Isolation of Extrachromosomal Deoxyribonucleic Acids from Extremely Thermophilic Bacteria

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Eight strains of thermophilic bacteria were examined for the presence of covalently closed circular deoxyribonucleic acid molecules by caesium chloride-ethidium bromide density gradient centrifugation. Four of the eight strains tested, *Thermus flavus* BS1, AT61, AT62 and *Thermus thermophilus* HB8 carried covalently closed circular DNA molecules. *Thermus flavus* BS1 harboured two species of plasmids with molecular weights of 6.1×10^6 and 17.0×10^6 as determined by electron microscopy. *Thermus thermophilus* HB8, *T. flavus* AT61 and *T. flavus* AT62 carried plasmids with molecular weights of 6.2×10^6 , 6.6×10^6 and 6.6×10^6 , respectively. Plasmids from *T. flavus* AT61 and AT62 were indistinguishable in their electrophoretic patterns in agarose or acrylamide gel after digestion with various restriction endonucleases. This is the first evidence for the presence of plasmids in extremely thermophilic bacteria, though their functions are unknown.

INTRODUCTION

Recently, techniques for introducing recombinant DNA molecules into *Escherichia coli* cells as autonomous replicons have been developed (Cohen, 1975). The use of these techniques has been of major importance in the advance of fundamental knowledge, and has already contributed to our understanding of the structure and function of genes (Cohen & Chang, 1975; Cohn, Lowry & Kedes, 1976; Efstratiadis, Kafatos & Maniatis, 1977; Brutlag *et al.*, 1977; Hershey *et al.*, 1977; Tonegawa *et al.*, 1977). At the same time, there has been concern over potential biohazards and attention has focused upon the need for DNA cloning vectors and bacterial hosts with a restricted capacity to multiply and transmit genetic information outside the laboratory.

The establishment of *in vitro* recombinant DNA techniques in an extreme thermophile should reduce the risks since these bacteria are obligate aerobes, are not parasitic, not spore-forming, and grow only above 45 °C with an optimum growth temperature of 60 to 72 °C (Oshima & Imahori, 1971; Saiki, Kimura & Arima, 1972). Furthermore, Lindsay & Creaser (1975) reported that thermostability of an enzyme was a transformable property between *Bacillus* species. It would be of interest to test whether genes from mesophilic bacteria could function in the thermophiles and if they do, whether products of the genes are converted to heat resistance.

We have looked for plasmids which would serve as vectors for the construction of recombinant DNA molecules in extreme thermophiles. This report describes the demonstration of covalently closed circular (CCC) DNA molecules in four *Thermus* strains by the technique of CsCl-ethidium bromide density gradient centrifugation. The isolation and characterization of these plasmids is reported.

METHODS

Bacterial strains and growth conditions. Thermophilic bacteria were kindly supplied by Dr T. Oshima of this Institute. The extreme thermophiles, *Thermus thermophilus* HB8 (Oshima & Imahori, 1971), *Thermus* HB27 (Sakaki & Oshima, 1975), and *Thermus flavus* strains BS1, BK1, AT61 and AT62 (Saiki *et al.*, 1972), were cultured at 75 °C with shaking in nutrient broth (pH 7.0) containing (g l⁻¹): polypeptone (Daigo Eiyo Chemical Co., Osaka, Japan), 5; yeast extract (Difco), 4; NaCl, 2; and glucose, 1 (Hishinuma, Hirai & Sakaguchi, 1977). A moderate thermophile, *Bacillus stearothermophilus* IAM1035 was grown at 60 °C in a medium consisting of (g l⁻¹): yeast extract, 5; polypeptone, 5; K₂HPO₄, 1; and glucose, 2. An acidophilic and thermophilic bacterium, *Bacillus acidocardarius* TA6, was grown at 65 °C in the medium described by Oshima, Arakawa & Baba (1977).

Isolation of plasmids. Methods for the isolation of CCC DNA from the bacteria and the isotopic labelling of DNA were as described previously for the isolation of plasmids from *Bacillus subtilis* (Tanaka, Kuroda & Sakaguchi, 1977) except that the initial density of the CsCl-ethidium bromide solution was adjusted to 1.600 g ml⁻¹ and [³H]adenosine (10 µCi ml⁻¹; The Radiochemical Centre, Amersham) was used instead of [³H]thymidine since thymidine was not incorporated into the DNA of the extreme thermophiles even when deoxyadenosine was present in the culture medium. Plasmid DNA was collected from the gradient, extracted three times with 1 vol. *n*-butanol to remove ethidium bromide, and dialysed against 0.1 × SSC (SSC buffer is 0.15 M-sodium chloride/0.015 M-sodium citrate) plus 1 mM-EDTA. The plasmid DNA preparation was incubated with ribonuclease A (10 µg ml⁻¹; Sigma) and ribonuclease T1 (5 µg ml⁻¹; Sankyo Co., Tokyo, Japan) to digest the ribonucleic acids.

Sucrose density gradient centrifugation. ³H-labelled plasmid DNAs were centrifuged in neutral 5 to 20 % (w/v) linear sucrose gradients in buffer consisting of 0.03 M-Tris/HCl pH 8.0, 0.005 M-EDTA and 1 M-NaCl. ¹⁴C-Labelled λ phage DNA (obtained by heat induction of *E. coli* M65 lysogenized with λcl857S7) was added to each sample as an internal marker for measuring S values of the plasmids. The samples were centrifuged at 50000 rev. min⁻¹ for 90 min in a Hitachi RPS65T rotor. Gradients were fractionated, and 0.1 ml portions of each fraction were applied to Whatman GF/C filters, dried and counted in a liquid scintillation counter.

Determination of the molecular weights of plasmid DNA by agarose gel electrophoresis. Plasmid DNAs were incubated with freshly diluted deoxyribonuclease I (0.01 µg ml⁻¹; Worthington Biochemical Corp.) for 10 min at 20 °C in 10 mM-Tris/HCl pH 8.0 containing 5 mM-MgCl₂ and 20 mM-NaCl. The reaction was stopped by addition of 0.033 vol. 0.5 M-EDTA (pH 8.0). Approximately 80 % of the plasmid molecules were converted to the linear form by this treatment; the remaining 20 % were in the open circular form. The incubated mixture was added to *Eco*RI digests of λ phage DNA, RSF1010 DNA [mol. wt 5.5 × 10⁶ (Tanaka & Weisblum, 1975)] and RSF2124 DNA [mol. wt 7.2 × 10⁶ (So, Gill & Falkow, 1975)] as internal markers, and the samples were electrophoresed in 0.7 % (w/v) agarose gels.

Electron microscopy. The procedure has been described previously (Tanaka *et al.*, 1977). The electron microscope used was model JEOL-100B (JEOL, Tokyo, Japan). The molecular weight of each plasmid was determined by comparison with Cole1 DNA [4.2 × 10⁶ (Bazaraal & Helinski, 1968)] added as an internal standard.

Digestion of plasmid DNAs with restriction endonucleases. Plasmid DNAs were digested with restriction endonucleases *Eco*RI (Tanaka & Weisblum, 1975), *Hind*III (Smith & Wilcox, 1970), *Hpa*I (Sharp, Sugden & Sambrook, 1973), *Hind*II (Landy *et al.*, 1974), *Bam*NI (identical with *Bam*I) (Shibata & Ando, 1976) and *Sma*R (Tanaka & Weisblum, 1975) for 30 min at 37 °C, and heated at 65 °C for 5 min to inactivate the enzymes. The resultant DNA fragments were separated by electrophoresis in agarose or acrylamide gels. Gels were formed in an electrophoretic buffer (pH 8.0) consisting of 40 mM-Tris base, 20 mM-sodium acetate, 1 mM-EDTA and 0.5 µg ethidium bromide ml⁻¹.

RESULTS

Isolation of plasmids

Four of the eight thermophiles tested, *T. thermophilus* HB8, and *T. flavus* AT61, AT62 and BS1, harboured plasmids. For quantitative analysis, cells were labelled with [³H]adenosine and the whole lysates were centrifuged in CsCl-ethidium bromide gradients. Satellite peaks were observed in these four strains to an extent of 1.9 %, 1.2 %, 2.0 % and 2.8 % of the chromosomal DNA, respectively.

Fig. 1. Sucrose density gradient centrifugation (5 to 20%, w/v) of [^3H]adenosine-labelled plasmid DNA from: (a) *T. flavus* BS1; (b) *T. thermophilus* HB8; (c) *T. flavus* AT61; (d) *T. flavus* AT62. Plasmid DNAs were labelled as described in Methods. Arrows indicate the position of [^{14}C]thymidine-labelled λ DNA included as a molecular weight marker. The top of the gradient is to the right. The radioactivity in the top of each gradient is due to small fragments of RNA generated by RNAase A and RNAase T1 treatment.

Sucrose density gradient centrifugation

The ^3H -labelled plasmid DNAs obtained from the CsCl -ethidium bromide density gradients were subjected to neutral 5 to 20% (w/v) sucrose density gradient centrifugation with ^{14}C -labelled λ DNA as a marker (33.6S) for determination of molecular weight. The gradient from *T. flavus* BS1 gave two peaks with S values of 28.5 and 40.6 (Fig. 1a) indicating that there are two plasmids (pTF1 and pTF2, respectively) in the cell. Plasmids of *T. thermophilus* HB8, and *T. flavus* AT61 and AT62 sedimented at 28.8S (pTT8), 28.8S (pTF61) and 29.0S (pTF62), respectively (Fig. 1b to d). The molecular weights of these plasmids, as calculated by the equation of Hudson, Clayton & Vinograd (1968), are presented in Table 1.

The number of copies of each plasmid can be calculated from the molecular weight and the amount of plasmid present in the CCC form per chromosome. On the assumption that the molecular weight of the chromosomal DNA of these extreme thermophiles is the same as that of *E. coli* [2.5×10^9 (Cooper & Helmstetter, 1968)] and that most of the plasmids are obtained in the CCC form, the numbers of copies were estimated and are summarized in Table 1.

Fig. 2. Electron micrographs of circular plasmid DNA: (a) pTF1; (b) pTF2; (c) pTT8; (d) pTF61; (e) pTF62. Bar markers correspond to a mol. wt of 1.0×10^6 .

Table 1. *Molecular weights and number of copies of the plasmids from extreme thermophiles*

Strain	Plasmid	$10^{-6} \times$ mol. wt, determined by:			No. of copies per chromosome
		Sucrose gradient centrifugation*	Agarose gel electrophoresis†	Electron microscopy‡	
<i>T. flavus</i> BS1	pTF1	6.2	5.9	6.1	9
<i>T. flavus</i> BS1	pTF2	13.5	—§	17.0	1
<i>T. thermophilus</i> HB8	pTT8	6.3	5.9	6.2	8
<i>T. flavus</i> AT61	pTF61	6.3	6.6	6.6	5
<i>T. flavus</i> AT62	pTF62	6.4	6.6	6.6	8

* λ DNA was included as an internal marker.

† Linear plasmid DNAs were obtained by digestion with deoxyribonuclease I as described in Methods, and electrophoresed with *Eco*RI digests of λ DNA, RSF1010 and RSF2124 as internal standards.

‡ ColE1 DNA was used as an internal standard.

§ The molecular weight of pTF2 was estimated to be larger than 13.7×10^6 (the largest fragment of *Eco*RI digests of λ DNA) and was not determined by this method.

Table 2. Number of cleavage sites with restriction endonucleases

Plasmid DNAs were digested with restriction endonucleases for 30 min at 37 °C, heated at 65 °C for 5 min, and the resultant fragments were separated by electrophoresis in agarose (digests with *EcoRI*, *HindIII*, *HpaI*, and *HindII*) or acrylamide (digests with *BamNI* and *SmaR*) gels.

Enzyme	No. of cleavage sites			
	pTF1	pTT8	pTF61	pTF62
<i>EcoRI</i>	0	0	0	0
<i>HindIII</i>	0	0	2	2
<i>BamNI</i>	5	11	9	9
<i>SmaR</i>	16	10	12	12
<i>HpaI</i>	0	1	1	1
<i>HindII</i>	0	5	2	2

Fig. 3. Cleavage patterns of plasmid DNAs with *BamNI* restriction endonuclease. Digests were fractionated by electrophoresis in 5 % (w/v) acrylamide gels. (a) Mixed sample of pTF1 and pTF2; (b) pTT8; (c) pTF61; (d) pTF62. DNA fragments from pTF61 and pTF62 were superimposable when electrophoresed in one tube (data not shown).

Determination of molecular weights of plasmids

The linear forms of plasmid DNAs were electrophoresed in agarose gels with internal markers to determine their molecular weights (Table 1). Each plasmid was further examined by electron microscopy. Twisted molecules were observed in plasmid samples, and they were converted to open circular forms during storage for more than 1 month at 4 °C or upon nicking with 1 ng deoxyribonuclease I ml⁻¹ (Fig. 2*a* to *e*). With added ColE1 DNA (mol. wt 4.2×10^6) as an internal standard, molecular weights of the five plasmids were determined (Table 1).

Digestion with restriction endonucleases

The number of cleavage sites after restriction enzyme digestion are summarized in Table 2. pTF61 and pTF62 were indistinguishable in their molecular weights (Table 1), in the number of cleavage sites with five restriction endonucleases (Table 2), and in the electrophoretic pattern of the small fragments generated by *SmaR* or *BamNI* digestion (Fig. 3).

Properties of the plasmid-harboursing strains

The plasmid-harboursing strains did not show resistance to ampicillin, streptomycin, kanamycin, tetracycline, or chloramphenicol at $10\ \mu\text{g ml}^{-1}$. The ability to produce bacteriocins was examined by the double-layer technique of Fredericq (1957), using six extreme thermophiles as indicators. No clear zone was observed around the colonies of the six strains, indicating that the presence of plasmids could not be correlated with bacteriocin production. The six extreme thermophiles used in this study had a common biological nature: yellow-pigmented, heterotrophic and optimum growth at 60 to 72 °C. The biological functions specified by these plasmids have not been identified so far.

DISCUSSION

This paper describes the isolation and characterization of five plasmids from extremely thermophilic bacteria isolated from hot springs in Japan. *Thermus flavus* AT61 and AT62 carried indistinguishable plasmids as determined by their molecular weights and cleavage patterns with restriction endonucleases, though the two strains were different in their phage susceptibility. Bacteriophage YS40 isolated by Sakaki & Oshima (1975) infects *T. thermophilus* HB8 and *T. flavus* AT61, but not *T. flavus* AT62 (Sakaki & Oshima, 1975). These observations suggest that pTF61 (pTF62) was transferred by some mechanism from one strain to another, although we cannot rule out the possibility that one of the two strains mutated in terms of phage host-specificity.

The plasmids from the extreme thermophiles were not cleaved by *Eco*RI and were more sensitive to *Bam*NI and *Sma*R which cleave sites with the sequence GGATCC (Shibata & Ando, 1976) and CCCGGG (McParland, Brown & Pearson, 1976), respectively. This suggests that the plasmid DNAs have high deoxyguanosine and deoxycytosine contents like their chromosomal DNA (69 to 70%) (Oshima & Imahori, 1971; Saiki *et al.*, 1972).

The large plasmid (pTF2) was present as only a single copy, whereas there were many copies of the small plasmids (pTF1, pTT8, pTF61 and pTF62). The fact that the latter plasmids were small and that there were many copies of them should facilitate molecular cloning in extreme thermophiles.

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