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Isolation and Partial Characterization of a Plasmid in the Extremely Halophilic Archaebacterium *Halococcus morrhuae* CCM 537

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The extremely halophilic archaebacterium *Halococcus morrhuae* CCM 537 was found to contain a plasmid, pHM2, of 6·0 kb. A restriction map was constructed. Southern hybridization of pHM2 DNA to DNA extracted from other *Halococcus* sp. strains revealed the presence of a similar plasmid in one other strain. A plasmid of *Halobacterium trapanicum* shared common sequences with pHM2. Other plasmids from halobacteria displayed no homology with pHM2. The characterization of pHM2 may make it useful for the development of cloning vectors for extremely halophilic archaebacteria.

INTRODUCTION

The majority of extremely halophilic bacteria are included in one of the three primary phylogenetic groupings, the so-called archaebacteria (Woese & Fox, 1977). They can grow in saline media with salt concentrations ranging from 12% (w/v) to saturation, and grow optimally at approximately 25% (w/v) total salts (Kushner, 1985).

Several plasmids have been identified in halobacteria (Paton *et al.*, 1981; Pfeifer *et al.*, 1981*a*; Ebert *et al.*, 1984; Tindall *et al.*, 1984; Ross & Grant, 1985; Gutiérrez *et al.*, 1986). The main plasmid species from these extremely halophilic rods are usually large, especially those from *Halobacterium halobium* (pHH1, pHH2, pHH3), *Halobacterium salinarium* (pHHR1) and *Halobacterium cutirubrum* (pHC1), but smaller plasmids in *Haloferax volcanii*, *Halobacterium trapanicum* and *Halobacterium* sp. strains SB3, GN101 and GRB have also been described (Pfeifer, 1988). Plasmids of *Halobacterium halobium* and closely related strains show extensive homology but have a high degree of structural variation (Pfeifer *et al.*, 1981*b*; Sapienza & Doolittle, 1982). Two small plasmids from *Halobacterium* sp. strains SB3 and GRB have been studied in detail and proposed as potential cloning vectors for halobacteria (Ebert *et al.*, 1984).

The majority of molecular studies of these bacteria have been done in halobacteria, but recent work has examined the genus *Halococcus* (Leffers & Garret, 1984; Montero *et al.*, 1988). As yet, no studies have been reported on the presence of plasmids in *Halococcus morrhuae* (Ross & Grant, 1985; Pfeifer, 1988). The paucity of information may be attributed to the fact that halococci display some of the toughest envelopes known among bacteria, being very difficult to break, though they can be disrupted by shaking with glass beads (Schleifer *et al.*, 1982).

Here we report the isolation and partial characterization of a small plasmid from *Halococcus morrhuae* CCM 537 (type strain). The homology of this plasmid to other halobacterial plasmids, as well as its occurrence in other halococci recently isolated, was tested.

METHODS

Bacterial strains. Halococcus morrhuae CCM 537 was used as reference strain. *Halococcus* sp. N-207 and *Halococcus* sp. P-423 were isolated from ponds of salterns located in Alicante, Spain (Montero *et al.*, 1988). *Halobacterium halobium* NRC 34020, *Halobacterium trapanicum* CHB-83, *Haloferax volcanii* NCMB 2012, and *Halobacterium* sp. SB3 were also used.

For molecular cloning experiments, *Escherichia coli* K12 strain D1210 (r_k^- m_k^- *recA13 ara-14 proA2 lacY1 galK2 supE44 lacI*^{Q}) was used as host and was kindly supplied by Dr F. Pfeifer, Max Planck Institut für Biochemie, FRG.

Growth conditions. All halobacteria and halococci strains were grown in HM medium (Ventosa *et al.*, 1982), containing (%, w/v): yeast extract (Difco), 1; Proteose–peptone no. 3 (Difco), 0·5; glucose, 0·1; Bacto agar (Difco), 2. This medium was supplemented with a balanced mixture of sea salts giving a final concentration of 25% (w/v) (Rodríguez-Valera *et al.*, 1980). *E. coli* was grown in Luria medium (Maniatis *et al.*, 1982) or in M9 medium (Miller, 1972). Tetracycline was added when required as a filter-sterilized concentrate in water to a final concentration of 25 mg l^{-1}. All cultures were incubated at 37 °C.

Plasmid isolation. Cultures of *H. morrhuae* were incubated for one week. Glycine was then added to the growth medium to a final concentration of 1% (w/v) and the culture was incubated for another 2 d. The cells were harvested by centrifugation at 15000 *g* for 10 min in a Sorvall GSA rotor and resuspended in distilled water. Cell lysis was achieved by the addition of 20% (w/v) Sarkosyl (Sigma) in water to a final concentration of 1% (w/v). The cell lysate was centrifuged at 20000 *g* for 20 min. To the supernatant were added 2 vols of ethanol and 5 M-NaCl to a final concentration of 0·1 M. The mixture was left for 10 min in dry ice, and then centrifuged at 12000 *g* for 10 min. The pellet was resuspended in Tris/EDTA (10:1) buffer. Plasmid DNA was obtained from CsCl/ethidium bromide gradients after centrifugation in a Beckman Ti 50 rotor at 55000 *g* for 2 d. Plasmids were separated by electrophoresis at 4 V cm^{-1} for 16 h on 1·0% (w/v) agarose vertical gels in Tris/acetate EDTA buffer. A small plasmid was electroeluted from the gels by the direct method of Maniatis *et al.* (1982). Plasmid isolation from halobacteria was achieved as previously described (Pfeifer *et al.*, 1981*a*).

Restriction endonuclease digestion and gel electrophoresis. Restriction endonuclease digestions of chromosomal and plasmid DNA were done under the conditions recommended by the manufacturers of the enzymes used. DNA fragments were separated by electrophoresis at 4 V cm^{-1} for 16 h on 1·0–2·0% (w/v) agarose horizontal gels. The gels were run in Tris/acetate/EDTA buffer, stained with ethidium bromide (5 μg ml^{-1} for 10 min) and destained with water (30–60 min). Restriction enzymes were purchased from Boehringer. Agarose type II was from Sigma. *Cla*I-digested φH DNA was obtained from Dr F. Pfeifer.

Plasmids, ligation, transformation and plasmid constructions. The small plasmid isolated from *H. morrhuae* CCM 537 was cleaved with *Eco*RI, and the resulting fragments were ligated into the *Eco*RI site of pUM121 (Nilsson *et al.*, 1983) by standard techniques (Maniatis *et al.*, 1982) and recovered in *E. coli*. Two recombinant plasmids, each one harbouring a different *Eco*RI fragment from pHM2, were used for detailed mapping of restriction sites on the plasmid. General molecular methods and experiments specific to *E. coli* were used according to Maniatis *et al.* (1982). pBR322 was obtained from Boehringer.

Hybridization. Electrophoretically separated and stained DNA fragments were denatured in the gel by submersion for 30 min in 0·5 M-NaOH containing 1·5 M-NaCl. The alkali was neutralized by gentle agitation for 30 min in 0·5 M-Tris/HCl, pH 7·2, containing 1·5 M-NaCl. Transfer of DNA to nitrocellulose membranes (Schleicher & Schüll), hybridization with nick-translated plasmid and subsequent washing, first with 2 × SSC containing 0·1% SDS (2 × 15 min at 68 °C) and then with 0·2 × SSC (2 × 15 min, 68 °C), were done according to Southern (1975). Nick translation of plasmid DNA (Rigby *et al.*, 1977) generated ^{32}P-labelled DNA with a specific radioactivity of 7·4 MBq (μg DNA)$^{-1}$.

RESULTS AND DISCUSSION

pHM2 from H. morrhuae CCM 537

Standard lysing procedures for other bacteria were not suitable for *H. morrhuae*. We were unable to obtain DNA from cells treated with lysozyme, sarkosyl, SDS, Triton X-100 or sodium deoxycholate, or from cells disrupted by mechanical methods. Other methods previously described for isolation of DNA from halobacteria (Pfeifer *et al.*, 1981*a*) or from halococci (Kocur & Bohacek, 1972; Leffers & Garrett, 1984) also were not suitable. Addition of glycine to culture media impairs cell wall biosynthesis and results in a weakened cell wall (Owen & Pitcher, 1985). Accordingly, we added glycine to the growth media to see if it facilitated extraction and purification of DNA from these organisms (see Methods). Using this method of cell culture, we were subsequently able to isolate both total DNA and plasmid DNA from strains of the genus *Halococcus*. In this study, we tested the type strain *H. morrhuae* CCM 537. Two plasmids (pHM1 and pHM2) were detected in this strain. The approximate molecular masses of these plasmids were 48 and 6 kb, respectively. The small plasmid, pHM2 was selected for further study because of its low molecular mass, which makes it potentially useful for the development of cloning vectors.

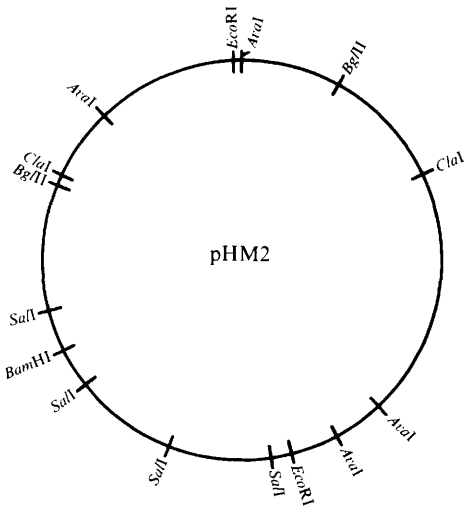

Fig. 1. Restriction map of pHM2.

Restriction analysis with *Eco*RI of pHM2 from *H. morrhuae* CCM 537 showed two fragments. These two *Eco*RI restriction fragments were ligated into the *Eco*RI site of the plasmid vector pUM121, and the ligation mixtures were used to transform *E. coli* K12 strain D1210. Recombinant plasmids were isolated from Tc^r transformants and were named pCM1 and pCM2. The inserted fragments in pCM1 and pCM2 were 3·2 and 2·8 kb in size, respectively, corresponding to the two *Eco*RI fragments of pHM2. The orientation of these fragments in pHM2 was deduced from single and double digestions of pHM2 with *Eco*RI and *Cla*I. The rest of the map (Fig. 1) was obtained from the analysis of pCM1 and pCM2. The following restriction enzymes were used: *Alu*I, *Ava*I, *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Hpa*II, *Pst*I, *Sal*I, *Taq*I and *Xho*I. More than five restriction enzyme sites each for *Alu*I, *Hae*III, *Hpa*II and *Taq*I were found on pHM2, but these have not been mapped. No sites were found for *Eco*RV, *Hin*dIII, *Pst*I or *Xho*I. It should be noted that of the restriction enzymes tested only *Bam*HI cleaved pHM2 once (Fig. 1).

An ideal plasmid from which to develop a cloning vector for halobacteria would: (i) be stably maintained in halobacteria, (ii) be small, (iii) be present in high copy number, (iv) contain unique restriction enzyme sites for cloning purposes, and, (v) possess a selectable marker (Old & Primrose, 1985). The plasmid identified, pHM2, satisfies conditions (ii), (iii) and (iv).

Southern blot hybridization analysis

pHM2 was ^{32}P-labelled and used to probe total DNA isolated from other strains of the genus *Halococcus* (Fig. 2) and to determine its relationship to plasmids harboured by other halobacteria (Fig. 3). Genomic DNA from *Halococcus* strains P-423 and N-207 was digested with *Sst*I. Probe DNA hybridized only to N-207 DNA, and in this case the band pattern obtained was indistinguishable from that seen with *H. morrhuae* CCM 537 from which pHM2 originated. These results indicate that *H. morrhuae* CCM 537 and *Halococcus* sp. N-207 harbour the same plasmid. In a previous numerical taxonomic study (Montero *et al.*, 1988) both strains were included in the same phenon, despite the fact that they were isolated from different places (one is a culture collection strain, the other was isolated from salterns in Alicante, Spain). *Halococcus* sp. P-423 has been placed in a different phenon, with very different phenotypic features from CCM 537. It is worth noting that the former strain shows a completely different genomic pattern from the latter.

To test whether pHM2 has sequences in common with other halobacterial plasmids, we examined four characterized halobacterial plasmids (Pfeifer *et al.*, 1981*a*). One plasmid from

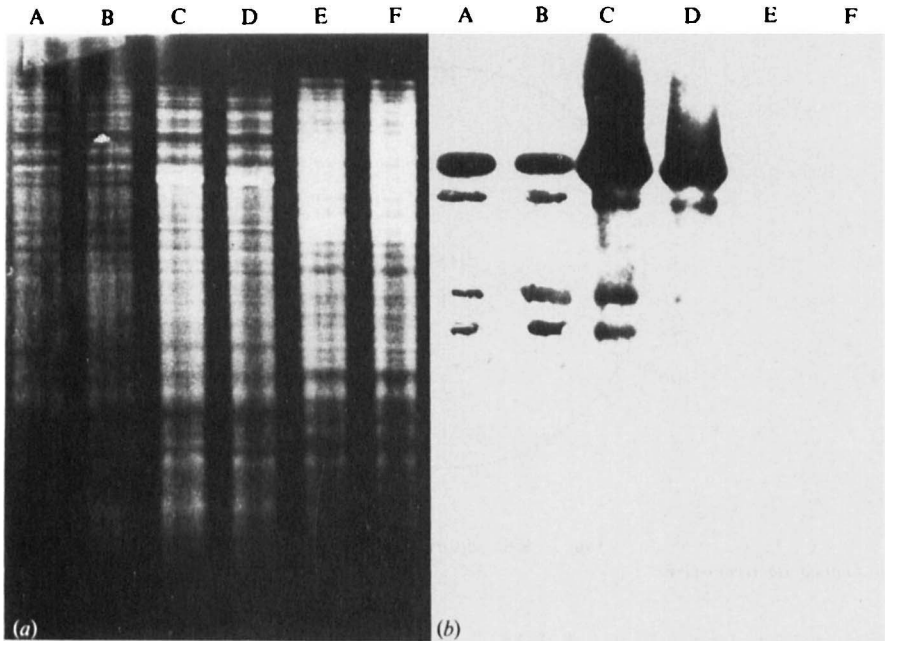

Fig. 2. (*a*) DNA fragment patterns of *Halococcus* sp. genomic DNA after digestion with the restriction enzyme *Sst*I. (*b*) Hybridization pattern of ^{32}P-labelled pHM2 probe DNA to *Halococcus* sp. *Sst*I-digested DNA. Tracks: A and B, *Halococcus morrhuae* CCM 537; C and D, *Halococcus* sp. N-207; E and F, *Halococcus* sp. P-423.

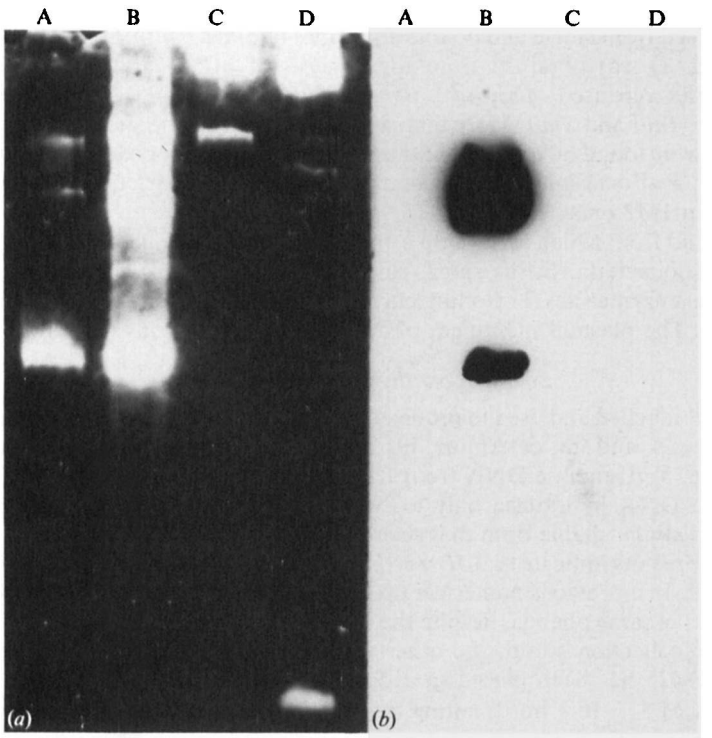

Fig. 3. (*a*) Halobacterial plasmids. (*b*) Hybridization of ^{32}P-labelled pHM2 to halobacterial plasmids. Plasmids were isolated from: A, *Haloferax volcanii* NCMB 2012; B, *Halobacterium trapanicum* CHB-83; C, *Halobacterium halobium* NRC 34040; D, *Halobacterium* sp. strain SB3.

Halobacterium trapanicum displayed homology with pHM2 (Fig. 3). This finding may indicate plasmid exchange in this archaebacterial group. Gene exchange by conjugation (Mevarech & Werczberger, 1985) and by transfection (Cline & Doolittle, 1987) have been reported which indicates that archaebacteria share similar genetic exchange systems to other bacteria.

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