Plasmid curing in *Thermus thermophilus* and *Thermus flavus*

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1. INTRODUCTION

The elimination of plasmid DNA from plasmid-carrying host strains, or curing, has been described for several species of mesophilic bacteria. Techniques for this purpose involve growth of the strains at elevated temperatures [1], or in the presence of sodium dodecyl sulphate [2], intercalating drugs [3,4], inhibitors of DNA replication [5,6] or transcription [7], and the formation of protoplasts [8,9]. In contrast, curing of extremely thermophilic microorganisms has not been reported so far in the literature.

Authors in [10] isolated several circular deoxyribonucleic acid molecules from 4 different strains belonging to the genus *Thermus*. We have characterized and cloned in *Escherichia coli* two of such molecules: pTT8 (6.0 MDa) (we had referred to this plasmid as pTT1; however, following the nomenclature proposed in [10], hereinafter we shall call it pTT8) from *Thermus thermophilus* HB8 [11]; and pTF62 (6,8 MDa) from *Thermus flavus* AT-62 [12].

Several agents known to promote curing of mesophilic bacteria, including ethidium bromide, acridine orange, rifampicin and novobiocin, were tested with *T. thermophilus* HB8 and *T. flavus* AT-62. Of these, only the gyrase inhibitor novobiocin was effective in eliminating pTT8 and pTF62 from their host strains, although at a low frequency.

In the course of these experiments, a second plasmid (pVV8, 47 MDa) present in *T. thermophilus* HB8 was identified.

2. MATERIALS AND METHODS

2.1. *Cells and growth conditions*

Thermus thermophilus HB8 was obtained from the American type culture collection (ATCC 27634). *Thermus flavus* AT-62 was a generous gift of Dr T. Saiki, Japan. Cells were grown under conditions described in [13].

2.2. *Plasmid elimination*

To cure the strains from their plasmids, cultures were started with an initial concentration of 5000 cells/ml and were left to grow to stationary phase in the presence of the curing agent as indicated in table 1. Cells were then plated in 2% agar plates containing growth media, but devoid of the curing substance, and colonies were screened for the presence of pTT8 or pTF62 by electrophoresis in agarose gels. Since pVV8 could not be visualized using direct colony screening, its presence had to be scored by electrophoresis of total DNA preparations from *T. thermophilus* HB8 in agarose gels. Cell lysates were prepared as in [11,12].

2.3. *Agarose gel electrophoresis*

Agarose (1%, Seakem) vertical slab gels were run in 50 mM Tris–HCl, 40 mM sodium acetate and 1 mM EDTA (pH 8.0). Gels were stained with ethidium bromide (1 μg/ml) and photographed under ultraviolet light.

2.4. *Electron microscopy*

DNA was prepared for electron microscopy as in [14] using pBR322 as a standard. Photographs of

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Table 1

Effect of intercalating dyes, rifampicin and novobiocin on plasmid elimination from *Thermus thermophilus* HB8

Curing agent	Conc. (μg/ml)	MIC (μg/ml)	pTT8-less colonies/no. colonies	pVV8-less colonies/no. colonies
Ethidium bromide	0.2	0.4	0/150	0/18
Acridine orange	1.0	5.0	0/150	0/22
Rifampicin	25.0	50.0	0/300	0/40
Novobiocin	0.1	0.5	2/400	0/55

Minimal inhibitory concentrations are the same for *T. thermophilus* HB8 and *T. flavus* AT-62

pVV8 molecules were enlarged and the contour lengths were measured.

3. RESULTS AND DISCUSSION

When a total DNA preparation obtained from *T. thermophilus* HB8 is subjected to agarose gel electrophoresis, in addition to pTT8 a band migrating slower than chromosomal DNA is always detected. This material, electroeluted from the gel and analyzed by electron microscopy, represents a high M_r plasmid which we have named pVV8 (fig.1). The size of pVV8 was calculated by electrophoresis in 0.8% agarose gels utilizing plasmids R40A (96 MDa), R1 (62 MDa), RP4 (36 MDa) and SA (25 MDa) as M_r-standards [15]. Following this procedure, a value of 47 MDa was obtained, which is in agreement with contour length measurements (not shown). To our knowledge, this is by far the largest extrachromosomal element found in an extremely thermophilic bacterium. The possibility that pVV8 might be a multimer of pTT8 was ruled out by

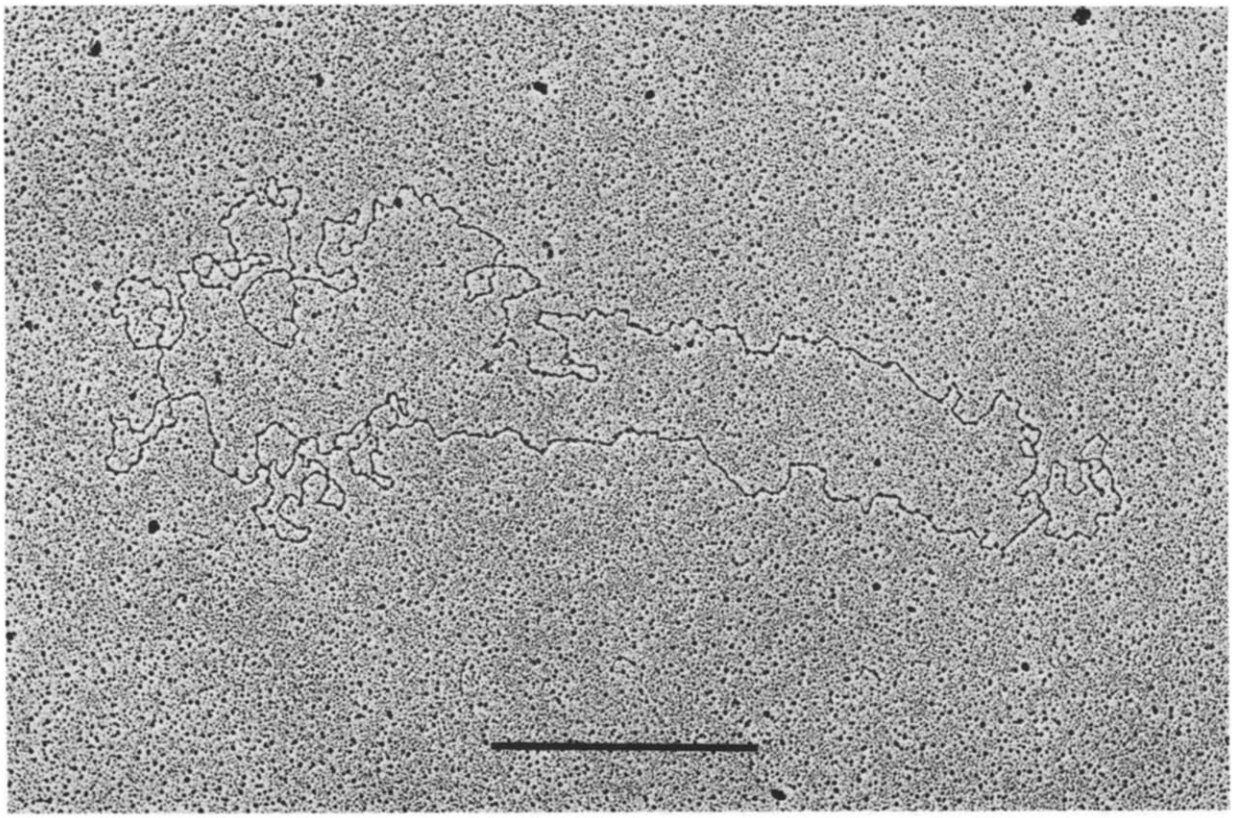

Fig.1. Electron micrograph of plasmid pVV8. The bar indicates 1.0 μm.

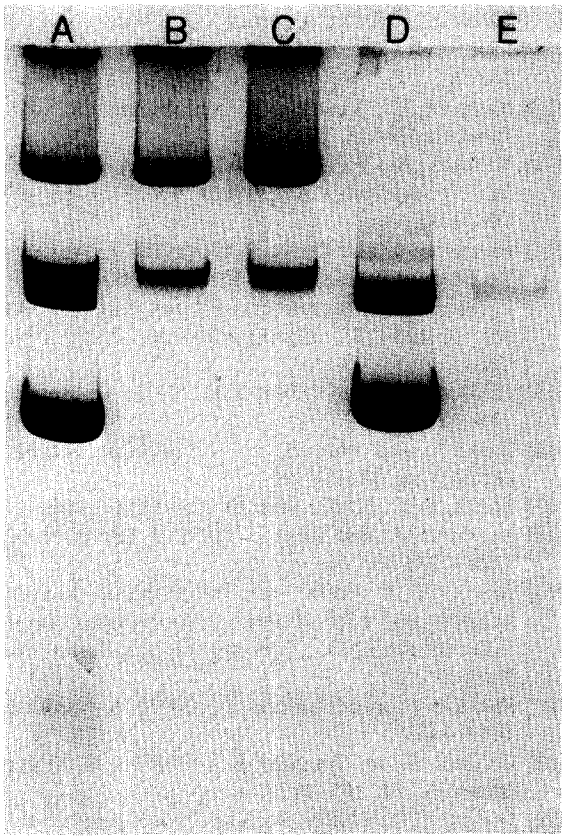

Fig.2. Electrophoretical separation of total DNA from cell lysates in 1% agarose gels: (A) *T. thermophilus* HB8; (B) and (C) *T. thermophilus* HB8 lacking pTT8; (D) *T. flavus* AT-62; (E) *T. flavus* AT-62 lacking pTF62.

restriction enzyme analysis. In turn, exhaustive analysis of *T. flavus* AT-62 lysates have confirmed the presence of only pTF62.

As an attempt to find a genetic marker in these plasmids, we have tried to find conditions for their elimination from their host strains. Curing agents utilized included ethidium bromide, acridine orange, rifampicin and novobiocin. Although *T. thermophilus* HB8 is about 100-times more sensitive to intercalating drugs and novobiocin than mesophilic strains [3–6], only the latter was effective in pTT8 elimination (table 1). The same results were obtained in curing experiments carried out with *T. flavus* AT-62, being pTT8 and pTF62 eliminated at the same frequency. The search of pVV8 elimination was more difficult due to the tedious screening procedure for this plasmid. However, a total of 135 different cultures derived from an equal number of colonies were analyzed and found to contain pVV8. Fig.2 shows the pattern obtained after agarose gel electrophoresis of total DNA preparations of *T. thermophilus* HB8 and *T. flavus* AT-62 strains, both harboring and lacking the small plasmids.

Based on their behavior in the presence of curing agents, pTT8, pVV8 and pTF62 appear to be quite stable. However, we have so far been unable to find any phenotypic expression that could be ascribed to the small plasmids. Strains both harboring and lacking pTT8 and pTF62 exhibit the same minimal inhibitory concentration (MIC) with a variety of antibiotics including ampicillin, gentamicin, chloramphenicol, sisomicin, amoxicillin, streptomycin, penicillin, lincomycin, bacitracin, thianphenicol and kanamycin. Both *Tth*I and *Tfl*I, which are heat-stable restriction enzymes that we [12,13], as well as others [16], have isolated from *T. thermophilus* HB8 and *T. flavus* AT-62, respectively, are present in their hosts after plasmid elimination, showing that they are chromosomal-coded enzymes. Several plasmid-carrying strains, including *T. thermophilus* HB8 and *T. flavus* AT-62 have been tested for the ability to produce bacteriocins [10]. The finding of the lack of bacteriocin production by these strains has been confirmed by us; it is therefore not possible to correlate the presence of pTT8 and/or pTF62 with bacteriocin production.

Some bacterial plasmids code for functions that confer resistance to heavy metal salts [17,18]. Since the presence of heavy metals has been reported in most hot springs and geysers [19], the natural habitat of *Thermus* strains, the MIC of some of these metals was tested. No difference in the MIC for the wild type and cured strains was found when salts of Hg^{2+}, Ag^{+}, Al^{3+}, Tl^{3+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Co^{2+} and Ba^{2+} were added to the cultures at 10^{-4}–10^{-6} M. Finally, phage ϕYS 40, which is infectious to some *Thermus* strains [20], exhibits the same infectivity to *T. thermophilus* HB8, irrespective of the presence or absence of pTT8.

With respect to plasmid pVV8, its large size would suggest that it might code for transfer functions. However, electron microscopic observations under various conditions of cells belonging to the genus *Thermus* have shown that these bacteria lack extracellular appendices, including pilus-like structures [21–23].

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