Sizing chromosomes and megaplasms in haloarchaea

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PFGE was used for the genomic analysis of different species and strains belonging to four out of the six recognized haloarchaeal (halobacterial) genera. All of them were found to carry one chromosome from 1·8–3 Mb, and usually several, but at least one, large plasmids of approximately 90–680 kb, which were detected in supercoiled and linear forms. From the data gathered, chromosomal size appears to be conserved at genus level, whereas plasmid composition and size seems to be subjected to certain variability.

Keywords: megaplasms, haloarchaea, chromosome, pulsed-field gel electrophoresis

INTRODUCTION

Extremely halophilic Archaea have been classified into six recognized genera (*Halobacterium*, *Haloarcula*, *Haloferax*, *Halococcus*, *Natronobacterium* and *Natronococcus*) (Juez, 1989) to which two new genera, *Halorubrum* and *Natrialba*, have been added recently (Kamekura & Dyll-Smith, 1995). Genetic and genomic studies have been carried out mainly with *Halobacterium* and *Haloferax* species (Doolittle, 1985; Pfeiffer, 1988; Schalkwyk, 1993). The complete genomic maps of *Haloferax volcanii*, *Haloarcula* sp. GRB and *Halobacterium salinarum* have been reported (Charlebois *et al.*, 1991; Saint Jean *et al.*, 1994; Bobovnikova *et al.*, 1994), as well as the chromosomal physical map of *Haloferax mediterranei* (López-García *et al.*, 1992; Antón *et al.*, 1994). In both *Haloferax* species the genome is composed of a circular chromosome of approximately 2·9 Mb, and three large extrachromosomal elements ranging in size from 86 to 690 kb. *Hf. volcanii* also carries an additional small plasmid (6·35 kb). In *Halobacterium* sp. GRB the genome also consists of several replicons, the chromosome (2·038 Mb) and four plasmids (305, 90, 37 and 1·8 kb), as is the case for *Hb. salinarum* NRC1 carrying one chromosome and two megaplasms (Bobovnikova *et al.*, 1994).

The existence of megaplasms in haloarchaea has been reported previously (Gutiérrez *et al.*, 1986) and an early attempt to estimate their sizes was carried out using classical electrophoretic analysis in spite of the inability of this technique to achieve accurate resolution of large

DNAs. Using PFGE, which allows the resolution of large DNA molecules, in either linear or supercoiled form (Smith *et al.*, 1988; López-García *et al.*, 1994), we have analysed the whole genomes of several species and strains of four haloarchaeal genera to detect the number of replicons present as well as their approximate sizes.

The objective of this work is to present new data and compare them with what is known about halobacterial genomic architecture. To date no general and systematic study regarding the genomic characteristics that are shared by the haloarchaea as a group has been performed.

METHODS

Organisms and growth conditions. The haloarchaeal species and strains used in this study are listed in Table 1. They are representatives of four non-alkaliphilic genera (*Halobacterium*, *Halorubrum*, *Haloarcula* and *Haloferax*) and the alkaliphilic genus *Natronobacterium*. *Halobacterium salinarum* (formerly *halobium*) CCM 2090 was obtained from two different sources (Dr A. Ventosa, University of Sevilla, Spain, and Dr M. Kohiyama, Institut Jacques Monod, France). *Natronobacterium pharaonis* was grown in the specific medium of Tindall (1991). Other strains were grown in SW 25% supplemented with 0·5% yeast extract at 37 °C (Rodríguez-Valera *et al.*, 1983) until late exponential phase, and then harvested by centrifugation at low speed.

DNA extraction. Intact genomic DNA was prepared as previously described (López-García *et al.*, 1992; Smith *et al.*, 1988).

PFGE. Orthogonal-field alternating gel electrophoresis (OFAGE) (Schwartz & Cantor, 1984) and contour-clamped homogeneous electric field electrophoresis (CHEF) (Chu *et al.*, 1984) were performed using a Pharmacia LKB Pulsaphor system. Some OFAGE gels were run using a 55 × 55 cm home-made system (López-García *et al.*, 1994). λ DNA concatemers (Pharmacia) and chromosomes of *Saccharomyces cerevisiae* YN295 and *Schizosaccharomyces pombe* DSM 70576 were used as linear size standards.

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Hybridization. For the hybridization experiment, DNA was transferred onto a Nylon membrane as described by Smith *et al.* (1988). The megaplasms used as probes were purified from PFGE gels, digoxigenin (DIG)-labelled directly in agarose by random primed synthesis and hybridized as described by the manufacturers (DIG-System, Boehringer Mannheim).

RESULTS AND DISCUSSION

Chromosomal size

Although circular chromosomes are unable to enter pulsed field gels due to their large sizes (Smith & Condemine, 1990), when intact DNA preparations are subjected to PFGE using high pulse times, and low electrical fields for long running times, some chromosomes randomly linearized by mechanical stress can be fractionated (López-García *et al.*, 1992). In this manner, the linearized chromosomes of three representative

species of the genus *Haloferax*, two of *Haloarcula*, two of *Halobacterium* (including three different strains of *Hb. salinarum*) and one of *Natronobacterium* (not shown) were resolved by PFGE (Fig. 1). Only a small proportion of total DNA was linearized and most of the chromosome remained in the well (compared with the intensity of the ethidium bromide staining of the fractionated chromosome). Their approximate sizes (Table 1) were calculated by comparison with linear DNA molecules of known sizes.

In the analysed genomes, chromosomal sizes from species belonging to the same genus are similar. Thus, the three *Haloferax* species harbour a chromosome of approximately 2.9 Mb. This size has been further confirmed by physical mapping in the cases of *Hf. volcanii* and *Hf. mediterranei* (Charlebois *et al.*, 1991; López-García *et al.*, 1992). Both *Haloarcula* species carry a chromosome of

Table 1. Number and approximate sizes of genomic replicons in haloarchaea

Haloarchaeal species	Chromosomal size (Mb)	Extrachromosomal elements	
		Approximate size (kb)	Number
<i>Halobacterium salinarum</i> (<i>halobium</i>) NRC 817	1.8	160 130 Small (< 50)	3
<i>Halobacterium salinarum</i> (<i>halobium</i>) CCM 2090*	1.8	350 160 70	3
<i>Halobacterium salinarum</i> (<i>halobium</i>) CCM 2090†	1.8	300 120	2
<i>Halobacterium salinarum</i> (<i>halobium</i>) CECT 396	1.8	250 160 70	3
<i>Halorubrum sodomense</i> ATCC 33755	2	100 70	2
<i>Haloarcula hispanica</i> ATCC 33960	3	400 370 130	3
<i>Haloarcula vallismortis</i> ATCC 29715	3	400 280 140 130	4
<i>Haloferax mediterranei</i> ATCC 33500	2.9	495 320 130	3
<i>Haloferax volcanii</i> NCMB 2012	2.9	680 410 90 6	4
<i>Haloferax gibbonsii</i> ATCC 33959	2.9	430 350 150	3
<i>Natronobacterium pharaonis</i> ATCC 35678	2	130	1

* Strain obtained from Dr A. Ventosa, Sevilla, Spain.

† Strain obtained from Dr M. Kohiyama, Paris, France.

Fig. 1. Linearized chromosomes of different haloarchaea. DNA was fractionated by PFGE using a pulse time of 2000 s at 3.3 V cm^{-1} for 120 h. Lanes: 1, *Hb. salinarum* NRC 817; 2, Spanish *Hb. salinarum* CCM 2090 (see Table 1); 3, French *Hb. salinarum* CCM 2090 (Table 1); 4, *Hb. salinarum* CECT 396; 5, *Hb. salinarum* (formerly *Hb. cutirubrum*) CCM 2688; 6, *Hb. salinarum* NRC 34002; 7, *Halobacterium* sp.; 8, *Hr.* (formerly *Hb.*) *sodomense* ATCC 33755; 9, *Ha. hispanica* ATCC 33960; 10, *Ha. vallismortis* ATCC 29715; 11, *Ha. vallismortis* (*marismortui*); 12, *Hf. mediterranei* ATCC 33500; 13, *Hf. volcanii* NCMB 2012; 14, *Hf. gibbonsii* ATCC 33959; 15, *Hc. morrhuae* CCM 537; 16, a natural halophilic isolate from La Mancha, Spain. Chromosomes of *Schiz. pombe* (A) and *Sacch. cerevisiae* (B) were used as linear size standards.

approximately 3 Mb, slightly larger than those from the *Haloferax* group. Finally, all the strains of *Hb. salinarum* tested carry a chromosome of similar size, around 1.8 Mb, which is smaller than the chromosome of *Halorubrum sodomense* (approximately 2 Mb), which for years has been classified into the genus *Halobacterium*. In this latter case, the difference in chromosomal size adds genomic evidence to the distinct nature of this species, which had been found to constitute a separate cluster by numerical taxonomy studies and polar lipid composition, and predicted to represent a new genus (Torreblanca *et al.*, 1986; Trincone *et al.*, 1990).

So far, more than 50 prokaryotic chromosomes have been physically mapped, nearly 10 of which belong to the archaeal domain. In spite of having greater similarities with eukaryotes, mostly at the protein level (Cole & Saint Girons, 1994), the chromosome and, in general, archaeal genome organization appears to be bacteria-like. Moreover, haloarchaeal chromosomes are not extreme in size when compared to other prokaryotes.

Although comparative genomics of prokaryotes is a new discipline, the first comparisons of chromosomal sizes and physical and genetic maps are producing useful information at both phylogenetic and structure/function levels. In this sense, knowledge of the sizes of haloarchaeal

chromosomes and of their degree of conservation at taxonomic levels constitutes an initial step, prior to physical and genetic mapping, in the exploration of the comparative genomics of this group.

Large extrachromosomal elements

Large haloarchaeal plasmids can be separated by PFGE in supercoiled (SC) or linear (L) forms, while open circular molecules either remain trapped in wells (if they are too large) or migrate short distances without being resolved (López-García *et al.*, 1994). PFGE of intact DNA from the haloarchaea used in this study (Table 1) revealed the presence of large plasmids, which were detected either in SC, L (as a consequence of random breakage) or both forms (Fig. 2).

Since supercoiled forms of large plasmids migrate with a certain dependence on the pulse time applied, but in a size-independent manner (López-García *et al.*, 1994), they usually migrate less than the linear molecules, and remain in the upper part of the gel. For haloarchaeal megaplasmids ranging between 100 and 600 kb, the best resolution of supercoiled molecules was achieved with pulses ranging from 50 to 200 s, although they could also be observed when longer pulses were used to separate Mb-sized linear molecules (Fig. 1). The largest megaplasmids linearize easily under mechanical stress and are, therefore, difficult to observe in the supercoiled form. This phenomenon is illustrated in Fig. 3. Fig. 3(a) shows a PFGE gel of different *Hf. mediterranei* genomic DNA

Fig. 2. Resolution of large haloarchaeal plasmids by OFAGE using a pulse time of 100 s at 10 V cm^{-1} for 24 h and a pulse time of 300 s at 8 V cm^{-1} for 24 h. Lanes: 1, Spanish *Hb. salinarum* CCM 2090; 2, French *Hb. salinarum* CCM 2090; 3, *Hf. mediterranei* ATCC 33500; 4, *Hf. volcanii* NCMB 2012; 5, *Hf. gibbonsii* ATCC 33959; 6, *Ha. hispanica* ATCC 33960; 7, *Ha. vallismortis* ATCC 29715; 8, a natural halophilic isolate from La Mancha, Spain. Chromosomes of *Sacch. cerevisiae* (A) and λ concatamers from Pharmacia (B) were used as linear size standards.

Fig. 3. Resolution of *Hf. mediterranei* megaplasms. Two sets of total DNA samples (1, 2 and 3, pre-run with a pulse time of 150 s at 10 V cm^{-1} for 40 h; 4, 5 and 6, not pre-run) were fractionated by OFAGE using a pulse time of 100 s at 10 V cm^{-1} for 42 h (a). Samples 1 and 4 were run without previous treatment; 2 and 5 were incubated in restriction buffer at 37°C overnight; 3 and 6 were digested with the macrorestriction enzyme *PacI*. After Southern blotting, the gel was hybridized with DIG-labelled pHM100 (b) and DIG-labelled pHM500 (c). *Sacch. cerevisiae* chromosomes were used as size standards (A).

samples (see legend). Samples 1, 2 and 3 had been pre-run and, therefore, exposed to shear damage. After Southern blotting, the gel was hybridized with pHM100 (130 kb) and pHM500 (495 kb), two of the three megaplasms present in this haloarchaeon. When hybridized with pHM100 (Fig. 3b), this element could be detected in the genomic DNA in two forms: linear (arrow in lanes 2 and 3) and supercoiled (lane 4). However, when pHM500 was used as probe (Fig. 3c), only the linear form could be observed. Megaplasms can be detected either in linear or both linear and supercoiled forms according to their sizes and, therefore, their susceptibility to shear stress and nuclease activity.

For approximate sizing, L forms, whose migration is size- and pulse-time-dependent, were fractionated by PFGE under the appropriate conditions for resolution in each case and compared with linear size standards (Fig. 2 shows an example). The number and approximate size of the extrachromosomal elements detected are given in Table 1. The most notable conclusion of this study is that all the haloarchaea tested contained megaplasms. The presence of these elements can account for up to 30% of the genome size. As mentioned above, the occurrence of megaplasms in haloarchaea has been reported previously (Gutiérrez *et al.*, 1986). Although some good estimates were given (Gutiérrez *et al.*, 1986), others differ greatly from the values we present here, which are more reliable since they have been obtained using a much more appropriate technology; in addition, some large megaplasms were not detected at all or even, in the case of *Hf. gibbonsii*, no plasmids were found, revealing that this early approach, although useful, had limitations.

Although the chromosomal size seems to be well conserved in a given genus, plasmid sizes and composition may vary not only among different strains from the same species, as is the case for *Hb. salinarum* or *Hf. mediterranei* (López-García *et al.*, 1993), but also in the same strain, as occurs in *Hb. salinarum* CCM 2090 which was obtained from two different sources. Therefore, genome variability seems to be maintained at extrachromosomal levels, where insertion sequences families are most abundant (Doolittle, 1985; Pfeiffer, 1988) and the rearrangements they mediate may take place readily.

The presence of megaplasms has been reported in different kinds of bacteria (Krawiec & Riley, 1990). Particularly, many specialized metabolic or interactive capabilities of bacteria depend on genes contained in megaplasms, such as *Rhizobium* symbiosis (Johnston, 1989), *Agrobacterium* tumour induction (Hooykaas, 1989), *Streptomyces* antibiotic biosynthesis (Kinashi *et al.*, 1992) and *Pseudomonas* organic compound degradation (Frantz & Chakrabarty, 1986). However, little is known about the genes on halobacterial megaplasms. Among the genes mapped in *Hf. mediterranei* (López-García *et al.*, 1992; Antón *et al.*, 1994) and *Hf. volcanii* (Cohen *et al.*, 1992) none have been found to present a copy only in megaplasms, since all the genes mapped in these replicons have at least one homologous copy in the main chromosome. The megaplasmid pHH1 (150 kb) and the

related pNRC100 (200 kb) from two distinct *Hb. salinarum* strains carry the genes for synthesis of buoyant gas-filled vesicles (Ng *et al.*, 1991; Horne *et al.*, 1991). Apparently copies of these genes were also found in the chromosome (Horne *et al.*, 1988). In *Halobacterium* sp. GRB, gas vesicle genes map only in the megaplasmid pGRB305 (Saint Jean *et al.*, 1994).

The advantage that bacteria might have from segregating their genetic material into several units has been explained on the basis of a mathematical model for the description of the growth of individual microbial cells (Stouthamer & Kooijman, 1993). The essential features of the model are that the uptake of nutrients is proportional to the surface area of the cell and maintenance costs are proportional to the volume. Growth of the individual cell continues until DNA replication of the genome is completed. The consequence is that cells with large genome size are bigger at the moment of cell division. In cells in which the total DNA is divided over several units which replicate simultaneously (e.g. a cell with more chromosomes or a cell with one chromosome and one or more megaplasms) a high surface to volume ratio can be maintained, which has a positive influence on the population growth rate.

According to this model, the presence of megaplasms should be more generalized than is so far known. Therefore, other parameters are probably important for the determination of this kind of distribution into distinct large genomic units. The haloarchaeal case appears remarkable: all of the studied species present a high fraction of their genomes as large extrachromosomal elements or megaplasms. What is the biological meaning of this genome partition? The identification of the genes present in megaplasms could shed some light on this problem. But for now it remains an open question.

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REFERENCES

- Antón, J., López-García, P., Abad, J. P., Smith, C. L. & Amils, R. (1994). Alignment of genes and *Sma*I restriction sites to the *Bam*HI genomic map of *Halobacterium mediterranei*. *FEMS Microbiol Lett* **117**, 53–60.
- Bobovnikova, Y., Ng, W. L., DasSarma, S. & Hackett, N. R. (1994). Restriction mapping the genome of *Halobacterium halobium* strain NRC-1. *Syst Appl Microbiol* **16**, 597–604.
- Charlebois, R. L., Schalkwyk, L. C., Hofman, J. D. & Doolittle, W. F. (1991). Detailed physical map and set of overlapping clones covering the genome of the archaeobacterium *Halobacterium volcanii* DS2. *J Mol Biol* **222**, 509–524.
- Chu, G., Vollrath, D. & Davies, R. W. (1984). Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science* **234**, 1582–1585.

- Cohen, A., Lam, W. L., Charlebois, R., Doolittle, W. F. & Schalkwyk, L. C. (1992). Localizing genes on the map of the genome of *Haloferax volcanii*, one of the Archaea. *Proc Natl Acad Sci USA* **89**, 1602–1606.
- Cole, S. T. & Saint Girons, I. (1994). Bacterial genomics. *FEMS Microbiol Rev* **14**, 139–160.
- Doolittle, W. F. (1985). Genome structure in archaeobacteria. In *The Bacteria, Vol. VIII*, pp. 545–560. Edited by C. R. Woese & R. S. Wolfe. London: Academic Press.
- Frantz, B. & Chakrabarty, A. M. (1986). Degradative plasmid in *Pseudomonas*. In *The Bacteria, Vol. X: The Biology of the Pseudomonads*, pp. 295–323. Edited by J. R. Sokatch. New York: Academic Press.
- Gutiérrez, M. C., García, M. T., Ventosa, A., Nieto, J. J. & Rufz-Berraquero, F. (1986). Occurrence of megaplastids in halobacteria. *J Appl Bacteriol* **61**, 67–71.
- Hooykaas, P. J. J. (1989). Tumorigenicity of *Agrobacterium* on plants. In *Genetics of Bacterial Diversity*, pp. 373–391. Edited by D. A. Hopwood & K. F. Chater. London: Academic Press.
- Horne, M., Englert, C. & Pfeiffer, F. (1989). Two genes encoding gas vacuole proteins in *Halobacterium halobium*. *Mol Gen Genet* **213**, 459–464.
- Horne, M., Englert, C., Wimmer, C. & Pfeiffer, F. (1991). A DNA region of 9 kb contains all genes necessary for gas vesicle synthesis in halophilic halobacteria. *Mol Microbiol* **5**, 1159–1174.
- Johnston, A. W. B. (1989). The symbiosis between *Rhizobium* and legumes. In *Genetics of Bacterial Diversity*, pp. 393–438. Edited by D. A. Hopwood & K. F. Chater. London: Academic Press.
- Juez, G. (1988). Taxonomy of extremely halophilic archaeobacteria. In *Halophilic Bacteria, Vol. II*, pp. 3–24. Edited by F. Rodríguez-Valera. Boca Raton, Florida: CRC Press.
- Kamekura, M. & Dyal-Smith, M. L. (1995). Taxonomy of the family Halobacteriaceae and the description of two new genera *Halorubrobacterium* and *Natrialba*. *J Gen Appl Microbiol* **41**, 333–350.
- Kinashi, H., Shimaji-Murayama, M. & Hanafusa, T. (1992). Integration of SCP1, a giant linear plasmid, into the *Streptomyces coelicolor* chromosome. *Gene* **115**, 35–42.
- Krawiec, S. & Riley, M. (1990). Organization of the bacterial chromosome. *Microbiol Rev* **54**, 502–539.
- López-García, P., Abad, J. P., Smith, C. L. & Amils, R. (1992). Genomic organization of the halophilic archaeon *Haloferax mediterranei*: physical map of the chromosome. *Nucleic Acids Res* **20**, 2459–2464.
- López-García, P., Abad, J. P. & Amils, R. (1993). Genome analysis of different *Haloferax mediterranei* strains using pulsed-field gel electrophoresis. *Syst Appl Microbiol* **16**, 310–321.
- López-García, P., Antón, J., Abad, J. P. & Amils, R. (1994). Halobacterial megaplastids are negatively supercoiled. *Mol Microbiol* **11**, 421–427.
- Ng, W. L., Kothakota, S. & DasSarma, S. (1991). Structure of the gas vesicle plasmid in *Halobacterium halobium*: inversion isomers, inverted repeats, and insertion sequences. *J Bacteriol* **173**, 1958–1964.
- Pfeiffer, F. (1988). Genetics of halobacteria. In *Halophilic Bacteria, Vol. II*, pp. 105–133. Edited by F. Rodríguez-Valera. Boca Raton, Florida: CRC Press.
- Rodríguez-Valera, F., Juez, G. & Kushner, D. J. (1983). *Halobacterium mediterranei* sp. nov., a new carbohydrate-utilizing extreme halophile. *Syst Appl Microbiol* **4**, 369–381.
- Saint Jean, A., Trieselmann, B. A. & Charlebois, R. L. (1994). Physical map and set of overlapping cosmid clones representing the genome of the archaeon *Halobacterium* sp. GRB. *Nucleic Acids Res* **22**, 1476–1483.
- Schalkwyk, L. C. (1993). Halobacterial genes and genomes. In *The Biochemistry of Archaea (Archaeobacteria)*, pp. 467–496. Edited by M. Kates, D. J. Kushner & A. T. Matheson. Amsterdam: Elsevier.
- Schwartz, D. C. & Cantor, C. R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gel electrophoresis. *Cell* **37**, 67–75.
- Smith, C. L. & Condemine, G. (1990). New approaches for physical mapping of small genomes. *J Bacteriol* **172**, 1167–1172.
- Smith, C. L., Klco, S. R. & Cantor, C. R. (1988). Pulsed-field gel electrophoresis and the technology of large DNA molecules. In *Genome Analysis: A Practical Approach*, pp. 41–72. Edited by K. Davies. Oxford: IRL Press.
- Stouthamer, A. H. & Kooijman, S. A. L. M. (1993). Why it pays for bacteria to delete disused DNA and to maintain megaplastids. *Antonie Leeuwenhoek* **63**, 39–43.
- Tindall, B. J. (1991). Cultivation and preservation of members of the family Halobacteriaceae. *World J Microbiol Biotechnol* **7**, 95–98.
- Torreblanca, M., Rodríguez-Valera, F., Juez, G., Ventosa, A., Kamekura, M. & Kates, M. (1986). Classification of non-alkaliphilic halobacteria based on numerical taxonomy and polar lipids pattern and description of *Haloarcula* gen. nov. and *Haloferax* gen. nov. *Syst Appl Microbiol* **8**, 89–99.
- Trincone, A., Nicolaus, B., Lama, L., De Rosa, M., Gambacorta, A. & Grant, W. D. (1990). The glycolipid of *Halobacterium sodomense*. *J Gen Microbiol* **136**, 2327–2331.

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