

SHORT COMMUNICATION

A Highly Conserved Plasmid from the Extreme Thermophile
Thermotoga maritima MC24 Is a Member of a Family
of Plasmids Distributed WorldwideT. Akimkina,* P. Ivanov,* S. Kostrov,* T. Sokolova,† E. Bonch-Osmolovskaya,† K. Firman,‡
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We have screened *Thermotoga* strains, isolated from hydrothermal vents near the Kuril Islands, for the presence of plasmid DNA. The miniplasmid pMC24 was isolated from the extreme thermophilic eubacteria *Thermotoga maritima* and sequenced, showing it to be a plasmid of 846 bp. It was found, from a search of the databases, to be closely related to the previously described *Thermotoga* miniplasmid pRQ7, isolated from a strain found on the Azore Islands, and was distinguished by only two point mutations. These changes resulted in two consecutive frameshifts altering a region encoding 9 amino acids in the Rep-coding region. We have also shown that pMC24, as with pRQ7, is negatively supercoiled. It seems that negatively supercoiled miniplasmids related to pRQ7 are spread worldwide and strongly maintained among *Thermotoga* strains. © 1999 Academic Press

Ecologically it appears that plasmids may be separated into two distinct clusters according to their supercoiled state (Drlica, 1992). The first large cluster includes highly negatively supercoiled plasmids isolated from eubacteria (both thermophilic and mesophilic) and mesophilic archaeobacteria. The second group includes slightly positively supercoiled or close-to-relaxed state plasmids from thermophilic archaeobacteria. The homeostatic maintenance of negative supercoiling is achieved by the antagonistic activities of at least two enzymes—ATP-dependent type II topoisomerase (DNA gyrase) that produces negative supercoils and an ATP-independent type I topoisomerase that removes them (Drlica, 1992; Luttinger, 1995).

The unusual topology of thermophilic archaeobacterial plasmids correlates with the presence of a unique topoisomerase type I reverse gyrase (Kikuchi and Asai, 1984), which introduces positive supercoils by an ATP-dependent process (Forterre *et al.*, 1985; Nadal *et al.*,

1986, 1988; Nakasu and Kikuchi, 1985). This enzyme was also detected in the cells of some thermophilic eubacteria (Bouthier de la Tour *et al.*, 1991), including a few *Thermotoga* strains. Members of the genus *Thermotoga* are strictly anaerobic and are among the most extremely thermophilic eubacteria; their physiology is similar to that of thermophilic archaeobacteria (Anhenbanch-Richter *et al.*, 1988). They represent one of the deepest known branches within the eubacteria (Stetter, 1996) and hence may possess features ancestral to this kingdom, of which the presence of reverse gyrase is probably one.

It has been suggested that the unusual topology of plasmid and viral DNA in hyperthermophilic archaeobacterium (i.e., relaxed or slightly positive supercoiling) may be important for function and that the presence of reverse gyrase serves as a hallmark of hyperthermophiles. However, the recent discovery of negative supercoiling in plasmid pRQ7, isolated from

Thermotoga maritima, one of the eubacteria hyperthermophiles, and the cloning of a gyrase gene show that this situation is not universal (Bouthier de la Tour *et al.*, 1995; Guipaud *et al.*, 1996, 1997; Palm *et al.*, 1993). Thus, *Thermotoga* possess a set of topoisomerases with antagonistic activities. Given the unique evolutionary position of this microorganism as ancestral, the intriguing question of the nature of the topology of *Thermotoga* plasmids arises. The cryptic miniplasmid pRQ7, discovered by Harriot *et al.* (1994), is the only known *Thermotoga* plasmid, and a negative supercoiling DNA topology has been determined for this plasmid.

Plasmid pRQ7 was found in a *Thermotoga* strain isolated from a region near the Azore Islands. Here we report the screening of *Thermotoga* strains, isolated from the distantly localized hydrothermal vents near the Kuril Islands region, for extrachromosomal DNA. This allowed the DNA sequencing of pMC24, a second *Thermotoga* miniplasmid very closely related to pRQ7, and showed that it is also negatively supercoiled. This plasmid was also found in two other *Thermotoga* strains, also isolated from the Kuril Island region, indicating that the plasmid is both highly conserved and widely distributed among these strains. The distant geographic locations of the isolates containing members of this family of plasmids suggest a worldwide distribution.

We screened 10 *Thermotoga* strains (9 marine strains of *T. maritima* and 1 freshwater strain *Thermotoga neapolitana*) for the presence of plasmid DNA. These strains were independently isolated from hydrothermal vents at different sites off the Kuril Islands and were maintained in the Microbial Museum of the Laboratory of Hyperthermophiles Microbial Communities (Institute of Microbiology, Russian Academy of Sciences). The strains were identified according to the standard scheme described in *Bergey's Manual of Determinative Bacteriology*, which involves hybridization of genomic DNA against samples of gDNA from known standard strains. Anaerobic cultures of different *Thermotoga* strains were cultivated at 80°C for 16 h (late exponential phase of

growth) in liquid Pfennig's medium containing 330 mg/L NH₄Cl, 330 mg/L KH₂PO₄, 330 mg/L MgCl₂·6 H₂O, 330 mg/L CaCl₂·2H₂O, 330 mg/L KCl, and 10 g/L sucrose with addition of vitamins and microelements (Pfennig and Lipirt, 1966; Wolin *et al.*, 1963). NaCl (2.5%) was added to the cultures of marine strains. Cells were collected by centrifugation at 4°C and plasmid DNA was isolated by the alkaline lysis method (Maniatis *et al.*, 1982) and analyzed by gel electrophoresis. Three strains (2 *T. maritima* and 1 *T. neapolitana*) were identified to contain plasmid DNA of the same size (about 800 bp). The corresponding plasmids were named pMC24, pMC26, and pMA1. A close relationship between these plasmids was demonstrated by Southern dot hybridization of each plasmid DNA, with plasmid pMC24 DNA serving as a probe (data not shown). Plasmid pMC24 was used for further investigations.

The ability of a number of restriction endonucleases to digest plasmid pMC24 was investigated. *EcoRV* and *HindIII* were capable of linearizing the plasmid, indicating the presence of single recognition sites for these enzymes. To determine the nucleotide sequence of pMC24, the *EcoRV*-linearized plasmid pMC24 was ligated to plasmid pTZ19 using the *SmaI* site in the polylinker. Plasmid DNA from one of the resultant recombinant plasmids was used to determine the partial nucleotide sequence of pMC24. On the basis of these sequence data, two inversely oriented oligonucleotide primers (5'-dCTTTGAGTACGTTCCCG-3' and 5'-dGAAGATATGGTAATTTTG-3') were synthesized. These were used to amplify the full-length DNA from plasmid pMC24. This work was carried out at Portsmouth University, ensuring no likelihood of contamination of the PCR product by other *Thermotoga* plasmids. Direct automatic sequencing of both strands of the obtained PCR product was used to determine the complete nucleotide sequence of pMC24 (courtesy of Cambridge BioScience, Ltd.). This strategy ensured that the full sequence of the native plasmid was obtained and obviated the possibility of losing regions of pMC24 containing more than one closely spaced recognition site for *EcoRV*. The plasmid

a) 445
 Y I W V R E R T G S G L V H M
 pMC24 TACATTTGGGTACGGGAACGTACTCAAAGTGGTTTGG-TGCATATG
 pRQ7 TACATTTGG-TACGGGAACGTACTCAAAGTGGTTTGGGTGCATATG
 Y I W Y G N V L K V V W V H M
 445

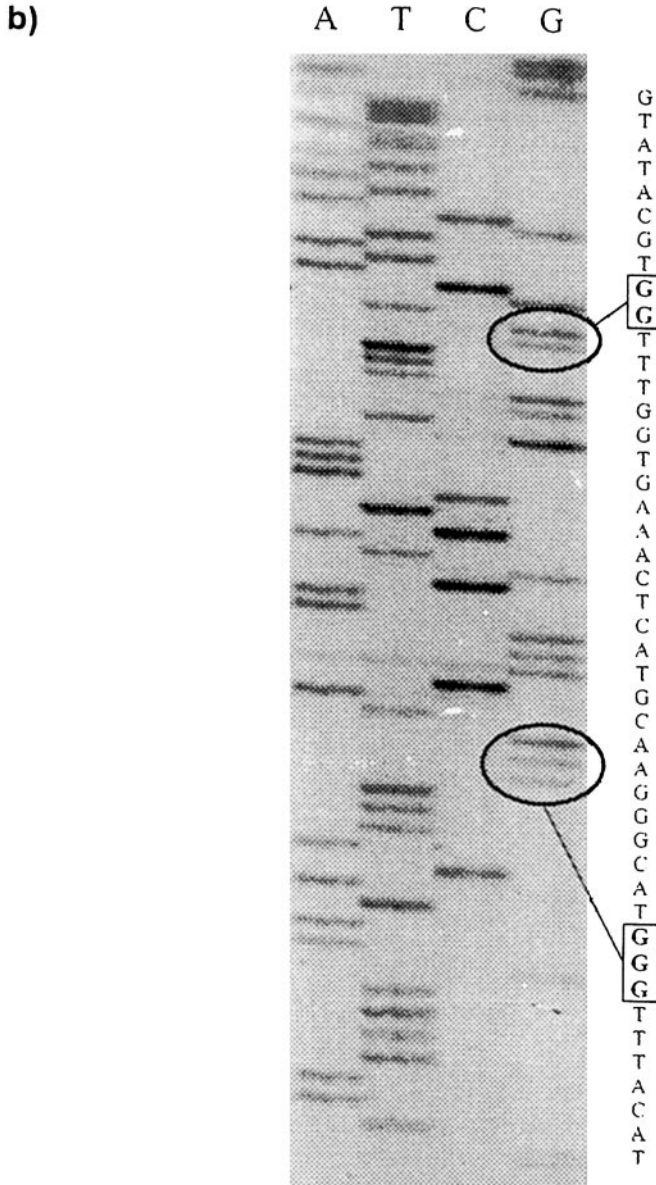


FIG. 1. Differences in the DNA sequence of pMC24 and pRQ7. (a) Alignment of the DNA sequence of the two plasmids pMC24 and pRQ7 in the region surrounding the observed differences. pMC24 has an additional G at position 454 and is missing a G at position 482. This results in the substitution of 9 amino acids within the coding sequence (shown in bold). (b) The DNA sequencing gel for the above region confirming the alterations described above.

consists of 846 bp. The DNA sequences of pMC24 and the previously described pRQ7 (Harriot *et al.*, 1994) were found to be highly conserved. pMC24 is distinguished from pRQ7 by only two point mutations (Figs. 1a and 1b). Both mutations are localized in the region of the large open reading frame, which is thought to encode the Rep protein (Guipaud *et al.*, 1997) required for plasmid replication. The first mutation is the introduction of an additional G nucleotide within the putative *rep* gene, which leads to a frameshift. The second mutation is the deletion of a G 27 nucleotides downstream of the first alteration. This mutation reconstitutes the reading frame of the Rep protein. This region, containing the two point mutations (compared to the pRQ7 sequence), was also sequenced manually using an Amersham sequencing kit to confirm the changes. These changes mean that the predicted Rep proteins encoded by plasmids pMC24 and pRQ7 are distinguished by a short nonidentical sequence of 9 amino acids in positions 63–71 (Fig. 1b). It is interesting that this part of the Rep protein, as encoded by pMC24, contains a set of 4 charged amino acids. This fact suggests that this region of the protein may be exposed on the surface of the molecule.

The above base changes were also identified on plasmids pMC26 and pMA1 by manual DNA sequencing of this region (data not shown). The lack of other changes to the DNA sequence, particularly the lack of variation at the third base of each codon of Rep protein, indicates a strong conservation among these plasmids, suggesting that further investigation of the sequence of family members may be required. Furthermore, the confirmation that all the plasmids we have isolated have the same base changes suggests that these changes are real. Other structural elements essential for plasmid replication, including the plus-strand replication origin and a set of direct and inverted repeats, are identical for both plasmids. It is interesting that the host *Thermotoga* strains carrying the novel plasmids pMC24, pMC26, and pMA1 and the strain with plasmid pRQ7 were isolated from distantly localized thermal sources (near the Kuril and Azore Islands, re-

spectively). Therefore, plasmids of this family seem to be spread worldwide among *Thermotoga* strains. The likelihood of contamination of the Kuril *Thermotoga* strains by pRQ7 is very remote, as the Azore strain has never been used, or held, in the collection used in these studies. We can speculate about the reasons for the high degree of conservation of the plasmid structure, suggesting that the degree of conservation in the DNA sequence is biologically important and/or may be related to the modest levels of spontaneous mutations due to the presence of efficient DNA repair mechanisms present in hyperthermophiles (Grogan, 1998). It is interesting that the plasmids encode only information sufficient for their own replication, and this suggests that the plasmid DNA may be extremely "selfish" and may even be parasitic. It is even possible that *Thermotoga* strains carrying this plasmid can "migrate" between thermal sources effectively "deep frozen" in the sea currents. However, this is far from proven and other explanations are possible.

We also investigated the DNA topology of this plasmid and found it to be negatively supercoiled, as is pRQ7 (Guipaud *et al.*, 1997), with a degree of supercoiling of -0.040 ± 0.006 . These values agree remarkably well with those obtained by (Guipaud *et al.* (1997)). It is interesting that we have found no other plasmids different from the pRQ7 analog in *Thermotoga* strains. Therefore, the existence of other topological forms (positively supercoiled or relaxed) of extrachromosomal DNA in *Thermotoga* cells remains an open question.

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