of the genome of moderate halophiles. To date, only the genome size of the moderate halophile *Salinivibrio costicola* has been determined (Mellado et al. 1997). The aim of the present study was to analyze several moderate halophiles by pulsed-field gel electrophoresis (PFGE) to determine their genome size and their typical fingerprinting pattern, so as to assess the use of this tool for a better definition of the taxonomy of this heterogeneous group of bacteria.

Materials and methods

Bacterial strains and culture conditions

The moderately halophilic bacteria used in this study are listed in Table 1. These strains were grown in a saline

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Table 1. Bacterial strains used in this study

Bacterial strain	Source	Reference
Halomonas:		
H. halodurans	ATCC 29686^T	Hebert and Vreeland 1978
H. eurihalina	ATCC 49336^T	Mellado et al. 1995
H. eurihalina	F 2-12	Quesada et al. 1990
H. elongata	ATCC 33173^T	Vreeland et al. 1980
H. elongata	ATCC 33174	Vreeland et al. 1980
H. halmophila	ATCC 19717^T	Dobson et al. 1990
H. israelensis	ATCC 43985^T	Huval et al. 1995
H. subglaciescola	UQM 2927^T	Franzmann et al. 1987
Chromohalobacter:		
C. marismortui	ATCC 17056^T	Ventosa et al. 1989
C. marismortui	A-492	Ventosa et al. 1989
C. marismortui	A-100	Ventosa et al. 1989

^{T}, type strain

medium containing 10% (w/v) total salts (Ventosa et al. 1982) to which 5 g/l yeast extract (Difco, Detroit, USA) was added. The composition of the salt solution was as follows (g/l): NaCl, 81; $MgCl_2$, 7; $MgSO_4$, 9.6; $CaCl_2$, 0.36; KCl, 2; $NaHCO_3$, 0.06; NaBr, 0.0026 (Ventosa et al. 1982), pH 7.2. Solid media contained 17 g/l Bacto-agar (Difco). All cultures were incubated at 37°C.

Preparation of intact genomic DNA and restriction digests

Pulsed-field gel electrophoresis (PFGE) was performed after preparation of intact genomic DNA and further restriction digestion. Gel inserts were prepared as described by Smith and Cantor (1987). Restriction endonuclease digests were performed in 150 µl of restriction buffer in a 1.5-ml eppendorf tube for each piece of gel insert, with 8–10 U of enzyme. The gel inserts were equilibrated in 150 µl of the corresponding buffer for 15 min on ice, the buffer was aspirated, the tube was filled with fresh buffer, and the appropriate enzyme was added. This mixture was maintained on ice for 10–15 min to allow the enzyme to diffuse into the agarose insert before digestion at 37°C. Digestions were performed in a shaking water bath for at least 4 h. After digestion, the buffer was aspirated, and the gel insert was dialyzed for at least 45 min at 37°C by adding 1.5 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) before placing the gel insert into the running gel. The sizes of the restriction fragments were determined by comparison with standard size markers consisting of a lambda ladder PFG marker (48.5–1018 kb; New England Biolabs, Beverly, USA), low-range PFG marker (0.1–200 kb; New England Biolabs), and *Saccharomyces cerevisiae* 334 chromosome plugs (Beckman; Palo Alto, USA).

Separation of DNA fragments

To separate DNA fragments, a contour-clamped homogeneous electric field (CHEF) electrophoresis system (Carle and Olson 1984) (Bio-Rad CHEF DRII system; Richmond, USA) was used. For this study, we used a 1% agarose gel (SeaKem GTG agarose; FMC Corp., Rockland, USA) and various pulse times depending on the range of resolution needed (Birren et al. 1988). For better separation of some particular fragments forming clusters of two or three bands, special electrophoresis conditions were used. Other bands were assumed to represent doublets or triplets on the basis of the results of at least two electrophoretical analyses in which the intensity of such bands was compared to that of the bands already probed to contain more than one fragment. The mean size of each band was determined from several independent measurements and at least at two different pulse times. The running buffer temperature was 11 ± 1°C. For the CHEF system, a constant voltage of 200 V and 0.08 A was selected. After electrophoresis, gels were stained with ethidium bromide (0.5 mg/ml) before photography with 302-nm UV light illumination.

Results

Restriction analysis of genomic DNA

Because the guanine plus cytosine content of the moderately halophilic bacteria studied ranged from 52 to 63 mol% (Dobson and Franzmann 1996; Mellado et al. 1995) and complete DNA sequences of these bacteria are not yet available, it was not possible to predict the restriction enzymes that could generate a number of fragments appropriate to be separated by PFGE. For this reason, the DNAs from 11 moderate halophiles belonging to the genera *Halomonas* and *Chromohalobacter* were treated with a large number of restriction endonucleases. The following restriction endonucleases did not digest the DNA: *Ase*I, *Dra*I, *Hpa*I, *Mbo*I, *Not*I, *Pac*I, *Sfi*I, *Ssp*I, and *Xba*I. We found that only digestion with *Swa*I and *Spe*I yielded an appropriate number of fragments.

Figure 1 shows the banding pattern of the moderate halophiles studied. *Swa*I patterns allowed a clear differentiation between the different moderate halophiles, and even within the same species there was a noticeable degree of polymorphism. The conditions used for the electrophoresis were different depending on the size ranges of the fragments to be separated (Birren et al. 1988); the typical running conditions were 200 V and 0.08 A (constant voltage). To obtain the optimal DNA fingerprinting, representing the complete genome of the bacteria, pulse times were ramped between 90 and 140 s during 24 h for DNA preparations with *Swa*I and between 30 and 60 s during 21 h for preparations digested with *Spe*I. The presence of DNA fragments smaller than 20 kb was studied by loading amounts of DNA larger than usual (0.7–0.8 µg) and running the electrophoresis at 200 V for 23 h with specific pulse times.

Genome size of moderately halophilic bacteria

The molecular size of the different genomes was determined by adding the sizes of the different fragments obtained after endonuclease digestion of the DNA and PFGE

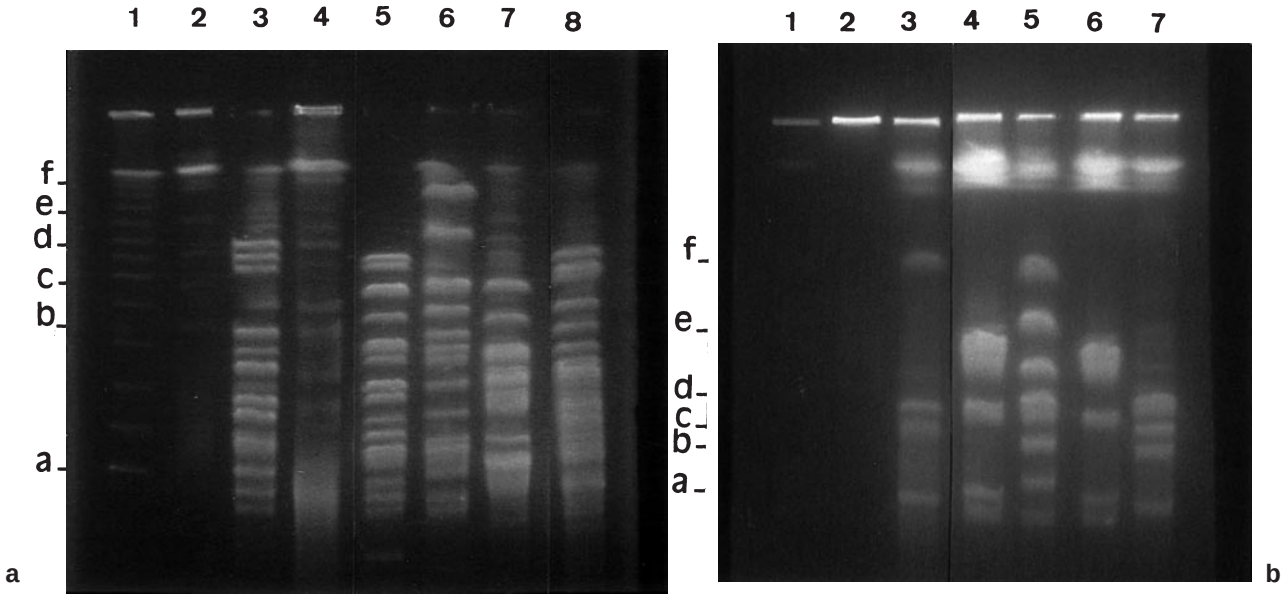

Fig. 1a,b. Ethidium bromide-stained pulsed-field gels of (**a**) *Spe*I-digested DNA from *Chromohalobacter marismortui* strain A-100 (*lane 3*), *Halomonas halophila* CCM 3662 (*lane 4*), *Chromohalobacter marismortui* ATCC 17056 (*lane 5*), *Halomonas israelensis* ATCC 43985 (*lane 6*), *Halomonas halmophila* ATCC 19717 (*lane 7*), and *Halomonas subglaciescola* UQM 2927 (*lane 8*). *Lane 1*, size standards of lambda DNA concatemers (*a*, 48.5 kb); *lane 2*, size standards of *Saccharomyces cerevisiae* strain YNN 295 chromosomes (*b*, 225 kb; *c*, 285 kb; *d*, 365 kb; *e*, 450 kb; *f*, 565 kb). **b** *Swa*I-digested DNA from *Halomonas halodurans* ATCC 29686 (*lane 3*), *Halomonas elongata* ATCC 33173 (*lane 4*), *Halomonas elongata* ATCC 33174 (*lane 5*), *Halomonas subglaciescola* UQM 2927 (*lane 6*), and *Halomonas eurihalina* ATCC 49336 (*lane 7*). *Lane 1*, size standards of *Saccharomyces cerevisiae* strain YNN 295 chromosomes (*b*, 285 kb; *c*, 365 kb; *d*, 610 kb; *e*, 825 kb; *f*, 945 kb); *lane 2*, size standards of lambda DNA concatemers (*a*, 145.5 kb)

Table 2. Number of bands detected and estimated size of the genome of representative strains of *Halomonas* and *Chromohalobacter*, digested with *Spe*I and *Swa*I restriction endonucleases

Bacterial strain	*Spe*I		*Swa*I	
	No. bands	Size (kb)	No. bands	Size (kb)
H. halodurans ATCC 29686^T	11	1837	4	1935
H. eurihalina ATCC 49336^T	14	2213	5[a]	2215
H. eurihalina F2-12	12	2279	6	2300
H. elongata ATCC 33173^T	13[a]	2245	6	2750
H. elongata ATCC 33174	11[b]	2400	7	2830
H. halmophila ATCC 19717^T	9[b]	1450	ND[c]	ND
H. israelensis ATCC 43985^T	14	2490	ND	ND
H. subglaciescola UQM 2927^T	14	2475	5	2510
C. marismortui ATCC 17056^T	15	2210	6	2295
C. marismortui A-492	8	1770	4	2000
C. marismortui A-100	12	2055	3	2270

[a] One double band
[b] Two double bands
[c] ND, not determined

analysis. The values obtained with the enzymes *Swa*I and *Spe*I are given in Table 2. Addition of the sizes of the *Spe*I bands suggests a size for the genome of the moderate halophiles studied ranging from 1450 to 2490 kb, while the size determined from *Swa*I digestion ranged from 1935 to 2830 kb. The genome sizes of *Halomonas elongata* ATCC 33173^T and *H. elongata* ATCC 33174, determined on the basis of *Spe*I, were significatively smaller than that estimated from the *Swa*I-generated fragments. The possible reason for this large difference could be the diffusion through the gel of those fragments smaller than 50 kb.

Discussion

The organization and stability of the genome of moderate halophiles have not been extensively investigated. In this study, the genome sizes of several moderately halophilic bacteria have been determined. There are several difficulties associated with determining DNA sizes by PFGE. A relatively minor problem is that the small fragments often leave the gel, especially with the longer times needed to separate the large fragments. However, such fragments add

little to the total genomic size and can be visualized by shorter electrophoresis times. Another problem is that fragments of approximately the same size may be interpreted as a single fragment. The possibility of doublet or triplet bands is suggested because of the increased density or width of the band, as was illustrated by *Spe*I digestion of *H. halmophila* in Fig. 1a. It is noteworthy the fact that genomes of different species of the same genus can have marked differences in size; in this way, *H. eurihalina* or *H. elongata* had larger genomes than *H. halmophila* (see Table 2).

For strains of the same species, the sizes are more homogeneous. The three strains of *Chromohalobacter marismortui* analyzed presented a calculated size of the genome of about 2000 kb. This genome size is similar to those recently determined of six moderately halophilic strains of the species *Salinivibrio costicola*, after digestion with the enzymes *Sfi*I or *Mbo*I (Mellado et al. 1997), for which the sizes ranged from 2100 to 2600 kb. However, they can be considered small in comparison with other bacterial species that have been so far studied (Cole and Girons 1994). We have recently sequenced the 16S rDNA of the type strain of *C. marismortui* ATCC 17056, as well as of other three isolates (A-65, A-100, and A-492). The sequences of the latter three strains showed a 16S rDNA homology of 99% (Mellado et al. 1995). However, comparison of the 16S rDNA of these three strains with the type strain ATCC 17056 resulted in a percentage of similarity of 97%. In this work, we show that the isolates A-492 and A-100 are clearly two different strains in spite of the high level of 16S rDNA similarity. Therefore, we showed that the PFGE technique is useful for distinguishing these isolates, which were very difficult to differentiate by the methods used so far.

In conclusion, our study has permitted the determination for the first time of the genome size of different moderate halophiles belonging to the genera *Halomonas* and *Chromohalobacter* and has shown polymorphism among these extremophilic organisms. On the other hand, we have shown that macrorestriction fingerprinting can be a useful tool for elucidating the taxonomic position of bacteria belonging to the *Halomonas–Deleya* complex, when the use of other taxonomic techniques has been less than satisfactory for this group. PFGE will also be useful in future studies for constructing physical and genetic maps of the chromosome of these interesting extremophilic microorganisms.

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