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Characterization of the Basic Replicon of pCM1, a Narrow-Host-Range Plasmid from the Moderate Halophile *Chromohalobacter marismortui*

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The moderately halophilic bacterium *Chromohalobacter marismortui* contains a 17.5-kb narrow-host-range plasmid, pCM1, which shows interesting properties for the development of cloning vectors for the genetic manipulation of this important group of extremophiles. Plasmid pCM1 can stably replicate and is maintained in most gram-negative moderate halophiles tested. The replication origin has been identified and sequenced, and the minimal pCM1 replicon has been localized to a 1,600-bp region which includes two functionally discrete regions, the *oriV* region and the *repA* gene. *oriV*, located on a 700-bp fragment, contains four iterons 20 bp in length adjacent to a DnaA box that is dispensable but required for efficient replication of pCM1, and it requires *trans*-acting functions. The *repA* gene, which encodes a replication protein of 289 residues, is similar to the replication proteins of other gram-negative bacteria.

The moderate halophiles are a group of extremophilic microorganisms defined as those which grow optimally in media containing 3 to 15% (wt/vol) NaCl (34). Besides the extremely halophilic aerobic archaea, the so-called halobacteria, the moderate halophiles are the most important microorganisms adapted to thrive in hypersaline environments and constitute a very diverse group of bacteria. Although extensive studies have been carried out on their taxonomy (56), physiology (34), and ecology (47), little information on their genetics is available so far. No studies on DNA transfer processes in these microorganisms have been reported, and very few mutants are available (30, 42). However, these halophiles are important not only for the ecology of hypersaline habitats but also for biotechnology. Their potential utility for environmental biotechnological applications in hypersaline habitats is evident, but in addition, many produce exoenzymes such as amylases, nucleases, and proteases of potential commercial interest (26), and the majority accumulate a variety of organic osmolytic compounds (''compatible solutes'') which might be used in the future as stabilizers of enzymes or whole cells (16). A detailed characterization of the physiology and biochemistry of these important bacteria and of their biotechnological applications requires the development of adequate genetic tools.

Chromohalobacter marismortui is a gram-negative, chemoorganotrophic, moderately halophilic bacterium isolated from the Dead Sea and marine salterns (57). It can grow within a wide range of salt concentrations (1 to 30% [wt/vol] salt), with an optimum of about 10%, and pHs (5 to 10), with an optimum of 7.5. This organism is a good model for genetic studies of moderate halophiles since in addition to its wide range of salt tolerance, it is simple to grow and maintain in the laboratory, utilizing carbohydrates, amino acids, and some polyols as sole carbon sources (57). In this article, we report the isolation and characterization of a narrow-host-range plasmid, pCM1, isolated from *C. marismortui* ATCC 17056.

Molecular mechanisms of plasmid replication have been extensively studied in recent years (33). Replication of broad-host-range plasmids in *Escherichia coli* as well as in other bacteria is well known (2, 7, 33, 55). Furthermore, replication of narrow-host-range plasmids has been widely examined in plasmids of *E. coli* such as ColE1, R1, R6K, and P1, etc. (5, 11, 33). These studies suggest that the inability of plasmids to be maintained in a particular organism is due to a lack of replication or segregation proficiency rather than to an inability to be introduced into or to express genetic markers in a distantly related host (50). There are some common structural features in the replication region of many replicons studied in gram-negative bacteria, such as the involvement of iterons (directly repeated sequences) in the replication of narrow- and broad-host-range plasmids of gram-negative bacteria (4), the presence of an A+T-rich region, and the presence of binding sites of host proteins required for plasmid replication, like DnaA (1, 25, 37, 39) and integration host factor (IHF) in the case of plasmid pSC101 (52, 53). The sequence of the basic replicon of the majority of the plasmids studied contains at least one open reading frame that encodes proteins implicated in plasmid replication. This fact suggests that a general mechanism may exist in the initiation reactions of different replicons. However, there is a lack of information on the plasmid replication mechanisms in bacteria not belonging to the family *Enterobacteriaceae*.

Studies of the molecular mechanism of replication of pCM1 may help to identify features that are associated with the narrow-host-range character of these plasmids and contribute to the construction of cloning vectors to be used in these biotechnologically important microorganisms. We describe herein the minimal replication region of the cryptic plasmid pCM1 from *C. marismortui*. The different features of this region and the sequence of the replication protein are also analyzed.

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TABLE 1. Organisms and plasmids used in this study

Organism or plasmid	Relevant characteristics	Reference or source
Organisms		
Vibrio costicola NCIMB 701^T	Wild type	17
Halomonas elongata ATCC 33173	Wild type	60
Halomonas subglaciescola UQM 2927^T	Wild type	13
"*Halomonas israelensis*" ATCC 43985	Wild type	59
Deleya halophila CCM 3662^T	Wild type	45
Chromohalobacter marismortui ATCC 17056^T	Wild type	57
Volcaniella eurihalina ATCC 49336^T	Wild type	44
Escherichia coli		
CC118(λ*pir*)	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-11 rpsE rpoB argE*(Am) *recA1*; lysogenized with λ*pir* phage	24
MV1190(λ*pir*)	Δ(*lac-proAB*) *thi supE* Δ(*srl-recA*) 306::Tn*10*(F′:*traD36 proAB lacI*^{q}*Z* ΔM15); lysogenized with λ*pir* phage	24
DH5α	F$^-$ *lacZ*ΔM15 *recA1 hsdR17 supE44* Δ(*lacZYA argF*)	Bethesda Research Laboratories
Plasmids		
pCM1	Cryptic plasmid (17.5 kb) isolated from *C. marismortui*	This study
pGP704	Ap^r; *ori* R6K; *mob* RP4; multiple cloning site of M13tg131	38

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria broth (48). All moderate halophiles were grown in saline medium containing 10% (wt/vol) total salts (58) and 0.5% yeast extract (Difco Laboratories, Detroit, Mich.) (SWYE medium). When necessary, the concentration of the salt solution in the SWYE medium was decreased to 4% (wt/vol). Solid medium contained 1.7% purified agar (Difco). As appropriate, the antimicrobial agents trimethoprim (Tp), chloramphenicol, and ampicillin were used for selection at concentrations of 500 (*E. coli*) and 150 (moderate halophiles), 25, and 100 μg/ml, respectively. When white/blue selection was possible, isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were used at concentrations of 110 μg/ml and 40 μg/ml, respectively. All cultures were incubated at 37°C.

Plasmid isolation. Screening for the presence of plasmid DNA from *C. marismortui* strains and small-scale preparation of plasmids from *E. coli* were done by the alkaline lysis method (48). Large-scale preparations of plasmid DNA were purified by CsCl-ethidium bromide density gradient ultracentrifugation (48). Plasmids for sequencing purposes were isolated with a QIAGEN plasmid kit (Diagen GmbH, Hilden, Germany).

Subcloning procedures and restriction analysis. DNA fragments were isolated from agarose gels with a Geneclean kit (Bio 101, La Jolla, Calif.) and subcloned into pBluescript II KS digested with the appropriate enzymes and treated with alkaline phosphatase. Transformation of *E. coli* was done by the rubidium chloride method (20). Cells were plated onto Luria broth agar containing ampicillin, X-Gal, and IPTG. Restriction analysis and recombinant DNA techniques were performed by standard methods (48) or as recommended by the reagent manufacturers. In order to obtain clear restriction patterns of recombinant plasmids present in halophilic bacteria, plasmids were reintroduced into *E. coli*, from which they were then isolated and subjected to restriction analysis.

Construction of deletions. Ordered deletions of subclones were made with exonuclease III and S1 nuclease, as described by Henikoff (23), with a kit from Promega (Madison, Wis.). The 1.6-kb *Eco*RI-*Eco*RV fragment was subcloned into pBluescript II KS, and the recombinant generated by this procedure was designated pB1. Nested unidirectional deletions from the *Eco*RI site towards the *Eco*RV site were generated by exonuclease III-S1 nuclease digestion of DNA which had been previously cleaved with *Eco*RI and *Sac*I; the fragments remaining after the deletions were designated ΔS. Deletions from the *Eco*RV site towards the *Eco*RI site were done on DNA previously cleaved with *Eco*RV and *Apa*I; the fragments remaining after the deletions were designated ΔA.

Construction of suicide vectors pEM10, pEM11, and pEMlac. The suicide vectors pEM10 and pEM11 (Fig. 1A) are derived from R6K plasmid-based pGP704 (38), which is able to replicate only in strains that produce the Pir protein, an R6K-specific essential replication protein. A 1.3-kb *Xba*I restriction fragment from pAS396 containing the gene conferring resistance to Tp was cloned in the corresponding sites in pGP704 to yield pEM10. Similarly, a 1.3-kb *Bam*HI fragment from pAS396 containing the Tp resistance gene was inserted into the single *Bgl*II site of pGP704, and the resulting construction was designated pEM11. For the construction of pEMlac, a 445-bp *Not*I restriction fragment from pNot18 containing the *lacZ* α-peptide was cloned in the *Pst*I-*Eco*RI site of pGP704. The resulting construction (pElac) was used to clone the 1.3-kb segment containing a Tp resistance gene in the *Bgl*II site to yield the plasmid pEMlac (Fig. 1B). This suicide vector includes the multiple cloning site of pUC18, the blue/white selection, and the Tp resistance gene as improvements on pGP704, making it useful for the isolation of replication regions from moderate halophiles specifically and from bacteria for which Tp and ampicillin are the best selection markers in general.

DNA sequencing. Double-stranded DNA sequencing was carried out by the dideoxy chain termination method (49). The standard protocols of the manufacturer for *Taq* DNA polymerase-initiated cycle sequencing reactions with fluorescence-labelled dideoxynucleotide terminators (Applied Biosystems Inc., Foster City, Calif.) were used. The products of the sequencing reactions were separated by denaturing polyacrylamide gel electrophoresis and analyzed with a 373A Automated DNA Sequencer (Applied Biosystems Inc.). Sequences were extended by the use of primers based on the sequences obtained. Sequence analysis and database searches and comparisons were done with the PC-Gene software package, version 6.7 (IntelliGenetics Inc., Mountain View, Calif.) and the GCG Sequence Analysis Software Package (Genetics Computer Group Inc., Madison, Wis.) (6).

Mating experiments. *E. coli* derivatives containing specific recombinant plasmids were mated with various moderately halophilic strains, with pRK600 being used as a helper plasmid (27). pRK600 is a derivative of pRK2013 (10) and contains the RK2 *tra* functions and a chloramphenicol resistance gene. This plasmid can mobilize the recombinant plasmids into the gram-negative moderate halophiles. Triparental matings in which *E. coli* HB101(pRK600) was used as the source of the mobilizing plasmid pRK600 were performed by a filter mating technique (24) employing a mixture of donor and recipient cells at a ratio of 1:1. Filters were incubated for at least 8 h at 37°C on the surfaces of 4% (wt/vol) saline medium plates; cells were then resuspended in 10 ml of sterile saline solution (0.85% NaCl), and appropriate dilutions were plated on selective medium. The frequency of transfer was estimated as the number of transconjugants per number of donor cells.

Assay of plasmid stability. The stability of plasmids pEM1, pEM2, pEM5, pED2, and pED4 in three moderate halophiles (*C. marismortui*, *Volcaniella eurihalina*, and *Halomonas elongata*) was assessed. Single colonies of plasmid-containing strains were inoculated in selective SWYE medium and cultured at 37°C to stationary phase. The cultures were diluted 10^3-fold in fresh, nonselective SWYE medium and incubated at 37°C. Afterwards, 10^3-fold dilutions into fresh nonselective SWYE medium after 7 or 10 generations, depending on the strain investigated, were made until the strain had been taken through up to 80 generations in nonselective medium. Cell counts were determined at intervals by removing 100-μl aliquots, diluting them 10^5- to 10^6-fold, and plating the dilutions on nonselective SWYE agar. From these plates, 100 colonies were screened for the presence of plasmids. The percentage of plasmid loss per generation was calculated from these data according to the method of Durland and Helinski (8).

Nucleotide sequence accession number. The sequence of the minimal autonomous replicon of pCM1 (see Fig. 4) has been assigned GenBank accession number X86092.

RESULTS

Isolation and restriction mapping of pCM1. Preliminary screening of several moderately halophilic strains revealed that some contain one or more plasmids of different sizes (data not shown). A cryptic plasmid from *C. marismortui* ATCC 17056,

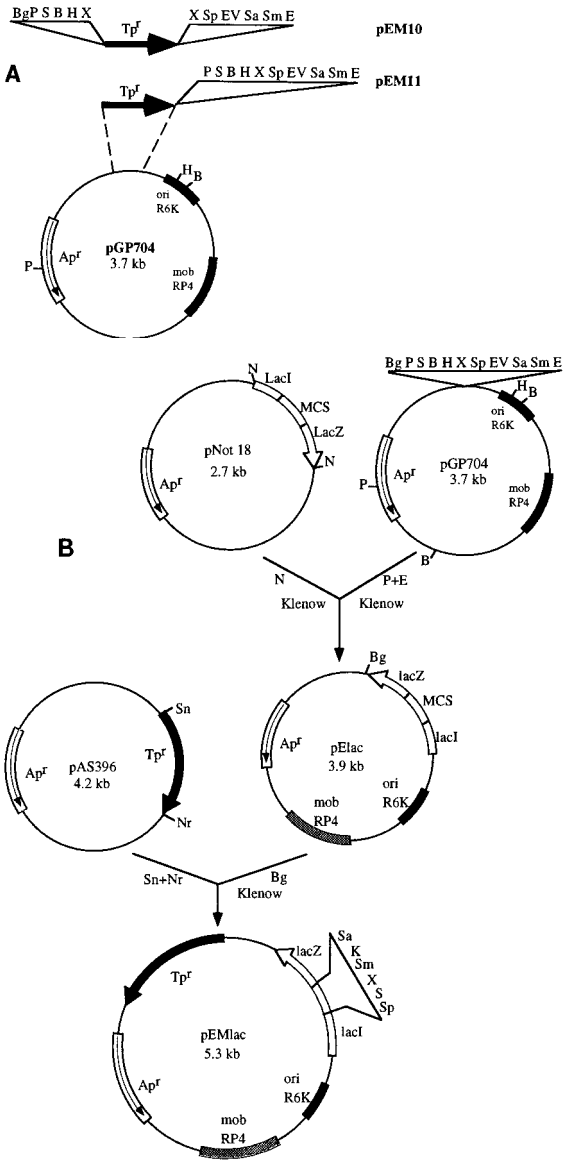

FIG. 1. (A) Suicide vectors pEM10 and pEM11. (B) Construction of the suicide vector, pEMlac, for cloning replication origins. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; N, *Not*I; Nr, *Nru*I; P, *Pst*I; S, *Sal*I; Sa, *Sac*I; Sm, *Sma*I; Sn, *Sna*BI; Sp, *Sph*I; X, *Xba*I; MCS, multiple cloning site.

designated pCM1, was isolated, purified, and used for further study. Restriction enzyme cleavage of pCM1 indicated unique sites for *Bgl*II, *Kpn*I, *Bam*HI, and *Xba*I (Fig. 2) and a molecular size of 17.5 kb.

Determination of the pCM1 minimal replicon. Lack of a selectable marker on this plasmid precluded an attempt at direct transformation into *E. coli*. Thus, the identification of plasmid regions competent for self-replication were identified by cloning into a suicide plasmid. Moderately halophilic bacteria are not susceptible to the majority of common antimicrobial agents which are totally or partially inactivated at high salt concentrations (42). An exception to this, however, is Tp. The Tp resistance gene from plasmid pAS396 was therefore used in the construction of a series of suicide vectors (pEM10, pEM11, and pEMlac) derived from the R6K plasmid-based pGP704 (38).

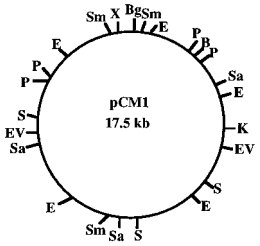

FIG. 2. Restriction map of pCM1 showing pertinent restriction sites. Bg, *Bgl*II; Sm, *Sma*I; E, *Eco*RI; EV, *Eco*RV; P, *Pst*I; B, *Bam*HI; Sa, *Sac*I; K, *Kpn*I; S, *Sal*I.

Fragments of pCM1 were cloned into Tp-resistant derivatives of the suicide vector pGP704, and the resulting recombinant plasmids were used to transform *E. coli* CC118(λ*pir*). These recombinant plasmids were subsequently transferred by conjugation into different moderate halophiles and tested for their ability to propagate themselves in these new hosts, which are not permissive for the R6K replicon of the vector (Fig. 3). Plasmid pEM1, consisting of the 6.1-kb *Sal*I-*Bgl*II fragment of pCM1 cloned into pEM10, was able to replicate as an autonomous Tp resistance replicon in different moderately halophilic strains. Constructions carrying other fragments of pCM1 were unable to replicate in the halophilic strains tested, even in *C. marismortui* carrying pCM1 (data not shown). This indicates that all functions necessary for the autonomous replication of pCM1 in the moderate halophiles tested were contained in the 6.1-kb *Sal*I-*Bgl*II fragment cloned in pEM1. In order to define the essential region more precisely, various subfragments of the 6.1-kb fragment were cloned into the suicide vectors. Hybrid plasmids were then transferred by conjugation to moderate halophiles containing (*C. marismortui*) or not containing (*Deleya halophila*, *H. elongata*, *Halomonas subglaciescola*, "*Halomonas israelensis*," and *V. eurihalina*) pCM1 in order to identify origin-containing fragments able to replicate in the presence of replicative factors supplied in *trans*. Plasmids pEM3 and pEM4 were unable to replicate even in bacteria carrying pCM1, indicating that deletions of the central 3.0-kb *Bam*HI-*Eco*RV fragment inactivated *oriV* (Fig. 3). Plasmid pEM5, which contains a 1.6-kb *Eco*RI-*Eco*RV fragment of pCM1, was able to replicate in all the moderate halophiles tested. The shorter 1.3-kb *Eco*RI-*Kpn*I fragment cloned in pEM6 was able to replicate only in *C. marismortui* containing the parental plasmid pCM1, indicating that the deleted 0.3-kb

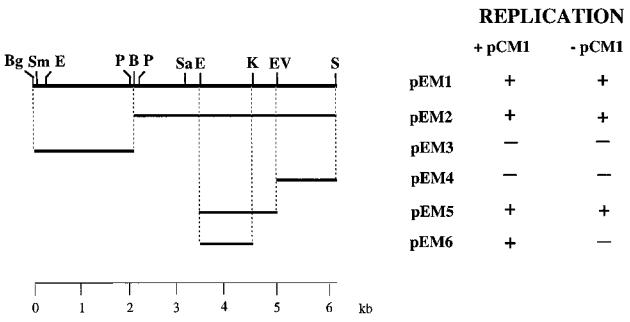

FIG. 3. Replication ability of DNA fragments generated from pCM1. pCM1 fragments of pEM1, pEM2, and pEM3 were cloned in pEM10; pEM4 and pEM5 were cloned in pEM11; and pEM6 was cloned in pEMlac. Only representative restriction sites are shown. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; EV, *Eco*RV; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sa, *Sac*I; Sm, *Sma*I; +, able to replicate; −, not able to replicate (in *C. marismortui*, *D. halophila*, *H. elongata*, "*H. israelensis*," and *V. eurihalina*).

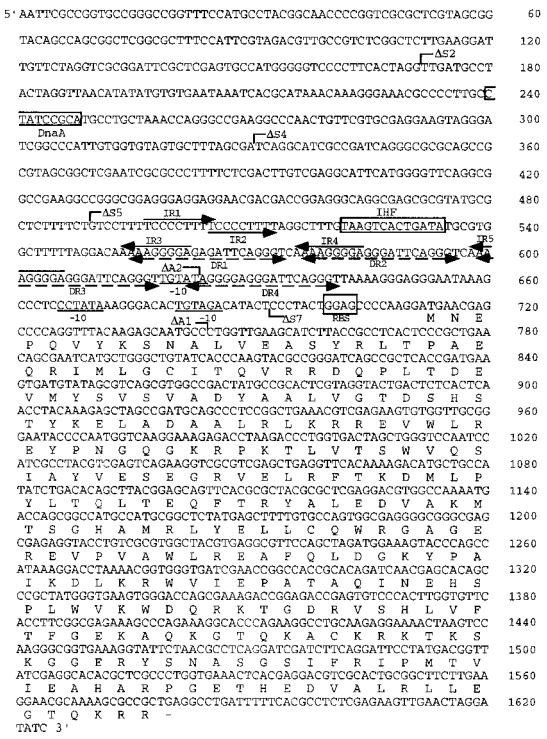

FIG. 4. Nucleotide sequence of the minimal autonomous replicon of pCM1. Potential features of the sequence are shown. Arrows indicate the extent and direction of deletions created and used in this study. Single-letter symbols for amino acids are aligned with the second nucleotide of each codon. Boxes show putative DnaA protein binding (DnaA), IHF-binding (IHF), and Shine-Dalgarno (RBS) sequences. Broken arrows indicate four direct repeats (DR1, DR2, DR3, and DR4), and solid arrows indicate five inverted repeats (IR1, IR2, IR3, IR4, and IR5). Potential −10 promoter regions are underlined.

*Kpn*I-*Eco*RV fragment contains a factor which is necessary for the replication and which can be supplied in *trans* (Fig. 3). Plasmids isolated from the various Tp-resistant exconjugants of the halophilic bacteria tested were of the expected sizes. No alteration of plasmid structure was detected after reintroduction into *E. coli* CC118(λ*pir*).

In order to test whether the plasmid replication system from *C. marismortui* is functional in *E. coli*, pEM5 was introduced into *E. coli* strains in which the suicide vectors are not functional because of an absence of the R6K Pir protein. In this genetic background, pEM5 was unable to replicate, indicating that the *C. marismortui* origin of replication is not functional in *E. coli*.

DNA sequence analysis of the pCM1 replicon and identification of relevant regions. DNA sequencing of the 1.6-kb *Eco*RI-*Eco*RV fragment from pEM5, which contains the functions necessary for autonomous replication, was done with pB1, a hybrid consisting of the 1.6-kb fragment cloned into pBluescript II KS (see Materials and Methods), as the DNA source and with universal primers as well as synthetic oligonucleotides.

The 1,624-nucleotide DNA sequence shown in Fig. 4 was scanned for open reading frames. One unique open reading frame, ORF1, extending from nucleotide positions 712 to 1581 and encoding a protein of 33.2 kDa was found. ORF1 starts with an ATG initiation codon located 8 nucleotides downstream of a Shine-Dalgarno-like sequence (5′-GGAG-3′). The deduced amino acid sequence shows a high content of basic (Arg and Lys) amino acids. Some of the best matches to promoter consensus sequences are from nucleotide positions 618 to 687, where there are three putative −10 regions (−10 TG TATA, −10 TGTAGA, and −10 CCTATA) with no corresponding −35 regions.

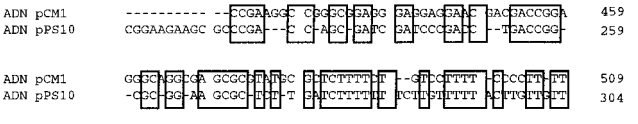

FIG. 5. Alignment of the *oriV* region of pCM1 with the *oriV* region of pPS10 from *P. syringae*. ADN, DNA.

In addition to this open reading frame, computer analysis shows a fragment of 673 bp in which the most notable features are (i) the presence of four conserved 20-bp tandemly repeated sequences (5′-AWAGGGGAGGRATTCAGGGT-3′) known as iterons, (ii) five inverted repeats of 9 nucleotides (5′-TC CCCTTTT-3′), (iii) a putative Dna box from nucleotide positions 240 to 248 with a sequence (5′-TTAT[A/C]CG[A/C]A-3′) similar to the DnaA-binding site sequence (15), and (iv) a consensus sequence of the IHF-binding site (5′-YAANNNNT TGATW-3′, where W is A or T, Y is a pyrimidine, and N is any nucleotide) (14) located in the middle of the inverted repeats, nucleotide positions 522 to 534. The 673-bp sequence of *oriV* was compared with those of the other reported minimal replicons by the FASTA program. A 65% identity between the nucleotide sequence of *oriV* of the narrow-host-range plasmid pPS10 from *Pseudomonas syringae* (42) and that of the *oriV* of pCM1 was found (Fig. 5).

ORF1 encodes a Rep protein. The ORF1-containing fragment downstream of the *oriV* fragment is necessary for autonomous replication of the plasmid, as the subcloning experiments showed that deletion of this fragment prevented replication of pEM6 except in the presence of pCM1. For this reason, ORF1, which could encode a Rep protein, was compared with other *rep* genes. A 52.9% identity between the central region of ORF1 and the *rep* gene of plasmid pFA3 from *Neisseria gonorrhoeae* was found (28). A comparison of the amino acid sequence of ORF1 with those of other Rep proteins revealed the highest homology (32% identical amino acids) with the replication protein of plasmid pFA3 from *N. gonorrhoeae* and slightly lower homologies with the RepE protein of the mini-F plasmid (25% identity) (35, 36) and the product(s) of ORF239 of pCU1 (24% identity) (31, 32). Alignment of these four Rep proteins revealed 22 amino acid residues conserved in all the proteins, localized mainly around residues 10 and 190 of the pCM1 RepA amino acid sequence (Fig. 6).

Deletion analysis of the 1.6-kb *Eco*RI-*Eco*RV fragment. Nested deletions of the 1.6-kb *Eco*RI-*Eco*RV fragment were made in order to define more precisely the replication region of pCM1. The deleted fragments were recovered as a *Pvu*II fragment from pB1 and cloned into the *Sma*I site of the suicide vector pEMlac. The deletion endpoints were determined by sequencing. These constructs were introduced into different

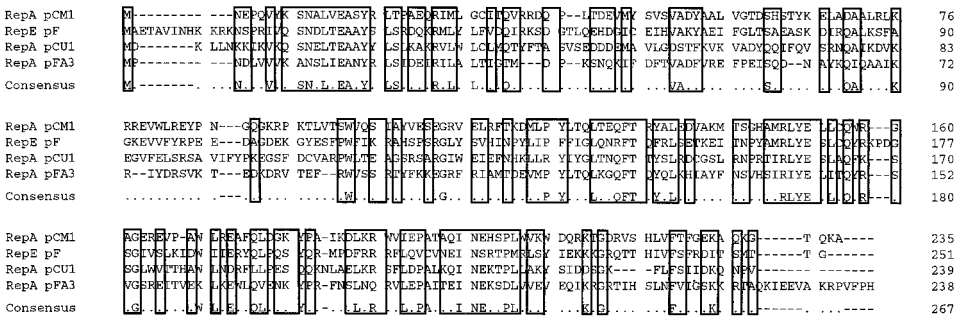

FIG. 6. Alignment of the sequences of the product of ORF239 of pCU1 (31, 32), RepE of pF (35, 36), RepA of pFA3 (28), and RepA of pCM1. Homologous amino acid sequences are boxed.

moderate halophiles with and without plasmid pCM1, and their ability to replicate was determined (Fig. 7B).

Four fragments generated by deletion from the *Eco*RI end were studied. Plasmids pED2 and pED4 containing fragments ΔS2 and ΔS4, respectively, with the latter lacking the putative DnaA box, were able to replicate but transferred to the halophiles with lower conjugation frequencies than pEM5-1.6-kb fragment-carrying plasmids. The sizes of exconjugant colonies carrying pED2 and pED4 were smaller and electrophoresis of plasmids prepared from these clones revealed a lower intensity of plasmid bands than the sizes and band intensity of exconjugant colonies carrying pEM5. Introduction of pED2 into *H. elongata* yielded only five or six exconjugants. Plasmid pED5 containing fragment ΔS5, which encompasses all of the identified repeat sequences, was unable to replicate even in the presence of pCM1. Plasmid pED7 containing fragment ΔS7, which carries only the replication protein, was, as expected, unable to replicate in either the presence or absence of pCM1.

Two fragments generated by deletion from the *Eco*RV end were also studied. Plasmid pED8 carrying fragment ΔA1, which lacks ORF1, can replicate only in bacteria harboring plasmid pCM1. Plasmid pED9 containing fragment ΔA2, which lacks one of the direct repeats, DR4, has lost the ability to replicate.

Investigation of the bacterial host range of pEM5. Plasmid pEM5 was transferred by conjugation from *E. coli* CC118(λ*pir*) to different moderate halophiles by means of the broad-host-range P group transfer functions. Plasmid DNA from Tp-resistant exconjugants was isolated and checked. As can be seen from Table 2, this plasmid can replicate and be stably maintained in most gram-negative moderate halophiles tested. An exception was *Vibrio costicola*, which is phylogenetically not closely related to the other moderate halophiles tested (37a). The plasmid is also unable to replicate in gram-negative bacteria (*E. coli* and *Pseudomonas putida*) not related to this group of halophilic bacteria. The highest transfer frequency was obtained with *C. marismortui* as the recipient. Cointegrate formation was not detected when plasmids were extracted from transconjugants, and no alteration of plasmid size or structure was observed by restriction enzyme cleavage analysis.

Plasmid stability. The stability of the different hybrid plasmids was examined in strains containing (*C. marismortui*) or lacking (*H. elongata* and *V. eurihalina*) the pCM1 plasmid. As Fig. 8 shows, similar results were obtained with the two strains lacking pCM1: all plasmids were stably maintained for more than 80 generations, except for pED4, which lacks the DnaA box. In contrast to the stability seen in pCM1-lacking strains, all plasmids, as expected, were unstable in *C. marismortui* containing pCM1, which is a result of the incompatibility of the two replicons. Plasmids containing larger fragments, pEM1 (6.1 kb) and pEM2 (4.0 kb), were lost in 45% of the cells after 80 generations in the absence of Tp selection, while plasmids containing the 1.6-kb or smaller fragments were completely lost after 35 generations.

DISCUSSION

Extremophilic microorganisms have assumed considerable importance in recent years and generated an increasing interest in their biology and biotechnological applications (16). The development of suitable recombinant DNA technology tools applicable to these organisms is a critical requirement for such endeavors. Until now, cloning vectors for the moderate halophiles have not been available. Recently, we reported that a

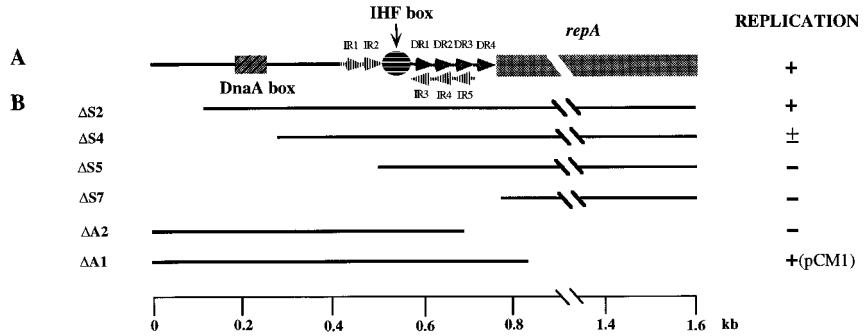

FIG. 7. Deletion analysis of the pCM1 replicon. (A) Schematic illustration of the 1.6-kb basic replicon of plasmid pCM1. (B) Consequence of deletions on replication. +, able to replicate; +(pCM1), able to replicate only in the presence of pCM1; −, not able to replicate; ±, defective replication.

TABLE 2. Bacterial host range of pEM5

Recipient strain	Transfer frequency (exconjugant per donor)[a]
Chromohalobacter marismortui ATCC 17056 ..	10^{-1}–10^{-2}
Deleya halophila CCM 3662	10^{-4}–10^{-5}
Halomonas elongata ATCC 33173	10^{-3}–10^{-4}
Halomonas subglaciescola UQM 2927	10^{-4}–10^{-5}
"*Halomonas israelensis*" ATCC 43985	10^{-4}–10^{-5}
Volcaniella eurihalina ATCC 49336	10^{-3}–10^{-4}

[a] The values shown are averages of three independent experiments.

plasmid, pMH1, is harbored by some moderate halophiles (9). This plasmid is, however, difficult to isolate and thus far can be detected only by transformation into *E. coli* cells. In contrast, in this article we show that pCM1 from *C. marismortui* ATCC 17056 can be readily isolated by a standard alkaline lysis technique. This enabled us to characterize its basic replicon, which is able to replicate in a number of moderately halophilic bacteria.

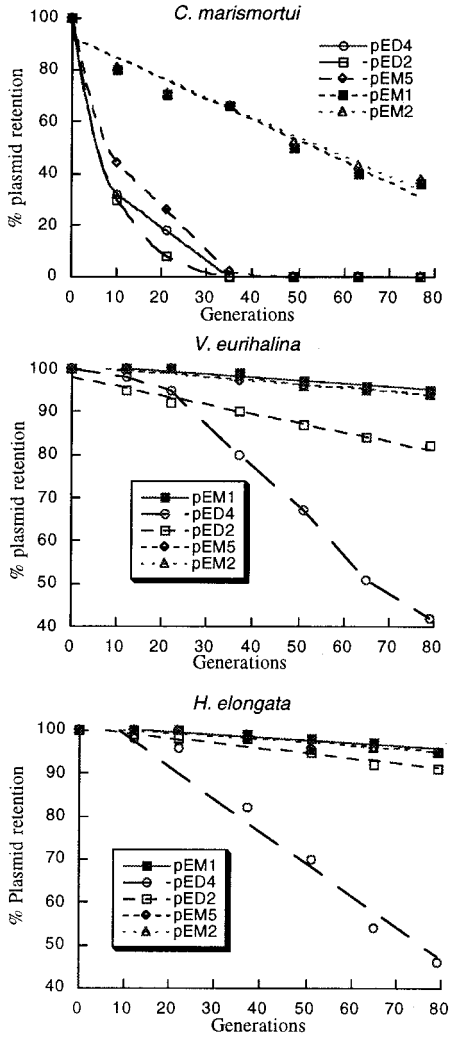

FIG. 8. Plasmid stability of constructions containing different pCM1 fragments. The assay is described in Materials and Methods. Percent plasmid retention means the percentage of the cell population which still harbors the plasmid. The generation times of *C. marismortui*, *H. elongata*, and *V. eurihalina* were calculated to be 90, 55, and 55 min, respectively.

Subcloning experiments demonstrated that the basic replicon of pCM1 is located in a 1.6-kb segment of the plasmid that contains a *cis*-acting region and *repA*, the gene of a protein required for replication. The origin region *oriV* has been defined to a 700-bp sequence that can function as an autonomous replicon in the presence of RepA. The organization of the basic replicon is similar to those of the pSC101 (54), mini-F (40), and mini-P (1, 61) replicons, with a DnaA box at the leftmost extremity of *oriV* followed by the iterons, an IHF-binding box, and the *repA* gene (Fig. 7A). Plasmid pED8 containing the region delimited by nucleotides 1 to 744 can replicate when the replication protein is supplied in *trans*. However, deletion of the leftmost 171 nucleotides of this fragment (Fig. 4 and 7) did not abolish replication, although the transfer frequency to the moderate halophiles was lower and the size of the resulting colonies was significantly smaller than those obtained when the whole replication region was present. This might be due to several reasons, including the removal of elements not essential for plasmid replication but somehow implicated in plasmid stability or the removal of regions implicated in the utilization of plasmid initiator protein. The former possibility is supported by the stability experiments in which those clones containing deleted plasmids lost the plasmid faster than did cells carrying the complete pEM5. The presence of a DnaA box in the *oriV* sequence suggests the participation of DnaA in the replication of pCM1, as has been reported for the organizationally related replicons of F (21, 29), pSC101 (22), P1 (1, 21, 61), RK2 (18), and pCU1 (31). Additional support for the involvement of this protein is indicated by the effect of the ΔS4 deletion which removed the DnaA box, resulting in a drastic reduction in the conjugation frequency and colonies of smaller sizes. If the DnaA protein is implicated in the replication of pCM1, it is not absolutely required. A similar finding has been obtained with *oriV* of pPS10 from *P. syringae* (41), although the DnaA protein is absolutely indispensable in the case of *oriC*, the replication origin of the *E. coli* chromosome, in which four DnaA boxes are scattered in the *ori* region and the deletion of one of them inactivates the activity of the origin (4). The role of the DnaA protein in pCM1 and pPS10 replicons, whose *oriV* regions share a high degree of homology (65%), may thus be different from that in *oriC*.

The *oriV* region of pCM1 contains four 20-bp direct repeats, or iterons. The involvement of such repeats of different lengths and numbers in the replication of both narrow- and broad-host-range plasmids of gram-negative bacteria is well documented (reference 41 and references therein). Iterons act as binding sites for the plasmid-encoded RepA protein and are absolutely necessary for replication initiation. This has been tentatively confirmed for pCM1 in our study by deletion of one of these iterons, DR4, which resulted in the loss of replication ability. The hexanucleotide sequence TCAGPuG has often been found within this family of direct repeats (12). In the mini-replicon of pCM1, a TCAGGG sequence is found at the right-hand ends of the iterons, as is the case with pPS10 (41).

During formation of the replisome of pSC101, DNA bending is necessary to bring together DnaA and RepA proteins bound to their recognition sites in the origin (3). This DNA bending is mediated by IHF, a sequence-specific, DNA-binding protein that belongs to the family of histonelike proteins of *E. coli* (43, 46, 51, 53). In pCM1, there is a putative IHF-binding site located just in the middle of the inverted repeats, suggesting that IHF might also be involved in bending the replicon to facilitate contacts between DnaA and RepA.

The expected size of the RepA protein (33.2 kDa) is similar to those of proteins expressed from other basic replicons (28, 32, 36). The expression of the RepA protein was attempted with both a prokaryotic in vitro DNA-directed transcription-translation system and an in vivo system (cloning the gene under the control of the ϕ10 promoter). In any case, no expression products were obtained. This fact might be due to product instability, considering that the protein is produced by a halophilic microorganism and the osmotic conditions could affect its stability.

Plasmids containing the 6.1-kb replication region of pCM1 or subfragments thereof are unstable in *C. marismortui* cells harboring pCM1 because of the incompatibility of the two homologous replicons. Plasmids carrying subfragments shorter than 4.0 kb are more unstable than those containing the complete 6.1-kb fragment, which might indicate the existence of a stability-related region upstream of the DnaA box. Alternatively, this effect may be due to the deletion of a sequence, *cmp*, a *cis*-acting plasmid locus that increases the interaction between replication origin and initiator protein, as has been described for the staphylococcal plasmid pT181 (19).

The information we have presented in this article constitutes a base for the development of versatile genetic tools for the analysis and manipulation of the ecologically and biotechnologically important group of moderately halophilic bacteria.

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