Characterization of a plasmid from moderately halophilic eubacteria

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A plasmid has been isolated for the first time from moderately halophilic eubacteria. *Halomonas elongata*, *Halomonas halmophila*, *Deleya halophila* and *Vibrio costicola* were found to harbour an 11·5 kbp plasmid (pMH1). The plasmid was isolated and characterized after transformation into *Escherichia coli* JM101 cells. A restriction map was constructed, and unique restriction sites for *Eco*RI, *Eco*RV and *Cla*I were detected. The occurrence of such a plasmid in the original halophilic strains was confirmed by Southern hybridization. The plasmid carries genetic determinants that mediate resistance to kanamycin, tetracycline, and neomycin. This property, together with its relatively small size, its stability in *E. coli* cells, and the presence of unique restriction sites, makes pMH1 a good candidate for the development of a cloning vector for moderate halophiles.

Introduction

Besides the extremely halophilic archaeobacteria, the moderate halophiles are the most important group of micro-organisms adapted to live in hypersaline habitats, and are defined as those micro-organisms which grow optimally in media containing 0·5 to 2·5 M-salt (Kushner, 1978). Despite extensive studies on their taxonomy, physiology, and ecology (Ventosa, 1988; Kushner & Kamekura, 1988; Rodriguez-Valera, 1986; Rodriguez-Valera *et al.*, 1985), the study of their genetics is still in its infancy. Little information is available on the occurrence of genetic exchange mechanisms in these halophiles, or on the isolation of stable mutants or autochthonous plasmids which could be used as cloning vectors, all of which are required for the development of molecular biology studies. The aim of the present study was to isolate plasmids which could be used as cloning vectors in further genetic studies of moderately halophilic strains. In this paper, we report the isolation and characterization of a plasmid, pMH1, present in four moderate halophiles, which displays suitable genetic features for use as a cloning vector. To our knowledge, this is the first plasmid described for moderately halophilic eubacteria, as well as the first demonstration of DNA transfer from moderately halophilic to non-halophilic bacteria.

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Methods

Bacterial strains. The moderate halophiles used in the majority of experiments were *Halomonas elongata* ATCC 33173, *Halomonas halmophila* ATCC 19717, *Deleya halophila* CCM 3662 and *Vibrio costicola* NCMB 701. Another 32 moderately halophilic strains belonging to the genera *Marinococcus*, *Salinicoccus*, *Volcaniella* and *Vibrio* were also used when screening for the presence of plasmids, and for hybridization experiments, the extremely halophilic archaeobacteria *Halobacterium salinarum* NRC 34002, *Haloferax gibbonsii* ATCC 33500 and *Haloferax volcanii* NCMB 2012. Furthermore, *Escherichia coli* strain JM109 (Yanisch-Perron *et al.*, 1985), which carried no detectable antibiotic resistance, was used as host for transformation procedures, conjugations and plasmid DNA preparations.

Growth conditions. All moderate halophiles were grown in a saline medium (SWYE) containing 10% (w/v) total salts (Nieto *et al.*, 1989) to which 0·5% yeast extract (Difco) was added. The extremely halophilic strains were grown in a similar medium but with 25% (w/v) total salts. *E. coli* was grown in Luria medium (LB) (Maniatis *et al.*, 1982). When needed, all media were solidified with 2% (w/v) Bacto-agar (Difco). Antibiotics were added when required as either filter-sterilized concentrate in water or ethanol (70%). All cultures were incubated at 37 °C.

DNA isolation. Routine small-scale isolation of plasmid DNA from *E. coli* was performed by the alkaline lysis method as described by Morelle (1989). Large amounts of plasmid DNA were obtained by CsCl/ethidium bromide density gradient ultracentrifugation at 142000 *g* for 36 h in a Sorvall AH-650 rotor. Genomic DNA was isolated according to Ausubel *et al.* (1989).

Transformation and screening for antibiotic resistance. Plasmid DNA obtained from 10 ml of culture was resuspended in 50 µl TE buffer (10 mM-Tris/HCl/1 mM-EDTA, pH 8) and used to transform 200 µl of competent *E. coli* JM101 cells. Preparation of competent cells and

transformation were performed according to Maniatis *et al.* (1982). To screen for plasmid-associated antibiotic resistance phenotypes, transformed cells of *E. coli* were tested for resistance to a range of antibiotics. Antibiotics were added to these final concentrations (μg ml^{-1}): ampicillin, 50; chloramphenicol, 125; erythromycin, 80; gentamycin, 50; kanamycin, 20; nalidixic acid, 30; neomycin, 30; spectinomycin, 80; streptomycin, 500; and tetracycline, 20 (Maniatis *et al.*, 1982). Parallel controls of the competent cells were also carried out to detect spontaneous resistant mutants.

Restriction endonuclease digestion and gel electrophoresis. Plasmid and chromosomal DNA were digested as recommended by the enzyme manufacturer (Boehringer). Digested DNA was separated by horizontal electrophoresis on 0·8% agarose gels (Sigma) in Tris/borate/EDTA buffer, pH 8·3. For physical mapping of the plasmid, the agarose concentration was either increased or decreased in order to detect smaller or larger DNA fragments, respectively. Gels were stained with ethidium bromide (Sigma) (5 μg ml^{-1} for 10 min), destained in water, and photographed under 254 nm ultraviolet light. The molecular masses of the plasmid fragments were estimated by comparison with *Hin*dIII-digested bacteriophage lambda DNA fragments.

DNA hybridization. Plasmid and genomic DNA were digested with restriction endonucleases, fractionated by agarose gel electrophoresis, and transferred to nylon filters (Amersham) as described by Southern (1975). [α-^{32}P]dCTP-labelled DNA was prepared by the multiprime DNA-labelling system (Amersham). Specific activities of 10^7–10^8 c.p.m. (μg DNA)$^{-1}$ were obtained. Hybridization conditions were as described by Vargas *et al.* (1990).

Results and Discussion

Preliminary screening for autochthonous plasmids from moderately halophilic eubacteria

The moderate halophiles are not only important in the ecology of hypersaline environments, but also very interesting from a genetic point of view. Their ability to grow in a broad range of saline concentrations might allow the transfer of DNA between non-halophilic eubacteria and extremely halophilic aerobic archaebacteria, the so-called halobacteria. The aim of this study has been to isolate autochthonous plasmids from moderate halophiles in order to use them as basic genetic elements for development of further cloning vectors. A number of strains with a broader range of tolerance growing well in media containing 0·5–25% (w/v) total salts, belonging to the genera *Halomonas*, *Deleya* and *Vibrio*, were selected for these studies.

Standard procedures for the detection of plasmids (Birnboim, 1983; Kado & Liu, 1981; Holmes & Quigley, 1981), as well as some modifications of the alkaline lysis method as recommended for *Halomonas* strains by Vreeland (1984) were performed, in which cell suspensions in SDS were heated to 65 °C during the lysis treatment. In a low proportion of preparations, thin DNA bands suggested the presence of plasmids (data not shown). When larger preparations of closed circular DNA extracted from strains in which a plasmid had been observed were purified further by CsCl/ethidium bromide density gradient, no plasmid DNA was recovered, despite several modifications of the alkaline lysis method. These difficulties might be due to thermostable nucleases which could cause rapid breakdown of DNA following cell lysis. The production of such enzymes has been reported in several moderate halophiles (Kamekura, 1986; Onishi *et al.*, 1983; Vreeland, 1984). Indeed, the majority of strains tested showed a remarkable DNAase activity. Therefore, standard methods seemed to be unsuitable for obtaining plasmid DNA from moderate halophiles.

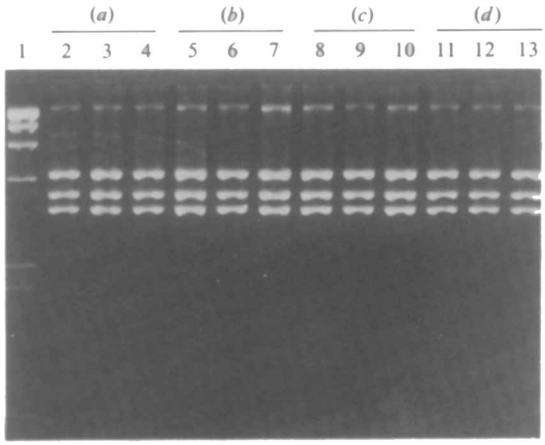

Fig. 1. *Hin*dIII-generated fragments of plasmid DNA from *E. coli* JM101 cells transformed with plasmid preparations from moderate halophiles. (*a*) *Halomonas elongata*; (*b*) *Deleya halophila*; (*c*) *Halomonas halmophila*; and (*d*) *Vibrio costicola*. Transformant selection was done on LB with tetracycline (lanes 2, 5, 8 and 11), kanamycin (lanes 3, 6, 9 and 12), or neomycin (lanes 4, 7, 10 and 13). Lane 1, *Hin*dIII digestion of bacteriophage lambda DNA.

Screening for plasmids from moderate halophiles which codify for antibiotic resistance: isolation of pMH1

In order to purify plasmid DNAs and to check whether plasmids carried useful genetic markers, we transformed competent *E. coli* cells with plasmid DNA from the strains investigated, and selected transformants on LB medium with a range of antibiotics. Using this approach, the only plasmids detected would be those maintained in *E. coli* cells that conferred antibiotic resistance. These are important genetic characteristics for plasmids to be used as basic elements for the construction of cloning vectors. This method has been employed elsewhere to facilitate the characterization of plasmids which can not be easily visualized (Maniatis *et al.*, 1982; Vargas *et al.*, 1990). With strains in which the presence of plasmids had been detected by the previous screening, a number of colonies were obtained on LB media containing kana-

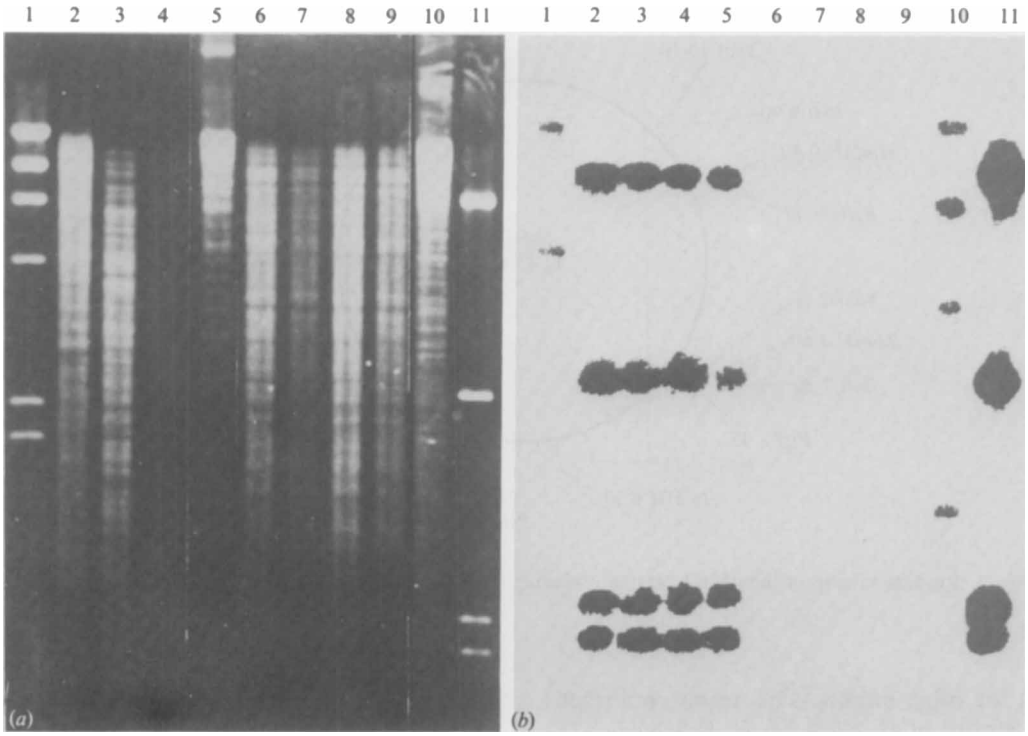

Fig. 2. (*a*) Agarose gel electrophoresis of *Pst*I-generated fragments of DNA from *H. elongata* (lane 2), *D. halophila* (lane 3), *H. halmophila* (lane 4), *V. costicola* NCMB 701 (lane 5), *Halobacterium salinarium* (lane 6), *Haloferax gibbonsii* (lane 7), *Haloferax volcanii* (lane 8), *V. costicola* AV22 (lane 9), *E. coli* JM109 (lane 10) and pMH1 (lane 11). Lane 1, *Hin*dIII-digested lambda DNA. (*b*) Hybridization patterns of the above DNA samples against a *Eco*R1-digested pMH1 DNA probe.

mycin (Km), neomycin (Nm), or tetracycline (Tc) when the donor DNA was extracted from *H. elongata*, *H. halmophila*, *D. halophila*, or *V. costicola*. Four sets of representative strains, each deriving from transformants with resistance to Km, Nm, or Tc and obtained with each of the four different original DNAs, were subsequently screened for their plasmid profiles after digestion with several endonucleases. In all cases, a single identical plasmid profile was detected for all the different representative strains, regardless of the parent strain from which the DNA was extracted, or the antibiotic used. The plasmid profiles after digestion with *Hin*dIII are shown in Fig. 1. The four moderate halophiles contained the same 11·5 kb plasmid, designated pMH1 (for moderate halophiles) and coding for resistance to kanamycin, neomycin, and tetracycline. The transformation procedures were repeated four times with the same results. To determine the maximum level of antibiotic resistance conferred, the transformed plasmid-containing strains were grown in LB medium with increased antibiotic concentrations. The highest resistances to kanamycin, tetracycline and neomycin were (μg/ml): 500, 100 and 50, respectively.

Additional conjugation and transformation experiments were carried out in order to determine the possible transfer of pMH1 to *E. coli*. A spontaneous rifampicin-resistant mutant of strain JM101 was used as recipient. The plasmid was transferred by both genetic transfer mechanisms, but a high frequency of transfer was found only with transformation. Therefore, pMH1 can be stably maintained and transferred into *E. coli* cells, and the multiple antibiotic resistance phenotype is associated with the presence of pMH1.

Presence of pHM1 in the genome of the parent moderately halophilic strains

The occurrence of pMH1 was confirmed by Southern hybridization analysis. Genomic DNA from the parent strains was prepared, digested with *Pst*I and isolated by agarose gel electrophoresis (see Methods). Total DNA from three halobacteria, another *Vibrio costicola* strain and *E. coli* JM109 were included as negative controls; pMH1 DNA also digested with *Pst*I was added as a positive control. As shown in Fig. 2, the same pMH1 *Pst*I hybridizing fragments were represented in digested genomic DNA from the four original strains as well as in *E. coli*, while in contrast they were absent in DNA

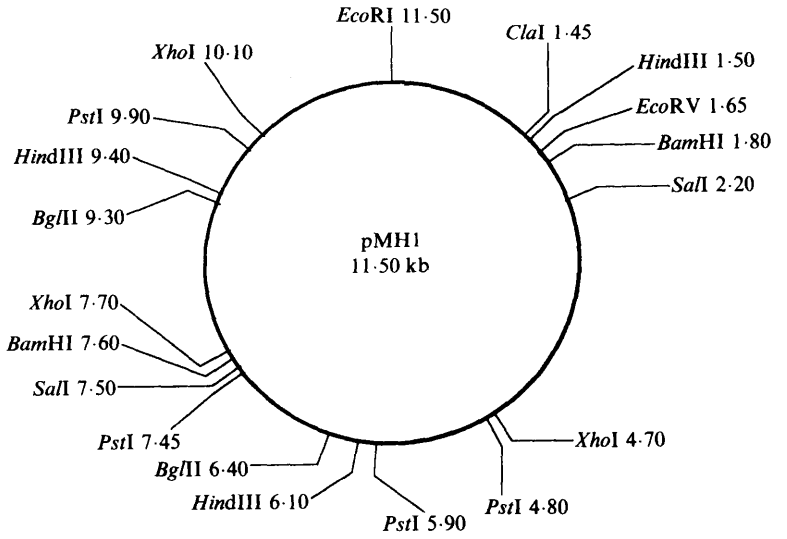

Fig. 3. Restriction map of the pMH1 plasmid, showing cleavage sites for the restriction endonucleases used.

extracted from the other strains. This result confirmed that the tested strains of *H. elongata*, *H. halmophila*, *D. halophila* and *V. costicola* harboured a homologous plasmid. The presence of homologous sequences in the total DNA of *E. coli* is not surprising, since this might explain the ease with which pMH1 can be maintained and transferred among *E. coli* cells. On the other hand, although the hybridizations were carried out under a high stringency, slight non-specific signals were detected with two of the *Hin*dIII fragments of lambda marker; this is not an unusual observation in hybridization experiments.

Restriction map of pMH1

In order to construct a pMH1 physical map, the plasmid from *E. coli* cells, which had been previously transformed with plasmid DNA derived from *H. elongata*, was prepared using a large-scale alkaline lysis method (Morelle, 1989), purified by CsCl/ethidium bromide density gradients, and digested with nine restriction endonucleases (as well as with their double and triple combinations). The enzymes used were: *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Sal*I, and *Xho*I, respectively. The map (Fig. 3) shows that of the restriction enzymes tested, only *Eco*RI, *Eco*RV, and *Cla*I cleaved pMH1 once. Moreover, pMH1 also contained cleavage sites for a number of other commonly employed site-specific endonucleases. These restriction sites are: *Sal*I, 2; *Bam*HI, 2; *Bgl*II, 2; *Hin*dIII, 3; *Xho*I, 3; and *Pst*I, 4.

We conclude that pMH1 is a good candidate for a cloning vector for moderately halophilic bacteria, since this plasmid satisfies the following conditions: (i) it is stably maintained and transferred in *E. coli* cells; (ii) it is relatively small; (iii) it possesses a selectable marker; (iv) it contains unique restriction enzyme sites for cloning purposes (Old & Primrose, 1989).

The common occurrence of pMH1 in the four moderately halophilic strains may reflect the very close relationship within the family Halomonadaceae (Franzmann *et al.*, 1988) of three strains. Although *V. costicola* was first isolated from cured meats (Smith, 1938), we have found that this species is one of the predominant micro-organisms which inhabit hypersaline waters (Ventosa *et al.*, 1982; García *et al.*, 1987; Márquez *et al.*, 1987) or soils (Quesada *et al.*, 1982) together with members of the Halomonadaceae. Since these moderate halophiles can be present in the same ecological habitats, pMH1 may codify functions which are common for these micro-organisms. This resembles the situation of their halophilic counterparts, the halobacteria, in which the general occurrence of cryptic plasmids of different sizes have been reported (Gutiérrez *et al.*, 1986). The presence of antibiotic resistance markers in pMH1 is not surprising, since very recently a high incidence of antibiotic resistance in bacteria which inhabit environments virtually free from anthropogenic influence has been found (Magee & Quinn, 1991). Experiments to determine some possible functions of pMH1 are in progress.

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