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Genetics and Potential Biotechnological Applications of Thermophilic and Extremely Thermophilic Micro-organisms

P.L. BERGQUIST*, D.R. LOVE*, J.E. CROFT*, M.B. STREIFF*, R.M. DANIEL† AND W.H. MORGAN†

Genetics Research Unit, Department of Cell Biology, University of Auckland, Private Bag, Auckland, New Zealand and †Thermophile Research Group, School of Science, University of Waikato, Hamilton, New Zealand

What are thermophiles?

THE DIVERSITY OF THERMOPHILES

Organisms capable of living at high temperatures have held a particular fascination for biologists and biochemists, as they exist at temperatures where their proteins and nucleic acids would be expected to be denatured. The topic of life at high temperatures and the ecology, physiology and biotechnological applications of thermophilic organisms has been reviewed recently (Zeikus, 1979; Brock, 1985; Weigel and Ljungdahl, 1986).

Three broad classes of organism are recognized on the basis of their optimal growth temperatures. No multicellular organisms live at temperatures higher than 50°C and eukaryotic micro-organisms appear to have a temperature limit of about 60°C. Thus, above 60°C, only prokaryotic micro-organisms are found, but not all micro-organisms can grow at these temperatures. For example, photosynthetic micro-organisms do not live at temperatures above 73°C. *Table 1* lists some typical bacterial species growing at various temperatures. With the notable exception of the recently described *Thermotoga maritima* (Huber *et al.*, 1986; Huser *et al.*, 1986), all bacteria capable of growth at temperatures of more than 90°C are archaebacteria

Abbreviations: BCI-glu, 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside; CMC, carboxymethylcellulose; DNA, deoxyribonucleic acid; IS, insertion sequence; MUC, 4-methylumbelliferyl-β-cellobioside; MUG, methylumbelliferyl-β-glucoside; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PEG, polyethylene glycol; RNA, ribonucleic acid.

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Table 1. Bacterial growth (after Brock, 1985; Donnison, Brocklesbury and Morgan, 1986)

Class of bacteria	Optimum growth temperature	Typical genera
Psychrophiles	< 20°C	*Bacillus globisporus* *Micrococcus cryophilus*
Mesophiles	20–40°C	*Escherichia coli* *Pseudomonas aeruginosa* *Bacillus subtilis*
Thermophiles	45–65°C	*Clostridium thermocellum* *Bacillus stearothermophilus*
Extreme thermophiles	> 70°C	*Bacillus caldotenax* *Thermus aquaticus* *Sulfolobus acidocaldarius* *Thermoproteus tenax*

Table 2. Growth characteristics of some thermophiles and extreme thermophiles (modified from Daniel, Cowan and Morgan, 1981; Donnison, Brocklesbury and Morgan, 1986; Huber *et al.*, 1986; Weigel and Ljungdahl, 1986).

Strain	T opt (C°)	T min (°C)	T max (°C)	pH opt
Eubacteria				
Bacillus stearothermophilus	55–60	40	78	
Bacillus acidocaldarius	60–65	45	78	2·0–5·0
Bacillus caldolyticus	72	—	76	6·3–8·5
Thermus aquaticus	70	40	79	7·5–7·8
Thermus sp. T351	75–80	44	?	8·7
Thermus sp. T4–1A	75–80	47	?	7·2
Caldocellum saccharolyticum	68	—	80	7·0
Thermotoga maritima	80	55	90	6·5
Archaebacteria				
Methanothermus fervidus	85	70	100	—
Sulfolobus acidocaldarius	70–75	—	85–90	—

although not all archaebacteria are thermophiles (*Table 2*). Many archaebacteria are dependent on sulphur transformations—sulphur oxidation by the aerobic acidophilic species and sulphur reduction to H_2S by anaerobic and frequently autotrophic species. The extremely thermophilic eubacteria show an even wider range of metabolic diversity (reviewed by Weigel and Ljungdahl, 1986). Many extreme thermophiles are obligate or facultative anaerobes, as might be expected from the low solubility of oxygen in water at elevated temperatures. Thus apart from the very hot (more than 80°C) and very acidic (pH less than 2) environments, colonization of hot pools by bacteria is likely to occur.

MOLECULAR BASIS OF THERMOPHILY

Early workers on thermophiles envisaged special mechanisms to stabilize the macromolecules, and especially the proteins, in organisms growing at high temperatures. The idea of survival by rapid synthesis of new macromolecules

(Gaughran, 1947; Allen, 1953) died early, with the discovery of still higher growth temperatures, but until quite recently workers have searched for evidence to show which factors were responsible for the exceptional stability exhibited by enzymes from extreme thermophiles (Koffler and Gale, 1957). It is now generally accepted that the enzymes of thermophiles are maintained in their native condition by their intrinsic stability rather than by the presence of additional factors conferring thermal stability, or by rapid turn-over. The thermal stability of purified enzymes from extreme thermophiles is quite dramatic in comparison to that of their mesophilic counterparts: for example, the half life of *Bacillus subtilis* α-amylase (EC 3.2.1.1) is a few seconds at 90°C but the α-amylase from *Bacillus caldolyticus* has a half life of about 20 minutes at 95°C.

In retrospect, the most satisfactory approach to the problem of protein stability is the comparison of proteins differing by only a single amino acid residue. Geneticists have made considerable use of temperature-sensitive mutants, in which a single amino acid substitution can lead to such a great loss of thermal stability that a temperature increase of a few degrees will cause the death rather than good growth of the organism. An early use of systematic mutation to investigate protein stability was made by Langridge (1968) who investigated the effect of 56 point mutations on the thermal stability of β-galactosidase (EC 3.2.1.23) of *E. coli*. He showed that some single amino acid replacements could drastically affect protein stability, whereas others had relatively little effect. This research appears to have been overlooked by most other workers in this field, but the findings have since been confirmed (e.g. Yutani *et al.*, 1977).

Later work involved a comparison of the sequences and three-dimensional structures of homologous proteins (most notably, ferridoxin) from closely related micro-organisms with different optimal growth temperatures (Perutz and Raidt, 1975; Walker, Wonacott and Harris, 1980; Ruegg, Ammer and Lerch, 1982). The additional stability of ferridoxins in thermophiles could be accounted for by a few extra salt bridges and hydrogen bonds (Perutz and Raidt, 1975).

Some recent experiments involving the analysis of DNA sequences are instructive. Matsumara *et al.* (1984) compared the DNA sequences of a kanamycin nucleotidyltransferase (gentamycin 2″-nucleotidyltransferase, EC 2.7.7.46), found on a plasmid in a mesophilic bacterium, with the sequence of the gene from a plasmid in a thermophile, *Bacillus stearothermophilus*. The nucleotide sequence of the thermophilic enzyme shows a single base difference, the replacement of a threonine (thr-130) by a lysine residue, which leads to a distinct enhancement of thermal stability. The introduction of a lysine at this position stabilizes the enzyme by permitting the formation of an effective salt bridge between the introduced lysine and any negatively charged amino acid (for example, glutamic acid or aspartic acid) without any change in the three-dimensional structure of the molecule. Sakaguchi *et al.* (1986) also have shown that the tetracycline-resistance gene of a plasmid from a thermophilic *Bacillus* differs from a similar gene of *Bacillus subtilis* by two adjacent base pairs.

Matsumara and Aiba (1985) used an *in vitro* mutagenesis approach to screen for thermostable mutants of the mesophilic kanamycin nucleotidyltransferase by exploiting the use of M13 single-stranded vectors, recovery of double-stranded DNA, and the *Bacillus stearothermophilus* plasmid transformation system (all described later). Of 12 mutants studied, 11 had the same G → T substitution (aspartic acid, GAT→tyrosine, TAT) at position 80 of the protein and one had a C→A substitution (threonine, ACG→lysine, AAG) at position 130. This latter mutation gave a sequence identical with the gene from the thermophilic plasmid pTB913 that Matsumara *et al.* (1984) had previously sequenced. They suggested that the lysine substitution contributed to additional electrostatic interactions with neighbouring glutamic acid and aspartic acid residues and that the aromatic ring of the tyrosine side chain may give an increase in hydrophobicity and packing interactions with other adjacent aromatic amino acids (phenylalanine, tyrosine). These two mutations were combined by *in vitro* recombinational techniques to give a double mutant with a 10·8°C enhancement of thermal stability (Matsumara, Yasumura and Aiba, 1986).

Other mutations at the carboxyl-terminus of the enzyme decreased thermal stability (Matsumara, Kataoka and Aiba, 1986). These results showed that alterations in thermal stability depend on the nature of the amino acid residue and its position in the protein. Furthermore, single base changes are sufficient to enhance thermostability (provided that the amino acid is altered) and these changes could be cumulative. An important consideration in these experiments is that a more thermostable protein could be obtained by simple mutagenesis techniques without precise knowledge of the three-dimensional structure of the enzyme.

This type of approach was taken to its logical conclusion by Matthews' group (Grutter, Hawkes and Matthews, 1979; Alber *et al.*, 1986), who coupled point mutation with an examination of three-dimensional structure, and confirmed that single amino acid changes could considerably alter the thermostability of proteins, without significantly affecting structure. Most random point mutations of proteins decrease their stability.

The theoretical basis for this finding had been proposed some years previously (Brandts, 1967). The free energy of stabilization of proteins is of the order of a few tens of kilojoules per mole, but is the resultant of much larger destabilizing forces (mainly due to chain entropy) and stabilizing forces (mostly due to hydrophobic, electrostatic, and hydrogen bond interactions). Thus even a small percentage change in these latter forces (which are of the order of a thousand kilojoules per mole) can dramatically alter the free energy of stabilization. The half life of a protein can be increased by an order of magnitude, at 70°C, by an increase in free energy of stabilization of 6·5 kJ/mole. Both theoretical studies and practical investigations confirm that a single additional hydrophobic, electrostatic or hydrogen bond interaction can add to the free energy of stabilization by amounts of this order (Fersht, 1971; Yutani *et al.*, 1977; Perutz, 1978; Daniel, 1986).

Although a given thermostable protein may be stabilized with respect to its less stable homologue by a specific type of intra-protein interaction (Perutz

and Raidt, 1975; Yutani *et al.*, 1977) there is no evidence that any one type of interaction is predominantly responsible for the additional stability possessed by proteins from extreme thermophiles.

Additional mechanisms for ensuring thermostability of cellular components include modification of tRNA molecules (reviewed by Oshima, 1986). *Thermus thermophilus* tRNA has a melting temperature of 87°C, compared with 81°C for *Escherichia coli* tRNA. *Thermus* $tRNA_n^{Met}$ differs from its *E. coli* counterpart in having a G-U base pair replaced by a G-C and by containing three modified bases, methylated ribose of G-19, A-59 and a thiolated residue at position 54 (Watanabe *et al.*, 1979, 1980). The 2-thioribosylthymidine at position 54 is important for thermal stability as a result of the steric effect of the 2-thiocarboxyl group and strengthened stacking interactions between neighbouring bases of the helix (Horie *et al.*, 1985). The modifications at positions 19 and 59 also appear to be important for thermostability (Oshima, 1986). The sequences for a number of major tRNAs have been determined and the first letter of the anticodon was found to be G or C, corresponding to the codon most frequently used in the coding sequences of *Thermus* (Hara-Yokoyama *et al.*, 1986; Oshima, 1986).

Mesophilic bacteria contain the polyamines putrescine and spermidine, but Oshima (1982, 1983) has shown that an extreme thermophile, *Thermus thermophilus*, contains at least 12 distinct polyamines of which the major components are thermine and thermospermine. Ohno-Iwashita, Oshima and Imahori (1976) showed that a cell-free protein synthetic system from *T. thermophilus* was largely inactive at high temperatures unless polyamines such as spermine and thermine were added to the reaction, and it was demonstrated that the polyamines stabilized a complex between ribosomes and aminoacyl-tRNA at high temperatures (Ohno-Iwashita, Oshima and Imahori, 1976; reviewed by Oshima, 1986).

The membranes of thermophilic bacteria are stable (or stabilized) at high temperatures whereas those of non-thermophilic bacteria are heat sensitive. Changes in the lipid content of thermophile membranes have been documented and their composition changes with increasing temperature (Esser, 1978; Sundaram, 1986). Analysis of lipids from the membranes of archaebacterial and eubacterial thermophiles shows that they possess methyl branching as a major characteristic of the apolar chains (reviewed by Langworthy and Pond, 1986). From model studies, such branching would be expected to aid in the fluidization rather than the stabilization of membranes. Langworthy and Pond (1986) point out that other features may be important, for example, the enrichment of the polar lipid head groups with carbohydrate residues, the manner in which the apolar chains are linked to glycerol, and whether or not a lipid bilayer or a covalently condensed bilayer can be formed. For example, isopropanoid glycerol diethers occur in mesophilic archaebacteria and are capable of forming a typical lipid bilayer membrane structure. Tetraethers comprise most of the glycerolipids in thermoacidophilic archaebacteria. The tetraethers can be thought of as extending from the inner to the outer face of the membrane, with a glycerol exposed on each face. This arrangement could provide, in essence, a 'monolayer' if the tetraethers were

regarded as diethers covalently condensed at the centre of the membrane. Thus the inner and outer faces of the membrane would be joined and thermophilic sulphur-dependent archaebacteria would possess covalently condensed bilayers composed of tetraether glycerolipids which cannot melt apart (Langworthy, 1985). Changes in the composition of membrane proteins may be necessary also for thermal stability but these alterations have not been extensively documented and some of the evidence is conflicting (reviewed by Sundaram, 1986).

The results summarized above suggest that macromolecular instability may not, after all, be the factor limiting the upper growth temperature for life. Life depends on the precise interconversion of small molecules, and the instability of some of these (e.g. glutamine: Ratcliff and Drozd, 1978; NAD: Walsh, Daniel and Morgan, 1983; ATP: Stetter, 1986) may limit life to below 150°C.

So far, there is little evidence of the existence of enzymes for metabolic activities which are restricted to, or dependent upon, life at high temperatures. Significant differences are now emerging for metabolism in extremely thermophilic archaebacteria (Budgen and Danson, 1986), but there is no evidence to show that these are obligate in extremely thermophilic eubacteria growing optimally at similar temperatures. This does not rule out the possibility that these differences are, indeed, adaptions to life at high temperatures but, if so, they are apparently not essential.

There is some direct evidence for more subtle differences, such as raised levels of unusual enzymes (*see*, for example, Guy and Daniel, 1982) or differing membrane compositions (Langworthy and Pond, 1986), but on the whole the surprises have not been regarding the differences but regarding the similarities of extreme thermophiles to other bacteria.

What is known about the genetics of thermophiles?

Very little work has been done on the genetics of extreme thermophiles and most reports have focused on *Thermus* sp. Of the three common systems of gene transfer in bacteria, only transformation has been demonstrated to occur and it is not known whether DNA can be transferred by conjugation or transduction. We discuss here results obtained with *Bacillus stearothermophilus* for, although it is not an extreme thermophile, it may be of value as an organism for the cloning of genes from other thermophilic species. The *Thermus* chromosomal DNA transformation system that is discussed has several similarities to genetic transformation in *Bacillus* sp., which may be a useful model. Very little information is available on genetic systems in archaebacteria although some genes have been expressed in *E. coli*.

The genus *Bacillus* is a source of secreted enzymes that are important industrially. Transfection allows direct expression of the introduced DNA without a requirement for recombination or other genetic events. Early attempts at developing genetic systems for thermophiles focused on *Bacillus stearothermophilus* and concentrated on the transfection of competent cells with DNA from a bacteriophage as a model system for a transformation assay

(Streips and Welker, 1971; Welker, 1978). A DNA restriction–modification system was found to operate and the competence factor was established to be another bacteriophage. Recent reports with mutants of *B. stearothermophilus* have established that protoplast fusion may be a useful method for genetic analysis of this organism (Chen, Wojcik and Welker, 1986).

Host–vector systems for *Bacillus stearothermophilus* have been described by Imanaka *et al.* (1984) and Imanaka and Aiba (1986). They transformed *B. stearothermophilus* with plasmid vectors that could also replicate in *Bacillus subtilis*. This system had the advantage that direct selection could be made for transformants carrying cloned genes by selection on agar containing antibiotics such as tetracycline or kanamycin. A shuttle vector that was able to replicate either in *B. subtilis* or *B. stearothermophilus* was generated by *in vitro* recombination using restriction enzyme fragments (*see also* Imanaka, 1983).

A chromosomal transformation system for extreme thermophiles has been developed recently (Koyama *et al.*, 1986; our unpublished observations). This system relies on the cells of *Thermus* becoming naturally competent for the uptake of linear chromosomal DNA with selection for prototrophic recombinants using auxotrophic mutants as recipients.

GENE TRANSFER SYSTEMS FOR THERMOPHILES

Current genetic manipulation techniques have been based to a large extent on plasmid manipulation and genetic transformation techniques. Several groups have examined thermophilic *Bacillus* and *Thermus* isolates for plasmids. Hishinuma, Tanaka and Sakaguchi (1978) first reported the isolation of small plasmids from four out of eight isolates of *Thermus flavus* and *Thermus thermophilus*. Two of these plasmids have been characterized by electron microscopy and restriction enzyme mapping (Vasquez, Gonzalez and Vicuna, 1984) but, together with similar plasmids isolated in our laboratory and by Munster, Munster and Sharp (1985), none could be demonstrated to encode resistance to antibiotics or heavy metal salts (*Table 3*). Raven and Williams (1985) have reported the isolation and restriction enzyme mapping of a small cryptic plasmid from *Thermus* sp. YS045 which may have potential as a cloning vector as it has three unique restriction enzyme cleavage sites.

Several plasmids have been isolated from *Bacillus stearothermophilus* and other thermophilic bacilli using their ability to replicate and express antibiotic resistance in *Bacillus subtilis* (Bingham, Bruton and Atkinson, 1980; Imanaka *et al.*, 1982; Hoshino *et al.*, 1985). Smaller derivatives were constructed after *in vitro* deletion using restriction enzymes. The essential replication genes and tetracycline resistance of pAB124 were found to be located on a 2·95 kb fragment and a similar fragment was found for this region in pTHT15. These small plasmids are potential shuttle vectors for the cloning and transfer of genes between the mesophilic *Bacillus subtilis* and the thermophilic *B. stearothermophilus* as they are present at high copy number in the cells and are stably maintained in both hosts without selection (Hoshino *et al.*, 1985).

Imanaka *et al.* (1982) constructed a shuttle vector (pTB90) which could

replicate in *B. subtilis* and in *B. stearothermophilus*. This plasmid was shown subsequently to have been derived fortuitously from the insertion of a 1·5 kb *Eco*RI fragment from a cryptic plasmid present in *B. stearothermophilus* (Imanaka *et al.*, 1984). This fragment allowed replication of pTB90 in *B. stearothermophilus* and permits a significant increase in the transformation frequency. This chimeric plasmid is relatively small and should be a suitable cloning vector for either *Bacillus* species. However, cloning of a penicillinase gene from *B. licheniformis* into pTB90 yielded an unstable plasmid (pLP11) that prevented continuous cultivation of transformed *B. stearothermophilus* at high temperatures (Aiba and Koizumi, 1984; Koizumi, Monden and Aiba, 1985). A mutant plasmid derived from pLP11 was able to integrate into the chromosome and supported growth in the presence of kanamycin at 63°C. A derivative plasmid was produced after a temperature shift down to 48°C (pTRZ117). This plasmid had acquired a 1·9 kb portion of chromosomal DNA and was maintained stably in an extrachromosomal state at 65°C (Koizumi *et al.*, 1986). It should be noted that the procedures for plasmid transformation of *Bacillus stearothermophilus* depend on protoplast formation and regeneration, and intact cells do not appear to become competent for plasmid transformation.

No *Thermus* strain that we have used carries antibiotic-resistance determinants. We have made preliminary attempts to transfer the wide host range plasmids R68-45, RP4 and pS-a from *E. coli* into *Thermus* sp.TOK3, which does not carry any large plasmid species. The *E. coli* donor was grown at 37°C, the *Thermus* recipient at 70°C, and the mating mixture was incubated at 44°C for varying lengths of time. The donor cells were counterselected at 70°C, but despite the use of several different experimental procedures, we have been unable to obtain transconjugants. There are a number of recognized reasons for the lack of success of conjugational transfer, including surface exclusion, failure of expression of replication and for antibiotic resistance genes, incompatibility and restriction/modification of the incoming DNA. This latter possibility is discussed later.

PLASMID TRANSFORMATION OF *THERMUS*

As indicated in *Table 3*, most strains of *Thermus* carry small plasmids of appropriate sizes for genetic manipulation as potential cloning vectors. We have isolated the 1·5 kb plasmid (pNZ1200) from *Thermus* T4-1A and pTT8 from *Thermus thermophilus* HB8 and constructed chimeric plasmids using plasmids able to replicate in *Escherichia coli* (*Figure 1*). We used the two *Thermus* plasmids because of their small size and known restriction enzyme sites: pTT8 was selected in particular because its host strain *Thermus thermophilus* HB8 does not produce α-amylase, protease and β-galactosidase, isolates 'cured' of pTT8 exist, and a defined minimal medium was available. We have been unable to maintain *Thermus* plasmids in *Escherichia coli*, and as we did not know if particular restriction enzymes sites were located in the essential replication region, we used alternative sites for the ligation of

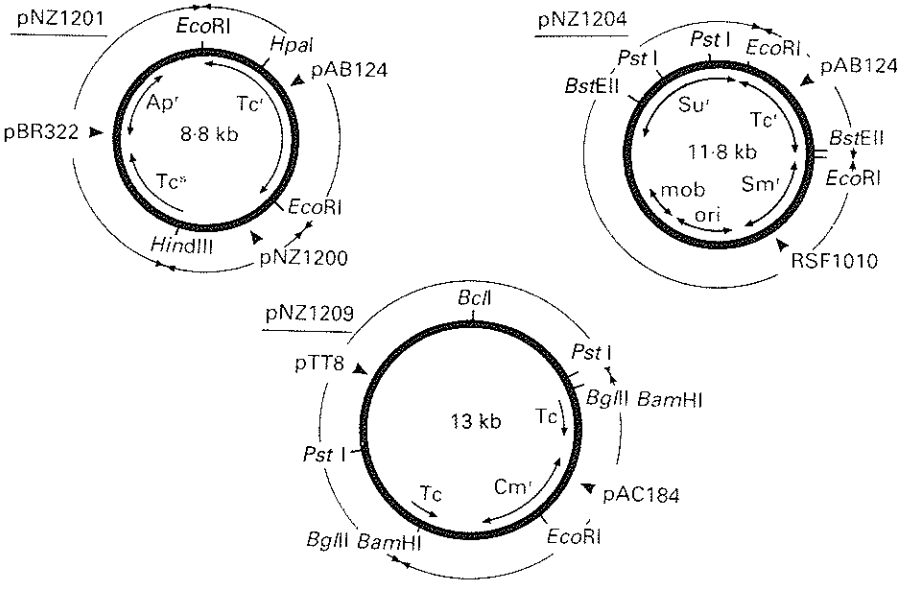


Figure 1. Potential shuttle and cloning vectors for *Thermus* sp. pNZ1201 was constructed by standard recombinant DNA techniques from the *E. coli* plasmid pBR322, the *Thermus* T4-1A plasmid pNZ1200 and the *B. stearothermophilus* plasmid pAB124 in two separate ligation steps. pNZ1204 was constructed from the wide host range plasmid RSF1010 and the *Eco*RI fragment from pAB124. pNZ1209 is one plasmid of a series constructed from the *Thermus thermophilus* HB8 plasmid pTT8. A *Bgl*II digest of pTT8 was ligated into the *Bam*HI site of the *E. coli* plasmid pAC184. Abbreviations: Ap', ampicillin resistance; Tc, tetracycline; Su', sulphonamide resistance; Sm', streptomycin resistance; Cm', chloramphenicol resistance; mob, mobilization site for conjugal transfer in *Escherichia coli*.

the thermophilic DNA into the *E. coli* vectors such as RSF1010, pBR322, pBR325 and pAC184 (for example, *see Figure 1*). Some shuttle vectors included the tetracycline-resistance gene of *Bacillus stearothermophilus* plasmid pAB124 which, we showed, was expressed at 37°C in *E. coli* and which we knew to be expressed at 60°C.

Procedures for the transformation of *Thermus* with these shuttle vectors have been based on those allowing DNA uptake in mesophilic systems that involve treating the cells with solutions of salts under conditions which make the cell membrane temporarily permeable. A comprehensive selection of mono-, di- and trivalent cations have been used for the treatment of the cells, with and without EDTA. No transformants for tetracycline resistance have been observed in any of the experiments although the frequency of detection was estimated to be 1 in 10^7–10^8 cells plated. Other experiments (J.E. Croft, unpublished work) demonstrated that pNZ1200 was unable to replicate in *E. coli* in the presence or absence of a mini-F derivative (pNZ049, Gardner *et al.*, 1980).

Table 3. Some plasmids from thermophilic and extremely thermophilic bacteria*

Strain	Plasmid	Marker†	Size (kb)	Reference
Thermus thermophilus HB8	pTT8	—	9·1	Vasquez, Gonzalez and Vicuna, 1984
	pVV8	—	70	
Thermus flavus AT-62	pTF62	—	10·3	Vasquez, Gonzalez and Vicuna, 1984
Thermus flavus BS-1	—	—	26; 9·2	Vasquez, Gonzalez and Vicuna, 1984
Thermus sp.T4-1A	—	—	50; 1·5	Croft, 1986
Thermus sp. OK6	—	—	>70; 60;13; 8; 6·4;	Croft, 1986
Thermus sp. TOK8	—	—	>70; 4·8; 3·0	Croft, 1986
Thermus sp. T351	—	—	40; 9·5	Croft, 1986
Bacillus stearothermophilus	pAB124	Tc^r	4·4	Bingham, Bruton and Atkinson, 1980
Bacillus stearothermophilus	pTB19	Tc^rKm^r	26	Imanaka *et al.*, 1982
	pTB20	Tc^r	4·2	Imanaka *et al.*, 1982
Bacillus sp.	pTH9	Tc^r	7·7	Hoshino *et al.*, 1985
	pTHT15	Tc^r	4·5	Hoshino et al., 1985
	pTHN1	Km^r	4·8	Hoshino *et al.*, 1985

* See also Munster, Munster and Sharp (1985)
† Abbreviations: Tc^r, tetracycline resistance; Km^r, kanamycin resistance

PROTOPLASTS FROM *THERMUS* AND TRANSFECTION PROCEDURES

Another approach is to use *Thermus* plasmids for transformation of *Thermus* strains. *Thermus* strains can be cured of the smaller plasmids that they carry by growth in the presence of the gyrase (DNA topoisomerase II, EC 5.99.1.3) inhibitor, novobiocin (Vasquez, Villanueva and Vicuna, 1983). It is possible to introduce *Thermus* plasmids into naturally competent cells (*see later*), but this procedure is limited by the fact that all plasmids found so far are cryptic. We have investigated the structure and organization of chromosomal genes involved in leucine biosynthesis for the purpose of using them as a selective marker once incorporated into a plasmid such as pTT8. C. Vasquez (personal communication) has cloned the gene(s) for resistance to tellurium salts (reduction of K_2TeO_3 to metallic tellurium) for use as a marker for *Thermus* plasmids. However, problems relating to the modification of thermophile DNA in *Escherichia coli* await resolution before vectors manipulated in *E. coli* can be used for studying gene expression at high temperatures.

We have demonstrated that protoplasts can be formed from *Thermus thermophilus* HB8 by treating exponential phase cultures with low concentrations of lysozyme (EC 3.2.1.17). These protoplasts can be regenerated on enriched or minimal agar plates containing sodium succinate (1–10% regeneration frequency; A. Chang, M.B. Streiff and P.L. Bergquist, unpublished work). A direct method for assaying for the introduction of DNA into a cell is to use transfection of protoplasts with DNA isolated from a suitable

bacteriophage. Two bacteriophages are known to infect *Thermus* sp.: bacteriophage YS40 is of similar size to bacteriophage T4, and the double-stranded DNA of molecular weight $1{\cdot}36 \times 10^8$ daltons is sufficiently large to pose technical difficulties in the isolation of intact molecules for transfection (Sakaki and Oshima, 1975). Bacteriophage W28P was isolated from the New Zealand North Island thermal region (Patel, 1985) and although it has a narrow host range, plating only on *Thermus* sp. W28P, it is morphologically similar to bacteriophage λ and has double-stranded DNA of molecular weight $2{\cdot}24 \times 10^7$ daltons. Preliminary experiments suggest that the phage DNA is capable of forming an open circular configuration and may have cohesive ends like λ. Purified DNA of this bacteriophage has been used to infect protoplasts of *Thermus thermophilus* HB8 that are capable of regeneration. However no plaques have been obtained using *Thermus* W28P, *Thermus thermophilus* HB8 or mixtures of these two bacteria on suitable plates. Phage DNA is unable to transfect naturally competent *Thermus thermophilus* HB8 which is transformable by chromosomal DNA (*see* next section). This system may hold promise for genetic transfer in a similar manner to the *E. coli*-λ systems but clearly requires further development with viable protoplasts prepared from *Thermus* W28P.

TRANSFORMATION OF *THERMUS* WITH CHROMOSOMAL DNA

In the course of experiments in which we attempted to achieve fusion between protoplasts of auxotrophic mutants of *Thermus thermophilus* HB8, we observed a high frequency of apparently prototrophic colonies which could not be attributed to cross-feeding. Frequencies of about 1% prototrophic colonies were observed, and their appearance could be prevented by the addition of DNAse I (deoxyribonuclease I, EC 3.1.21.1). We surmised that transformation was occurring as the result of the uptake of DNA released from protoplasts that did not regenerate. At the same time, Koyama *et al.* (1986) reported that auxotrophic mutants of *Thermus thermophilus* HB27 could be transformed with isolated DNA from several strains of *Thermus*. This system relies on the cells of *Thermus* becoming naturally competent for the uptake of linear chromosomal DNA, and, because no suitable drug resistance markers have been found in *Thermus*, auxotrophic mutants have been used with selection for prototrophic recombinants (Koyama *et al.*, 1986; M.B. Streiff, unpublished work). Transformation has been achieved for a variety of mutations in amino acid biosynthetic pathways at a relatively high frequency (about 1–12% of the total number of viable recipient cells).

We have shown that a Leu$^-$ and a Lys$^-$ mutant of *T. thermophilus* HB8 can be transformed with DNA isolated from *Thermus* sp. Fiji 3 and TOK3 as well as with *Thermus thermophilus* HB8 and we have confirmed that purified *Thermus* plasmid pTT8 can be cotransformed into a *Thermus* Fiji 3 Pro$^-$Str-r mutant at a frequency 1000-fold lower than chromosomal DNA. This plasmid transformation phenomenon of *Thermus* may be unusual, as plasmid DNAs are inefficient for transformation of competent cells of *Bacil-*

lus and *Haemophilus*. It is also unusual that *Thermus* cells are more or less competent for transformation throughout their growth period. Usually competence is a transient phenomenon related to the phase of growth, as seen in *Bacillus subtilis*.

We have shown that a Leu$^-$ mutant of *T. thermophilus* HB8 can be transformed to Leu$^+$ with chromosomal DNA isolated directly from a variety of *Thermus* strains and that the plasmid pTT8 can be cotransformed into a recipient cell. However, the *Thermus leu*B gene cloned into a shuttle vector incorporating pBR322 and pTT8 does not give transformants, nor does λ DNA incorporating about 20 kb of *Thermus* chromosomal DNA from the vicinity of the region coding for the leucine biosynthetic pathway (M.B. Streiff, D.R. Love and J.E. Croft, unpublished work).

Thermus thermophilus HB8 is the source of the restriction enzyme *Tth*I (Sato and Shinomiya, 1978; Venegas *et al.*, 1980) which is an isoschizomer of *Taq*I (EC 3.1.23.39). These enzymes (recognition sequence: TCGA) will not cut DNA that has been methylated at the A residue. Plasmid pTT8 isolated from *T. thermophilus* HB8 is protected from digestion by *Taq*I, presumably as a result of methylase activity. From our results cited above, it appeared that *Thermus* DNA is able to bring about transformation as long as it has not been passaged through *Escherichia coli*. *In vitro* methylation of DNA from the shuttle vector pNZ1209 with *Thermus aquaticus Taq*I methylase did not restore transformational ability. Why *Thermus* DNA cycled through *E. coli* fails to transform, remains to be explained. The usual type I restriction–modification systems (ATP-dependent endonucleases, EC 3.1.21.3) (Boyer, 1971) usually bring about a 10^3–10^4-fold reduction in bacteriophage plating efficiency or in chromosomal recombinants, but, as each molecule of the ATP-dependent endonucleases can catalyse only one DNA scission, some phage progeny or recombinant bacteria can be produced if sufficiently high DNA concentrations are employed (Bickle, 1982). Variations in DNA concentration over a wide range have not allowed us to generate transformants with either the shuttle vector or the λ derivatives mentioned above.

Most of the work that we have done on gene transfer systems for *Thermus* has been based on the supposition that it was a Gram-negative organism (a thermophilic *E. coli*!). A comparison of the biochemical properties of *Thermus* with those of typical Gram-positive and Gram-negative bacteria suggests that it cannot be classified unambiguously in either group (Hudson and Morgan, 1986; Donnison, Brocklesbury and Morgan, 1986; Hensel *et al.*, 1986). The chromosomal transformation system for *Thermus* is reminiscent of that operating for *Bacillus, Haemophilus* and other Gram-positive bacteria. Furthermore, the 5S RNA sequences of several species of *Thermus, Bacillus* and *Clostridium* show considerable homology. Such homologies have been used as a measure of the evolutionary relationships between different bacteria (de Wachter, Huysmans and Vandenberghe, 1985). Accordingly, Gram-positive bacteria, for example *Streptococcus thermophilus*, may prove to be more appropriate hosts for manipulating and studying the expression of cloned genes from *Thermus*.

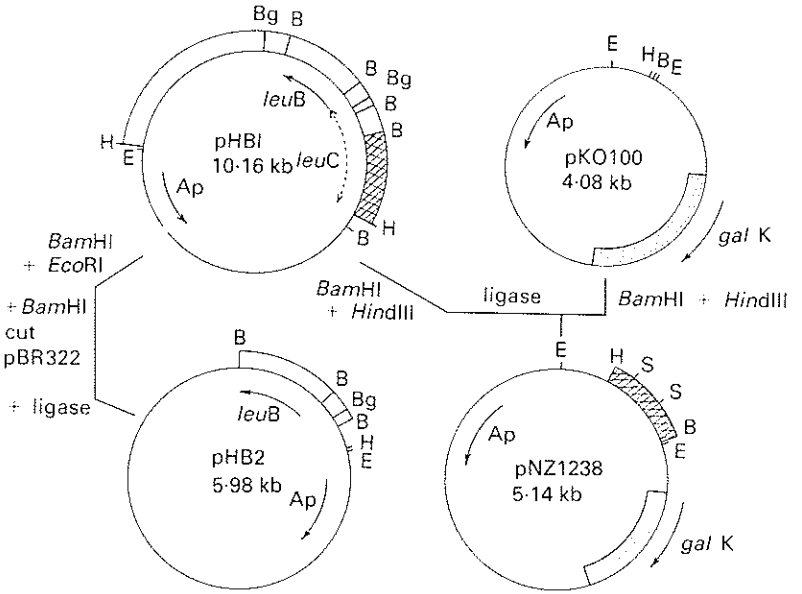
SURROGATE GENETICS OF THERMOPHILIC GENES BY CLONING IN *ESCHERICHIA COLI*

One approach to a genetic analysis for *Thermus* has been to clone restriction enzyme-generated fragments into vectors such as pBR322 and to examine the ability of the thermophilic DNA to complement mutations on the *E. coli* chromosome at 37°C. For example, Nagahari, Koshikawa and Sakaguchi (1980) reported the isolation of the *leu*B gene of *Thermus thermophilus* HB27 by shotgun cloning of *Hin*dIII-digested chromosomal DNA into pBR322. Tanaka, Kawano and Oshima (1981) independently cloned the *leu*B and *leu*C genes of *Thermus thermophilus* HB8 into *Escherichia coli* and showed that the resulting 3-isopropylmalate dehydrogenase (EC 1.1.1.85) maintained its thermostability. Subsequent DNA sequence analysis of the thermophilic *leu*B gene showed a different codon usage compared with *E. coli*. Although a typical ribosomal binding site was present in front of the open reading frame for the thermophilic 3-isopropylmalate dehydrogenase, there were no recognizable *E. coli* promoter-like sequences (Kagawa *et al.*, 1984; Oshima, 1986).

The pathway of leucine biosynthesis has been investigated extensively and *E. coli*, *Salmonella typhimurium*, *Neurospora crassa* and *Saccharomyces cerevisiae* each use the isopropylmalate pathway. In *E. coli* and *S. typhimurium*, the genes form part of an operon with transcription initiated at a single promoter in front of the *leu*A gene (Yang and Kessler, 1974). The operon is controlled at the physiological level by the presence of leucine in the medium and utilizes an attenuation mechanism, in which the transcript prior to the first translational start (leader transcript) provides a method of regulating transcription termination (Gemmill *et al.*, 1979). The *leu* operon in bacteria is noteworthy in that there are two genes, *leu*C and *leu*D, coding for the non-identical subunits of the isopropylmalate dehydratase enzyme (EC 4.2.1.33).

Tanaka, Kawano and Oshima (1981) cloned *Thermus* genes that complemented *leu*B and *leu*C mutants of *E. coli*. Cells carrying the plasmid pHB1 produced 3-isopropylmalate dehydrogenase and isopropylmalate dehydratase. A subcloned fragment produced only the 3-isopropylmalate dehydrogenase (pHB2, *Figure 2*). The nucleotide sequence of the *Thermus* DNA present in pHB2 was determined by Kagawa *et al.* (1984) and revised by Oshima (1986). The *leu*B gene was contained within the larger *Bam*HI fragment of the two present in the inserted DNA in pHB2; the 5′ region contained a typical *E. coli* ribosomal binding sequence but differed from the attenuated structure of the leucine operon in *E. coli* and *S. typhimurium*.

We took pHB1 and cloned the *Hin*dIII–*Bam*HI fragment of 5·8 kb (*Figure 2*) into the promoter-cloning vector pKO100 (McKenny *et al.*, 1981). Further deletions using restriction enzymes allowed the promoter to be located on a 115 bp *Hin*dIII–*Sma*I fragment. Sequence analysis of this region provided identification of −35 and −10 regions characteristic of *Escherichia coli* promoters although no obvious ribosomal binding site was present. There is a potential attenuation control region structurally similar to that region



The promoter activity was located to within 115 bp of the *Hin*dIII site

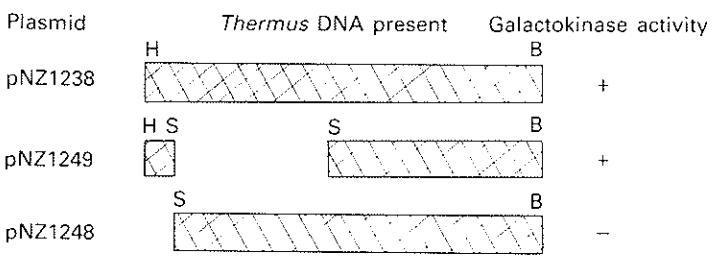


Figure 2. Identification of a promoter and chimeric plasmids composed of *Thermus thermophilus* HB8 DNA cloned into pBR322 and pKO100 (Croft *et al.*, 1986). pKO100 contains the *galK* gene of *E. coli* but does not express galactokinase activity due to the lack of a promoter. Hence expression of the *gal*K gene can be used as a means of detecting inserted DNAs which contain promoters. Translational stop codons in all three reading frames prevent translation which may originate in the inserted DNA. The *Thermus* DNA *Hin*dIII fragment of pHB1 was digested with *Bam*HI and ligated with pKO100 digested with *Bam*HI or *Bam*HI plus *Hin*dIII. pNZ1238 contains the 1·06 kb *Hin*dIII–*Bam*HI fragment of pHB1 ligated with pKO100. The stippled region represents the *galK* gene of pKO100. The open and hatched regions represent *Thermus* DNA; the latter region identifies the 1·06 kb *Hin*dIII–*Bam*HI fragment. pHB2 is the plasmid formed from pHB1 by partial digestion with *Bam*HI followed by ligation (Tanaka, Kawano and Oshima, 1981). The 1·62 kb portion of *Thermus* DNA which is present in pHB2 has been sequenced by Kagawa *et al.* (1984). The position of the *leu*B and *leu*C complementing activities are indicated.
Abbreviations: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Sma*I; Bg, *Bgl*II.

upstream of the *leu*A gene in *E. coli* and *S. typhimurium*, and which contains a second open reading frame with two leucine codons present. Computer analysis of the putative attenuation region shows that an mRNA produced could be folded to give secondary structures that are mutually exclusive, one of which is a potential transcriptional terminator. A second structure could be present if a ribosome was stalled as a result of leucine starvation (terminator pre-emptor structure, *see* Gemmill *et al.*, 1979).

We also investigated whether or not other genes of leucine biosynthesis were present on pHB1 DNA to form an operon analogous to that seen in the Enterobacteriaceae. *Thermus* DNA fragments cloned into suitable vectors were used to complement *leu*A,B,C and D mutations in *Salmonella typhimurium* and *Escherichia coli*. The results in *Figure 3* show that the promoter activity of the *Hin*dIII–*Sma*I fragment mentioned above is necessary for complementation of *leu*B,C,D mutants and that the gene products are part of a polycistronic transcriptional unit (compare lines 2, pNZ1230; and 3, pNZ1250). Internal deletions abolish the complementing ability for *leu*C and D mutations (line 6, pNZ1227). The smallest plasmid capable of complementation is pNZ1231 and line 5 shows that the 2.55 kb *Hin*dIII–*Bam*HI fragment of pHB1 does not carry genes involved in complementation of leucine mutations. Further support for the idea of a polycistronic mRNA is provided by the data in line 8, where it can be seen that provision of DNA in *trans* does not allow complementation of *leu*C and D mutants.

Where are the coding sequences for *leu*C and *leu*D in the *Thermus* DNA? The sequence of 1·62 kb of DNA including the *leu*B open reading frame is known (Kagawa *et al.*, 1984) and there are stop codons in all three reading frames in the 0·24 kb *Bam*HI–*Bg*III fragment (*Figure 2*). If we assume that the size of the *leu*C and *leu*D proteins in *Thermus* are the same as those in *Salmonella* (47·0 kdal and 23 kdal, respectively, Friedberg *et al.*, 1985), the only open reading frames downstream of the *Bg*III site in pNZ1231 which could code for a protein of greater than 100 amino acids are (1) the one proposed for the *leu*B gene itself and internal subsets of this reading frame, and (2) open reading frames initiated at valine codons 8, 50, 74 and 89 bases from the *Bg*III site. The composition of the codons in the latter reading frames make it unlikely to code for a genuine *Thermus* protein (Oshima, 1986). Thus the 196-base open reading frame internal to, and in phase with, *leu*B may be the *leu*D gene. This open reading frame is located seven nucleotides downstream from a potential ribosome-binding sequence (GGGAGCC). The alternative proposition is that the *leu*B gene codes for a multifunctional enzyme that by itself gives *leu*B activity (isopropylmalate dehydrogenase) but when associated with the *leu*C polypeptide, it gives isopropylmalate dehydratase function. A further feature of our results is our inability to complement *Escherichia coli leu*A mutations (*Figure 3*, line 1). Provision of up to 7 kb of *Thermus* DNA upstream of the *leu*B gene does not give complementation and we conclude that either the *leu*A product is not functional or it is not arranged in the same operon structure as in *E. coli* and *Salmonella*, and hence may be unlinked to *leu*B,C and D.

the enzymes using DNA sequences cloned from other organisms as probes. For example, there is no homology between the β-galactosidase of *E. coli*

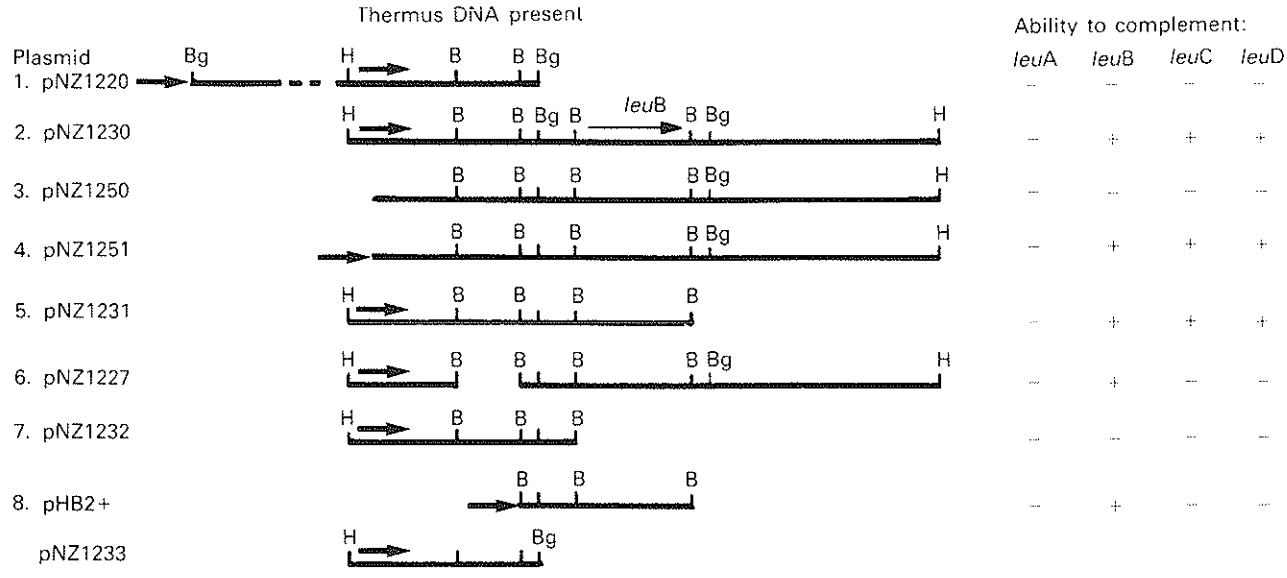


Figure 3. Complementation of leucine mutants of *E. coli* by *Thermus* DNA (Croft *et al.*, 1986). Only the *Thermus* DNA present in the cloning vector is shown. The arrow indicates the presence of a *Thermus* promoter sequence, except for line 4, pNZ1251, where the DNA is transcribed from the *E. coli lac* promoter and line 8, pHB2, where the DNA is transcribed from the pBR322 *tet* promoter. The dashed line shown in Line 1 represents upstream DNA not drawn to scale. The *Thermus* DNA is present on two compatible cloning vectors in the results shown in line 8 (pHB2 and pNZ1233 are pBR322 and pAC184 recombinant plasmids respectively). Abbreviations: +, complementation (= growth) of leucine mutation in *E. coli* after transformation and selection of plasmid antibiotic resistance markers; −, no complementation (no growth); H, *Hind*III; Bg, *Bgl*II; B, *Bam*HI.

There is significant homology between the *leu*B gene of *Thermus thermophilus* HB8 and two other *Thermus* species for which we have gene banks in λ vectors. The 1·62 kb region (*Figure 2*) sequenced by Kagawa *et al.* (1984) and cloned fragments from *Thermus* T351 (ATCC 31674) and *Thermus* sp. (ATCC 27737) have almost identical restriction enzyme maps and show substantial homology by Southern hybridization analysis. The sequence of the first 200 bases of the amino-terminal portions of each of the genes are identical. T. Oshima (personal communication) has reported that the nucleotide sequences of the 3-isopropylmalate dehydrogenase genes from a number of other isolates of *Thermus* and thermophilic bacilli are highly conserved at the amino-terminal end but are less strongly conserved at the carboxyl terminus.

A few other genes from *Thermus* sp. have been cloned and expressed in *E. coli* (reviewed by Brock, 1985). For example, the malate dehydrogenase (EC 1.1.1.37) of *Thermus flavus* AT62 was isolated as a 3·0 kb *Hin*dIII fragment in pBR322 after screening *E. coli* transformants for thermostable malate dehydrogenase activity by enzymatic assay. No direct selection is possible for this gene function. The thermophilic malate dehydrogenase was expressed from a vector promoter but its enzymatic and immunological characteristics were identical to the enzyme isolated from *Thermus* (Iijima, Uozomi and Beppu, 1986). An unusual application of recombinant DNA techniques to thermophile genes is the cloning of the restriction/modification system (*Taq*I) from *Thermus aquaticus* in *Escherichia coli*. The methylase gene product is produced as a fusion protein because it is transcribed from the pBR322 *tet* promoter. The enzyme is functional *in vivo* as DNA from the strain carrying the plasmid is fully methylated. The restriction endonuclease is produced, apparently because of recognition of a promoter-like sequence within the structural gene for the methylase. The system appears to be polycistronic but attempts to over-express the restriction endonuclease from the λP_L promoter were unsuccessful because of an efficient transcriptional terminator between the two genes (Slatko *et al.*, 1985; B. Slatko, personal communication).

However, this approach to thermophile genetics may not be universally applicable as it appears to depend on fortuitous *in vitro* recombination events generating a promoter sequence that can be recognized in *E. coli* and often relies on enzymes required in catalytic amounts for biosynthetic pathways. We have been unable to detect expression of a serine protease, pullulanase (EC 3.2.1.41) and β-galactosidase genes from *Thermus* in *E. coli* for reasons which have not been resolved. Mutants in the *lac*Z gene of *E. coli* could not be complemented and neither casein degradation nor pullulan hydrolysis could be detected after incubation of *E. coli* recombinants at elevated temperatures. These results could arise from a lack of transcriptional control sequences (for example, a ribosomal binding site), by the protein being produced but not correctly folded for activity, or being lethal to the cloning bacterium. In some cases it is not possible to identify the genes encoding the enzymes using DNA sequences cloned from other organisms as probes. For example, there is no homology between the β-galactosidase of *E. coli*

or an α-amylase from *Bacillus subtilis* and sequences present on the *Thermus* genome (our unpublished results).

GENES FROM ANAEROBIC THERMOPHILES

Few genetic studies have been reported for anaerobic bacteria because of the difficulty of applying to these organisms the extensive screening procedures developed for *Escherichia coli* and *Bacillus subtilis*. The genetics of anaerobic thermophiles is even more rudimentary because of the cumulative technical difficulties of anaerobic conditions and high temperatures. Few mutant strains have been isolated and hence genetically marked strains necessary for the development of gene transfer systems and the study of genetic recombination are not available. Mendez and Gomez (1982) reported the isolation of spontaneous and UV-induced auxotrophic mutants of *Clostridium thermocellum* after penicillin selection. Reversion of only one of the mutants to prototrophy was observed. Mutants that were resistant to 5-fluorouracil and rifampicin were isolated after treatment with UV light and gamma radiation (Gomez, Snedecor and Mendez, 1981). Measurements of the DNA content per cell of *Clostridium thermocellum* suggested that there was only a single genome per cell, and hence the paucity of mutants did not result from the cells possessing more than one chromosome.

Regulatory mutants of β-amylase in *Clostridium thermosulfurogenes* have been generated by *N*-methyl-*N'*-nitrosoguanidine (NTG) mutagenesis and selection for growth in the presence of 2-deoxyglucose. Hyun and Zeikus (1985a) have isolated mutants that were not catabolite-repressed for growth on starch in the presence of glucose, those that were constitutive for β-amylase production, and mutants that over-produced the enzyme. Derepression and overproduction of pullulanase and glucoamylase (EC 3.2.1.3) was found for mutants of *Clostridium thermohydrosulfuricum* generated in a similar manner (Hyun and Zeikus, 1985b,c). *Cl. thermohydrosulfuricum* shows some potential for development of a genetic transfer system for cloned genes. Soutschek-Bauer, Hartl and Staudenbauer (1985) have shown that cells of this bacterium could be transformed with the *Staphylococcus* plasmid pUB110 and a derivative, pGS13. The presence of polyethylene glycol (PEG) was necessary to obtain transformants but protoplasts could not be regenerated after PEG treatment. pUB110 has been used extensively in *Bacillus subtilis* and lends itself to a variety of genetic manipulations for the expression of cloned genes.

A more productive approach has been to examine the expression of genes from thermophilic anaerobes in *Escherichia coli*. Most work has been done with *Clostridium thermocellum* and initial attempts to use complementation of auxotrophic mutations of *E. coli* (in a similar fashion to the cloning of the *Thermus leu*B gene) resulted in the identification of an insertion element capable of mutating *E. coli* DNA and presumably acting in a similar manner in *Cl. thermocellum* (Gomez, Snedecor and Mendez, 1981; Snedecor, Chen and Gomez, 1982). Cornet et al. (1983a) constructed a gene bank of *Cl. thermocellum* DNA using the cosmid pHC79 (Hohn and Collins, 1980) as a

vector and the restriction endonuclease *Sau*3A to give a random digest of the genome. Complementation of the Trp^- and Leu^- phenotypes of the *E. coli* recipient was observed, and either the Trp^+ or Leu^+ phenotype was cotransducible with ampicillin resistance expressed by the cosmid as the selective marker. These results confirm that the prototrophic phenotype observed did not result from the insertion of an IS. Two cosmids were shown to produce enzymes that could hydrolyse carboxymethyl cellulose (CMC) and cell-free extracts of one reacted with antiserum to an endo-β-1,4-glucanase (endocellulase; EC 3.2.1.4) which had been isolated previously from *Clostridium thermocellum* (Pétre, Longin and Millet, 1981). Subcloning of the *Clostridium* fragments from these two cosmids showed that endocellulase activity could be located on a 3·4 kb *Hin*dIII fragment in one case (*cel*A) and a 3·6 kb *Hin*dIII fragment in the other (*cel*B). Expression of these enzyme activities was orientation-independent, implying that they were being expressed from a promoter-like sequence in the inserts. Studies using *E. coli* minicells showed that the inserted DNA encoded proteins of the expected molecular weight that reacted with antisera to endocellulase A and B. The availability of the cloned genes allowed them to be used as probes for related sequences. It was found that *cel*A and *cel*B are not re-iterated, show no homology with each other and are not contiguous on the chromosome of *Cl. thermocellum* (Cornet *et al.*, 1983b; Pétre *et al.*, 1986).

A further gene bank (Millet *et al.*, 1985) was constructed using sized *Eco*RI fragments from a partial digest cloned into the plasmid pACYC184 (Chang and Cohen, 1978) using direct detection of endocellulase expression (CMC^+) with Congo Red (Teather and Wood, 1982) and putative exo-β-1,4-glucanase activity (exocellulase, MUC^+, *see later*). Besides the *cel*A and *cel*B genes previously characterized, five additional restriction enzyme fragments coded for endocellulases and three different fragments were identified which were MUC^+. Two of the CMC^+MUC^+ isolates have been characterized in detail. The protein from one, encoded by the gene *cel*C, has been purified to homogeneity and shown to differ in substrate specificity and to have a lower optimal temperature (65°C) than endocellulase A (80°C, Pétre *et al.*, 1986). The availability of an antiserum to the *cel*C protein synthesized in *E. coli* allowed the demonstration by Western blot analysis that this enzyme was secreted into the medium during the growth of *Clostridium thermocellum*. The cloned DNA for *cel*C does not show significant homology with *cel*A. Another CMC^+MUC^+ isolate was subcloned in phase into the vector pUC8 to form a translational fusion which resulted in the high level expression of the *cel*D gene which allowed facile purification and crystallization of the endocellulase (Joliff *et al.*, 1986a,b). The enzyme is denatured above 65°C but has a high specific activity—428 IU/mg—higher than the other endocellulases from *Clostridium* and significantly higher than *Trichoderma* endocellulase I. The *cel*D gene is not reiterated on the genome and shows no significant homology with the other two *cel* genes (Joliff *et al.*, 1986a).

The nucleotide sequence of the *cel*A, B and D genes has been determined (Béguin and Aubert, 1985; Grépinet and Béguin, 1986; Joliff, Béguin and

Aubert, 1986). Each gene has a potential signal peptide sequence which resembles the signal sequences typical of Gram-positive bacteria, although they show little similarity to each other. All share a conserved re-iterated domain coding for the carboxy-terminus of the gene and have recognizable promoter-like regions and ribosomal binding sites. The start point for the synthesis of *cel*A mRNA has been mapped by S1 and primer extension mapping (Béguin *et al.*, 1986). Availability of the cloned gene and its sequence allowed the analysis to be extended to *Clostridium thermocellum* so that the initiation and transcription of *cel*A mRNA could be compared with the transcripts isolated from *E. coli*. There was one transcriptional start site 134 bases upstream from the initiation codon and adjacent to sequences resembling an *E. coli* and *Bacillus subtilis* promoter sequence. A second and major transcript in *Clostridium* initiated at position −57. The transcription of *cel*A appears to be monocistronic and there is a sequence present similar to an *E. coli* rho-independent terminator (Rosenberg and Court, 1979). The presence of two promoter-like sequences and two mRNA transcripts for *cel*A mRNA suggests that the gene may be differentially regulated under particular conditions of growth. Although *Clostridium thermocellum* is a Gram-positive anaerobic thermophile, it is noteworthy that the transcriptional control signals are similar to those of aerobic Gram-positive and Gram-negative bacteria.

Others have reported the cloning of *Clostridium thermocellum* endocellulases using a bacteriophage λ vector and a *Sau*3A partial digest of genomic DNA (Schwarz, Bronnenmaier and Staudenbauer, 1985). A bacteriophage carrying DNA analogous to *cel*A was identified, and the gene library was screened for β-glucosidase (EC 3.2.1.21) and β-glucanase activity using specific substrates. For example, plaques producing β-glucosidase were identified by their fluorescence under ultraviolet light after growth on plates containing methylumbelliferyl-β-glucoside (MUG). Subcloning of restriction enzyme fragments allowed the identification of two different β-glucosidases and an endo-β-1,3-glucanase (laminarinase, EC 3.2.1.6 and 3.2.1.39) on small *Eco*RI fragments. Schwarz, Grabnitz and Staudenbauer (1986) showed that the endocellulase A from this gene bank (*cel*A) produced in *E. coli* differs in molecular weight but retains the heat stability of the enzyme produced in *Clostridium thermocellum*. The kinetic and chemical characteristics of the enzyme were unchanged after prolonged maintenance in *E. coli*, in contrast to a report that the *leu*B gene product of *Thermus* showed a lower optimal temperature under such conditions (Nagahari, Koshikawa and Sakaguchi, 1980).

Caldocellum saccharolyticum is a thermophilic obligatory anaerobic bacterium that has an optimum growth temperature of 68°C but which will continue to grow at 80°C. It is able to degrade cellulose but is unrelated to *Clostridium thermocellum* as shown by the lack of DNA–DNA hybridization (Donnison, Brocklesbury and Morgan, 1986). We have constructed a gene bank of this bacterium in bacteriophages λ1059 and λ2001 using a sized partial *Sau*3A digest of genomic DNA and have isolated DNA fragments encoding a number of enzymes involved in cellulose breakdown. These

fragments have been subcloned in plasmid vectors in *Escherichia coli* using direct identification of the gene of interest on plates containing either a chromogenic substrate or a specific staining reaction. Colonies of *E. coli* carrying the cloned *Caldocellum* DNA were grown at 37° and shifted to 70°C for 4–12 hours. The *E. coli* cells lyse and the active enzyme diffuses into the substrate layer in the plate. β-Glucosidase activity was recognized using 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (BCI-glu) and carboxymethylcellulase (CMC^+, endocellulase) activity by staining plates with Congo Red (Teather and Wood, 1982). Exocellulase activity was assayed by overlaying colonies or plaques with soft agar containing 4-methylumbelliferyl-β-cellobioside and observing those which fluoresced at 340 nm after 2 hours incubation at 70°C (MUC^+ phenotype).

β-Glucosidase hydrolyses cellobiose to form glucose. BCI-glu was used for the *in situ* detection of cloned β-glucosidase and phages were isolated from plaques which were blue due to the hydrolysis of BCI-glu. β-Glucosidase activity was isolated on a 1·78 kb *Hin*dIII fragment after subcloning in pBR322 (pNZ1001). Expression in *E. coli* was orientation-dependent, and we assume that it was expressed from a vector promoter. A partially purified preparation of the enzyme can be prepared by heating the host strain to 80°C for 30 minutes and removing the precipitated proteins. The enzymatic characteristics of β-glucosidase activity in the *E. coli* cell extract are virtually identical to those of the native β-glucosidase of *Caldocellum* (*Table 4*). A protein of $Mr = 52\,000$ was found in *E. coli* maxicells transformed with pNZ1001 (Love and Streiff, 1987; Love, Streiff and Bergquist, 1986). The *Caldocellum* β-glucosidase has been sequenced by the dideoxy-chain terminating method of Sanger, Nicklen and Coulson (1977), and an open reading frame of appropriate size has been located by computer analysis. The translational start-point has been assigned with reference to the amino-terminal sequence of the partially purified enzyme (*Figure 4*, D.R. Love, R. Fisher and P.L. Bergquist, unpublished work). This gene has been cloned in pKK223-3 (Amann, Brosius and Ptashne, 1983) under the control of the *E. coli* p*tac* promoter (de Boer, Comstock and Vasser, 1983): this construct gives a 12-fold increase in the amount of enzyme produced.

The *Caldocellum* gene bank has been assayed for phage that express cellulase activity by *in situ* hydrolysis of CMC^+ and MUC^+. Phage with all three possible genotypes have been isolated and the cellulase activity subcloned in some cases (Streiff *et al.*, 1986). Incorporation of BCI-glu into CMC plates allows the *in situ* assay of cellulases and β-glucosidase. Results of this *in situ* assay show that the β-glucosidase and endocellulase genes of *Caldocellum* are not linked. *Figure 5* shows diagrams of the restriction enzyme maps for some of the λ phage and plasmid vectors carrying the genes for cellulase activity. The inserted DNA in bacteriophages λNZP2 and λNZP5 overlap as shown: λNZP6 and λNZP7 overlap neither with each other nor with λNZP2 etc. The CMC^+ activity of λNZP2 has been subcloned into pBR322 and into pUC8. Its CMC^+ phenotype is independent of orientation and we assume that the gene is being transcribed from a sequence recognized as a promoter in *E. coli*. Sequence analysis of the appropriate

Table 4. Characteristics of β-glucosidases

Organism	MW	pH max	T max	Thermostability	K_mpNPG	K_m cellobiose	K_i glucose	Reference
Alcaligenes faecalis: mesophilic bacterium	120 000–160 000 (gel filtration)	6·0–7·0	ND	90% loss, 60°C 6·5 min, pH 6·5	0·125 mM (40°C, pH 6·6)	ND	3 mM	Han and Srinivasan, 1969
Clostridium thermocellum: thermophilic anaerobic bacterium	43 000 (SDS-PAGE) (gel filtration)	6·0–6·5	65°C	40% loss, 60°C 420 min, pH 6·0	2·6 mM (60°C, pH 6·0)	83 mM	135 mM	Ait, Creuzet and Cattanéo, 1979, 1982
Thermomonospora sp.: thermophilic filamentous bacterium	ND	6·5	55°C	50% loss, 60°C < 60 min	ND	ND	ND	Hagerdal, Ferchak and Pye, 1980
Caldocellum saccharolyticum: extremely thermophilic anaerobic bacterium*	52 000 (SDS-PAGE)	6·25	85°C	50% loss, 70°C 2280 min, pH 6·25	0·66 mM (70°C, pH 6·25)	21 mM	300 mM	Love and Streiff, 1987
Alternaria alternata: mesophilic fungus	45 000	4·5–5·0	70–75°C	50% loss, 70°C 7·8–10 min, pH 5·0	0·14 mM (40°C, pH 5·0)	0·81 mM	ND	Macris, 1984
Trichoderma koningii: mesophilic fungus	39 800	ND	ND	60% loss, 45°C 30 min, pH 6·0	0·37 mM (37°C, pH 5·0)	1·18 mM	1·05 mM	Wood and McRae, 1982
Talaromyces emersonii: thermophilic fungus	ND	4·1	70°C	50% loss, 70°C 410 min, pH 5·0	0·14 mM (37°C, pH 5·0)	0·58 mM	0·71 mM	McHale and Coughlan, 1981

ND = not done
* Cloned enzyme expressed in *Escherichia coli*

fragment from λNZP2 shows that a typical *E. coli* promoter sequence and ribosomal binding site is present.

Both the β-glucosidase gene and the endocellulases have been cloned and expressed in *Bacillus subtilis*, the former in the pC194 vector, the latter as part of a shuttle vector composed of pBR322 and the *B. subtilis* vector pUB110.

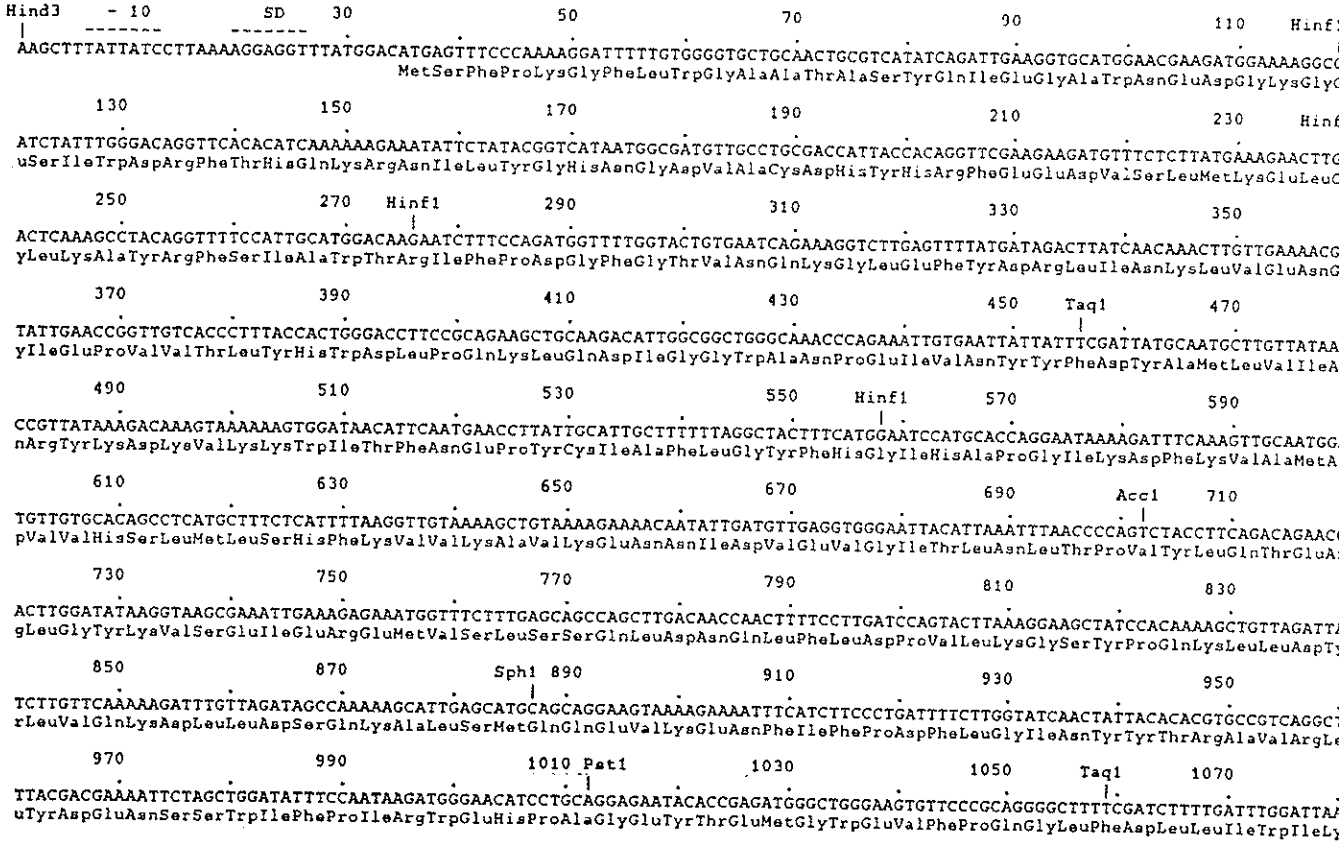
The *Caldocellum* gene bank has yielded a number of other DNA fragments with genes expressed in *E. coli*. α-Amylase degrades starch to limit dextrins by cleavage of the α-1,4-linkages. Pullulanase (debranching enzyme) cleaves at α-1,6-linked glucose residues in the starch molecule. We identified a λ clone that gave clearing of starch (*in situ* test with iodine/KI) and a clone that gave clearing of the surrounding agar in plates incorporating Remazol Brilliant Blue complexed to pullulan. The pullanase has been studied in some detail after subcloning in pBR322 and Tn5 transposition analysis has located the gene to a 3·5 kb fragment. The enzyme from the mesophile *Klebsiella pneumoniae* has been cloned in *E. coli* (Michaelis *et al.*, 1985) and the DNA encoding it is of similar size to the *Caldocellum* fragment specifying pullulanase.

Caldocellum will also grow on xylans and on xylose as a sole carbon source. We identified a λ clone that degraded xylan incorporated into agar plates in the same manner as clones producing cellulases may be observed on plates containing CMC. Only preliminary experiments have been done with this isolate, but the enzyme synthesized in *E. coli* is as stable to heat as that produced by the parental anaerobic thermophile.

OTHER EXAMPLES OF CLONED GENES FROM THERMOPHILES

The cloning and expression of *Thermus* genes for leucine biosynthesis has been described above. The 3-isopropylmalate dehydrogenases from *Bacillus coagulans*, *B. caldotenax* and *B. caldolyticus* have been cloned in *Escherichia coli* using pBR322 as a vector (Sekiguchi *et al.*, 1986a, b). Each of these genes showed significant homology to each other by Southern hybridization and showed some homology to the gene from *Thermus thermophilus* HB8, but they did not hybridize with *leu*B DNA from mesophiles. Comparison of the deduced amino acid sequences of the *B. coagulans*, *Thermus thermophilus* HB8 and *Saccharomyces cerevisiae* enzymes showed about 40% homology between pairs of enzymes and 32% between the three enzymes.

Other examples have been reported such as the cloning and expression of genes from *Bacillus stearothermophilus* in *Bacillus subtilis* and *Escherichia coli*. Most of these experiments (including the *leu*B gene results above) have been designed to examine crucial amino acids responsible for thermostability, but the degree of expression of a particular gene, or whether it is expressed at all because of an unfortunate choice of cloning sites, has not been addressed. For example, a *Hin*dIII restriction enzyme fragment from *Bacillus stearothermophilus* DNA expressed α-amylase when cloned in *Escherichia coli* using pBR322 as vector. The α-amylase produced had virtually identical


Hind3
- 10
SD
30
50
70
90
110
Hinf1
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MetSerPheProLysGlyPheLeuTrpGlyAlaAlaThrAlaSerTyrGlnIleGluGlyAlaTrpAsnGluAspGlyLysGlyGl
130
150
170
190
210
230
Hinf1
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uSerIleTrpAspArgPheThrHisGlnLysArgAsnIleLeuTyrGlyHisAsnGlyAspValAlaCysAspHisTyrHisArgPheGluGluAspValSerLeuMetLysGluLeuGl
250
270
Hinf1
290
310
330
350
ACTCAAAGCCTACAGGTTTTCCATTGCATGGACAAGAATCTTTCCAGATGGTTTTGGTACTGTGAATCAGAAAGGTCTTGAGTTTTATGATAGACTTATCAACAAACTTGTTGAAAACGG
yLeuLysAlaTyrArgPheSerIleAlaTrpThrArgIlePheProAspGlyPheGlyThrValAsnGlnLysGlyLeuGluPheTyrAspArgLeuIleAsnLysLeuValGluAsnGl
370
390
410
430
450
Taq1
470
TATTGAACCGGTTGTCACCCTTTACCACTGGGACCTTCCGCAGAAGCTGCAAGACATTGGCGGCTGGGCAAACCCAGAAATTGTGAATTATTATTTCGATTATGCAATGCTTGTTATAAA
yIleGluProValValThrLeuTyrHisTrpAspLeuProGlnLysLeuGlnAspIleGlyGlyTrpAlaAsnProGluIleValAsnTyrTyrPheAspTyrAlaMetLeuValIleAs
490
510
530
550
Hinf1
570
590
CCGTTATAAAGACAAAGTAAAAAAGTGGATAACATTCAATGAACCTTATTGCATTGCTTTTTTAGGCTACTTTCATGGAATCCATGCACCAGGAATAAAAGATTTCAAAGTTGCAATGGA
nArgTyrLysAspLysValLysLysTrpIleThrPheAsnGluProTyrCysIleAlaPheLeuGlyTyrPheHisGlyIleHisAlaProGlyIleLysAspPheLysValAlaMetAs
610
630
650
670
690
Acc1
710
TGTTGTGCACAGCCTCATGCTTTCTCATTTTAAGGTTGTAAAAGCTGTAAAAGAAAACAATATTGATGTTGAGGTGGGAATTACATTAAATTTAACCCCAGTCTACCTTCAGACAGAACG
pValValHisSerLeuMetLeuSerHisPheLysValValLysAlaValLysGluAsnAsnIleAspValGluValGlyIleThrLeuAsnLeuThrProValTyrLeuGlnThrGluAr
730
750
770
790
810
830
ACTTGGATATAAGGTAAGCGAAATTGAAAGAGAAATGGTTTCTTTGAGCAGCCAGCTTGACAACCAACTTTTCCTTGATCCAGTACTTAAAGGAAGCTATCCACAAAAGCTGTTAGATTA
gLeuGlyTyrLysValSerGluIleGluArgGluMetValSerLeuSerSerGlnLeuAspAsnGlnLeuPheLeuAspProValLeuLysGlySerTyrProGlnLysLeuLeuAspTy
850
870
Sph1
890
910
930
950
TCTTGTTCAAAAAGATTTGTTAGATAGCCAAAAAGCATTGAGCATGCAGCAGGAAGTAAAAGAAAATTTCATCTTCCCTGATTTTCTTGGTATCAACTATTACACACGTGCCGTCAGGCT
rLeuValGlnLysAspLeuLeuAspSerGlnLysAlaLeuSerMetGlnGlnGluValLysGluAsnPheIlePheProAspPheLeuGlyIleAsnTyrTyrThrArgAlaValArgLe
970
990
1010
Pst1
1030
1050
Taq1
1070
TTACGACGAAAATTCTAGCTGGATATTTCCAATAAGATGGGAACATCCTGCAGGAGAATACACCGAGATGGGCTGGGAAGTGTTCCCGCAGGGGCTTTTCGATCTTTTGATTTGGATTAA
uTyrAspGluAsnSerSerTrpIlePheProIleArgTrpGluHisProAlaGlyGluTyrThrGluMetGlyTrpGluValPheProGlnGlyLeuPheAspLeuLeuIleTrpIleLy

Figure 4. Nucleotide sequence of a β-glucosidase from *Caldocellum saccharolyticum* (Love, Fisher and Bergquist, unpublished work). The ribosomal binding site (SD) and the −10 region of the promoter are shown. The start-point for translation has been confirmed by determination of the amino-terminal portion of the amino acid sequence of the purified β-glucosidase. An inverted repeat sequence (transcriptional terminator?) is indicated by the dotted arrows.

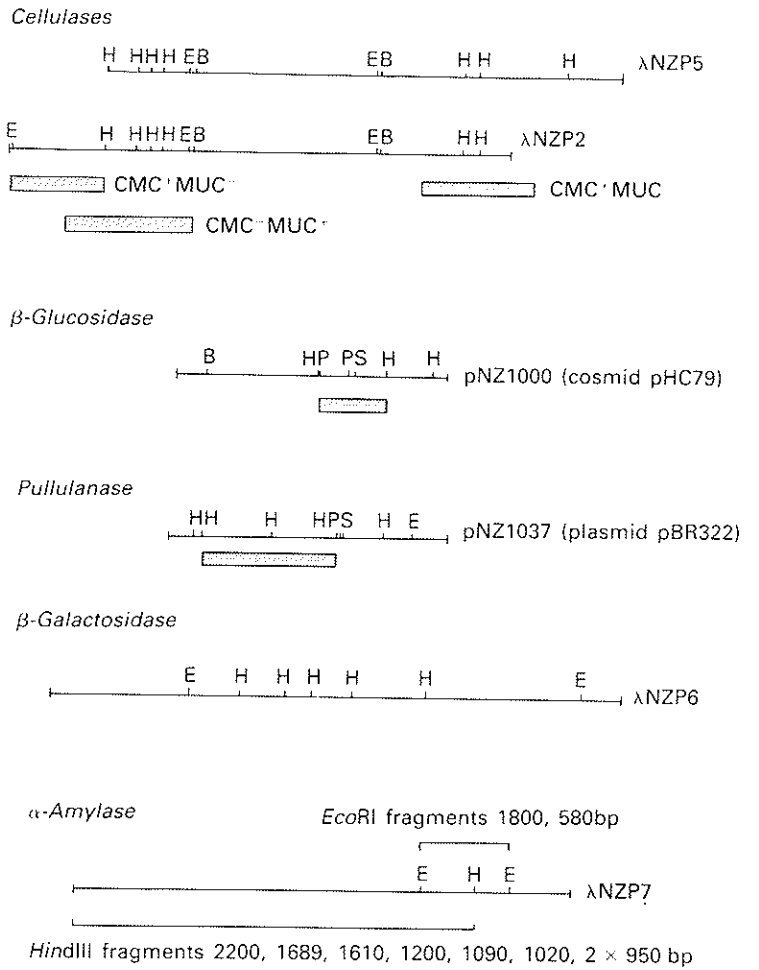

Figure 5. Restriction enzyme maps of λ clones from *Caldocellum saccharolyticum*.

properties to the enzyme isolated from *B. stearothermophilus* but was not secreted (Tsukagoshi *et al.*, 1984).

The nucleotide sequences of the α-amylase of *Bacillus licheniformis* and *Bacillus stearothermophilus* are largely homologous (60%) and each enzyme is functional at temperatures around 75°C. Gray *et al.* (1986) have cloned and sequenced the genes and constructed hybrid plasmids by homologous crossing over between the two genes cloned out of frame on the same vector. The point of cross-over was initially determined by hybridization to synthetic oligonucleotide probes designed to detect regions of minimal homology between the two genes, followed by sequence analysis. The residual enzymatic activities of the hybrid enzymes produced in *E. coli* were measured after incubation at 90°C. Increased temperature resistance was conferred by the amino-terminal portion of the *B. licheniformis* enzyme whereas increased

specific activity was associated with amino-terminal residues of the *B. stearothermophilus* α-amylase. The method for the generation of hybrids is applicable to genes with significant homology and avoids the necessity for the precise structural information required for oligonucleotide-directed site-specific mutagenesis.

A thermostable β-galactosidase from *B. stearothermophilus* encoded on a 3·0 kb *Pst*I fragment was cloned using the *Staphylococcus aureus* plasmid pUB110 which replicates at a high copy number in *Bacillus subtilis*. The gene *bga*B, has been sequenced and shows no homology with the *lac*Z gene of *Escherichia coli* (which is, itself, relatively thermostable). The enzyme was produced constitutively and retained its thermostability so that it could be purified readily from heated cell extracts of *Bacillus subtilis* (Hirata, Negoro and Okada, 1985; Hirata *et al.*, 1986).

Takagi, Imanaka and Aiba (1985) have analysed the promoter region of a neutral protease gene from *Bacillus stearothermophilus*, and determined the site of transcriptional initiation using S1 mapping (the nuclease degradation of single-stranded (non-hybridized) material from heteroduplexes made with *in vivo* or *in vitro* synthesized RNA and one strand of the DNA from a cloned gene). They found a promoter sequence homologous with the *spo*B gene of *Bacillus subtilis* and a ribosomal binding site nine bases upstream from the translational start codon. Ogawa *et al.* (1984) isolated *Bacillus stearothermophilus* promoter fragments using a shuttle promoter cloning vector capable of replication in *E. coli* and *Bacillus subtilis*. Sequences capable of acting as promoters in both organisms were isolated but it was not determined if these fragments were located suitably in front of open reading frames in the original DNA or whether this promoter-like activity was a fortuitous result of the cloning technique employed.

THERMOPHILIC ARCHAEBACTERIA

The archaebacteria comprise a third biological kingdom which was recognized initially by the application of molecular biological techniques to taxonomy. Two main phylogenetic branches of the kingdom include the strictly anaerobic methanogenic bacteria and the aerobic and anaerobic sulphur-oxidizing bacteria (Reeve *et al.*, 1986; Stetter, 1986). Some members of the archaebacteria are thermophiles and all bacteria except *Thermotoga maritima* that grow at temperatures of more than 90°C are archaebacteria (Brock, 1985). Some aspects of the molecular biology of thermophilic archaebacteria have been studied, particularly the organization and sequence structure of ribosomal and tRNA genes (Dams *et al.*, 1983; Neumann *et al.*, 1983; Willekens *et al.*, 1986) and DNA-dependent RNA polymerases (Zillig *et al.*, 1985a). However, the genetics of archaebacteria are at a rudimentary stage and have been pursued in depth only for some species of thermophilic methanogens and by cloning of their genes in *Escherichia coli* (Hamilton and Reeve, 1986).

Reeve and his collaborators have cloned genes from two thermophilic methanogens using their ability to complement auxotrophic mutations in *E.*

coli. A 1·6 kb fragment from *Methanobacterium thermoautotrophicum* which complemented *pur*E mutations has been sequenced and an open reading frame encoding a protein of about 36 300 daltons has been identified. A polypeptide of appropriate molecular weight was synthesized in *E. coli* minicells containing the thermophilic DNA fragment (Hamilton and Reeve, 1984, 1985a). Mutations in the *E. coli his*A gene are complemented by cloned DNA from *Methanococcus thermolithotrophicus*. Incomplete sequence data show that the gene is highly homologous to two mesophilic *Methanococcus his*A genes (Cue *et al.*, 1985; J.N. Reeve, personal communication). To date, the only other polypeptide-encoding gene from a thermophilic archaebacterium that has been cloned and expressed is the 3-isopropylmalate dehydrogenase of the extreme acidothermophile *Sulfolobus acidocaldarius* (Oshima, 1986). The other genes cloned have encoded RNAs involved with protein synthesis, such as tRNA genes for serine and leucine (Kaine, Gupta and Woese, 1983) and 16S ribosomal RNAs (for example, Olsen *et al.*, 1985). The most striking observation is the presence of introns within the tRNA genes, but no protein-encoding gene sequenced so far has contained an intron (Hamilton and Reeve, 1986).

DNA sequences from methanogens have been analysed for operon-like structures, ribosomal binding sites and transcriptional control sequences. The DNA cloned from *M. thermoautotrophicum* carries one complete and two open reading frames, one of which is presumed to be read in the opposite direction to the other two (Hamilton and Reeve, 1985a). Each of these open reading frames has sequence related to the consensus archaebacterial ribosomal binding sequence AGGTGA, and which has the potential to base pair with the 3′-terminal sequence of *M. thermoautotrophicum* 16S RNA (Hamilton and Reeve, 1985a, b). The DNA sequences recognized by archaebacterial RNA polymerases have not been established but some common sequences are found upstream from the translational initiation start sites, and hence are potential promoter sequences. In general, the DNA sequences 5′ to archaebacterial rRNA genes and polypeptide-encoding genes are not closely related. The putative promoter sequences 5′-GAANTTTCA-3′ and 5′-TTTTAATATAAA-3′ (where N = any base) have been identified in methanogens (Reeve *et al.*, 1986) and there must be acceptable versions of the −10 and −35 sequences found in *E. coli* (Hamilton and Reeve, 1986) because those genes which are expressed are orientation-independent (Hamilton and Reeve, 1984). Moreover, such sequences are much more distant from the ATG initiation codon than is commonly found in *E. coli* (35–95 bases, Hamilton and Reeve, 1986). It is not yet known whether any protein-coding genes in thermophilic archaebacteria contain introns, as identification by complementation demands a functional cloned gene product and selects against genes containing introns.

BACTERIOPHAGES AND PLASMIDS IN ARCHAEBACTERIA

So far, archaebacterial phage have been reported from *Halobacterium* (mesophile) and from *Thermoproteus* (Janekovic *et al.*, 1983) while plasmids

have been found in some methanogens, *Sulfolobus* and Halobacteria. A plasmid reported from *Sulfolobus* (Yeats, McWilliam and Zillig, 1982) subsequently was found to be a bacterial virus (SAV1) that appeared to be present in a prophage form, as it could be induced with ultraviolet light (Martin *et al.*, 1984). Three different viruses have been found in one isolate of *Thermoproteus tenax*, which also appear to be able to exist in a lysogenic state. There are no reports on their utility as agents of genetic transfer.

Methanobacterium thermoautotrophicum harbours a cryptic plasmid with single restriction enzyme cut sites for several enzymes (Meile, Kiener and Leisinger, 1983). Manipulation of this plasmid has allowed the construction of potential shuttle vectors for methanogens which are capable of replication in a number of bacteria and in yeast (Meile and Reeve, 1985). Development of a system for re-introduction of the plasmid into thermophilic methanogens is an obvious next step. A plasmid present in *Sulfolobus ambivalens* appears to encode the capacity for chemolithoautotrophic growth on sulphur and is of a suitable size for development as a cloning vector (Zillig *et al.*, 1985b).

Molecular genetics and the biotechnological applications of thermophilic bacteria

Interest in thermophilic bacteria in biotechnological applications has been based on a number of real or anticipated advantages of conducting processes at high temperature (*Table 5*). There is no inherent reason why the overall metabolism of thermophilic eubacteria should differ from that of their mesophilic counterparts. Thus similar types of regulatory mechanisms may operate in the biosynthetic and catabolic routes in thermophilic and mesophilic organisms, but this has not been confirmed in thermophiles because their metabolic pathways have been less thoroughly investigated than those of mesophiles. The limited amount of physiological biochemical engineering and process control information available with regard to thermophiles has been reviewed by Sonnleitner and Fiechter (1983) and by Sonnleitner (1984). Many thermophiles (e.g. *Thermus*) grow rapidly in liquid culture but with a very low yield of biomass. For such reasons, thermophilic organisms are

Table 5. Advantages of thermophilic bacteria or enzymes in biotechnology processes (*see also* Sonnleitner, 1984; Weigel and Ljungdahl, 1986)

1. Lower fermentation/feedstock costs—cooling of fermenter not required
2. Decreased viscosity of culture broth
3. Sterility requirements not as stringent as for mesophilic organisms
4. Possibility that low biomass production leads to high product: substrate ratios
5. Many enzymes secreted
6. Simplified recovery of volatile products
7. Stability of enzymes—refrigeration not required
8. Thermophilic enzymes more resistant than mesophilic to detergents and denaturants and more resistant to immobilization procedures
9. Some enzymes, for example, proteases, have higher specific activities than mesophilic enzymes

unlikely to be used generally as fermentation strains until their physiology and growth characteristics have been more fully investigated.

Up until now, the biotechnological applications of thermophiles have been limited by the very small number of products investigated even on the laboratory scale, namely, ethanol production, a protease and D-asparaginase (Daniel, Cowan and Morgan, 1981; Guy and Daniel, 1982; Hartley and Payton, 1983). The work of Daniel *et al.* (1986) has shown that many thermophilic enzymes display extraordinary stability not only to heat but also to other denaturing agents such as detergents (Cowan, Daniel and Morgan, 1985). These features make thermophilic enzymes very desirable for a number of industrial applications, for example, in biosensors, where long-term stability of response is important in situations where access or replacement is difficult and reliability imperative (inside the human body, or in large fermenters). Enzymes for cleaning applications may demand proteins that are stable to high pH, high temperatures, and oxidizing and bleaching agents. For these applications, it does not matter how or in what organism these enzymes are produced, whether in thermophiles themselves or in *Escherichia coli*. In fact, there are some advantages in producing the enzymes in a mesophilic organism which allow the facile purification of the thermophilic protein by a simple heating step (Daniel, 1986).

On the other hand, commercial applications for food technology would necessitate the production of the cloned gene product in an organism that is already regarded as suitable for the industry, such as yeast or *Aspergillus*. For example, thermophilic β-galactosidase (EC 3.2.1.23) for the modification of milk and milk products might be cloned in suitable yeast vectors (Skipper *et al.*, 1985). Other thermophilic enzymes of commercial importance that could be produced in mesophilic organisms include xylose isomerase (EC 5.3.1.5) and lipases (triacylglycerol lipase, EC 3.1.1.3) (reviewed by Ng and Kenealy, 1986).

Many existing processes in the food and beverage industry have been designed around the limitations of mesophilic enzymes and thus there are a number of potential applications of thermostable enzymes. The production of glucose and maltose from starch is an example of the desirability of using high temperatures during saccharification because of the viscosity of starch and sugar solutions. The majority of the starches used in current enzyme-based glucose syrup manufacture contain 75–85% amylopectin consisting of linear chains of α-1,4-linked glucose residues, joined together by α-1,6-glucosidic linkages. About 5% of amylopectin consists of 1,6-linkages which block the action of glucoamylases and α-amylases and which cannot be hydrolysed by α-amylases. The use of debranching enzymes in this process has been limited by the instability of pullulanases under the conditions used for saccharification (up to 105°C, using thermostable α-amylase, Jensen and Norman, 1984). The possibility of using a superior thermostable pullulanase has been discussed by Plant, Morgan and Daniel (1986).

A major attraction of using extremely thermophilic micro-organisms has been the possibility that they could be used to advantage for the production of desirable but toxic volatile products (ethanol, butanol, acetone; *see* Pay-

ton, 1984; Weimer, 1986). The evaporation of volatile products at high fermentation temperatures supplies a solution to the problem of removal of potentially inhibitory products in the culture medium because, in contrast to yeast and *Zymomonas*, thermophilic ethanol producers may be inhibited by as little as 1% ethanol (Hartley and Payton, 1983; Sonnleitner and Fiechter, 1983).

Protein engineering

Most organisms of biotechnological significance have been subjected to deliberate stepwise mutagenesis procedures or to selective enrichment for enhanced yields. Traditional mutagenesis has been limited by the requirement for a specific phenotype to be recognizable in order to identify rare mutations in a mutagenized population. A well-documented example is the development of high penicillin-producing strains of *Penicillium* via stepwise mutation and selection, which resulted in a dramatic increase in productivity over a period of 35 years (Hardy and Oliver, 1985).

The introduction of recombinant DNA methodology has meant that genes can be removed from their context and purified in milligram amounts so that it is now possible to alter the nucleotides in DNA efficiently and systematically. Some obvious applications for this technique are to use site-directed mutagenesis to alter a particular amino acid residue in order to make an enzyme more resistant to oxidation (Estell, Graycar and Wells, 1985), to re-design an enzyme so that the substrate specificity is altered (Craik *et al.*, 1985), to alter the pH dependence (Thomas, Russell and Fersht, 1985), or to alter a unique promoter that may be active only in a particular organism so that it is no longer subject to positive or negative regulatory controls. This topic has been the subject of a number of recent reviews and space does not permit a detailed description of the various techniques. The interested reader is referred to the reviews of Botstein and Shortle (1985), Craik (1985) and Leatherbarrow and Fersht (1986).

The availability of cloned genes allows the restructuring of segments of DNA as well as the possibility of changing individual bases (reviewed in Botstein and Shortle, 1985). *Figures 6* and *7* provide a schematic outline of possible mutagenesis strategies once a cloned gene is available. Two techniques of immediate interest in the alteration of the physical properties of thermophilic enzymes are localized random chemical mutagenesis and oligonucleotide site-directed mutagenesis. *Figure 6* broadly outlines the techniques available but the following points are important. First, random chemical techniques may be most useful initially where the three-dimensional structure of the protein is unknown. In this form, it is conceptually little different from classical mutagenesis techniques except for the concentration on the gene of interest and the specificity available as a result of the selection of the strand used for mutagenesis (Botstein and Shortle, 1985). Secondly, oligonucleotide-directed techniques (*Figure 7*) allow the precise alteration of individual residues but generally require a detailed knowledge of the crystal structure of the enzyme (Winter *et al.*, 1982; Winter and Fersht, 1984).

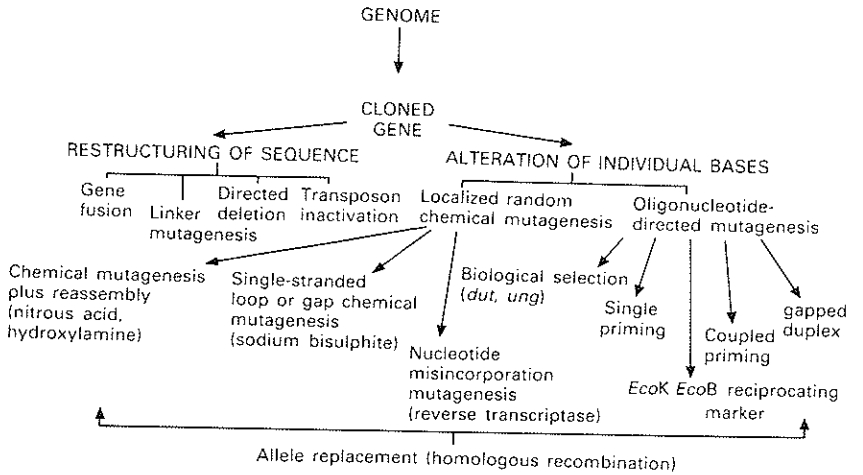

Figure 6. Mutagenesis strategies involving a cloned gene. The possibilities for mutation range from random chemical mutagenesis of cloned restriction fragments to precise alteration by oligonucleotide-directed mutagenesis. Restructuring of sequences is based on the alteration of double-stranded DNA, either *in vivo* using transpositional insertion or *in vitro* using restriction enzymes for deletion of fragments, fusions with other genes or the addition of linkers (Barany, 1985). This type of localized random mutagenesis *in vitro* then requires reassembly of complete molecules by DNA ligase. Alteration of individual bases is largely but not entirely based on the M13 single-stranded bacteriophage system. Chemical mutagenesis of a short single-stranded (gapped or looped out) region in an otherwise double-stranded region may be achieved by using a single-stranded mutagen, for example, sodium bisulphite. Chemical mutagenesis with hydroxylamine or nitrous acid can be carried out on partially single-stranded DNA as above or on double-stranded DNA plus reassembly into a non-mutated vector molecule. Nucleotide misincorporation by an error-prone DNA polymerase or incorporation of a nucleotide analogue during repair synthesis (Glover, 1984; Botstein and Shortle, 1985) is effectively conducted under conditions where there is a single-stranded template being copied. The scope of mutagenesis can be limited by the use of appropriate primers (Singh, Heaphy and Gait, 1986). Oligonucleotide-directed mutagenesis relies on the sequence of the gene to be mutated being available and a synthetic oligonucleotide primer. The various procedures are designed to increase the efficiency of the basic technique and to enhance the efficiency of mutant production. Once any of these techniques has yielded suitable mutant proteins, the gene(s) must be replaced in context. Various strategies have been exploited that rely on homologous recombination *in vivo* (for further details, see Botstein and Shortle, 1985).

Recent advances in computer graphics may be helpful where the sequence of the gene or protein is known, but not the three-dimensional structure of the enzyme (van Brunt, 1986).

Several examples of the use of these techniques for the alteration of proteins from thermophiles have been reported. The enzymes from thermophiles appear to be logical candidates for mutagenesis where increased stability is desired. Oshima (1986, and personal communication) has shown that it is possible to select for increased thermal stability of *Thermus thermophilus* 3-isopropylmalate dehydrogenase by chemical mutagenesis of the gene cloned in *Escherichia coli*. An increase in 10°C for the optimal temperature for enzyme activity was observed when serine 93 was changed to asparagine after hydroxylamine mutagenesis ($AG^{U}/c \rightarrow AA^{U}/c$). Generally, however,

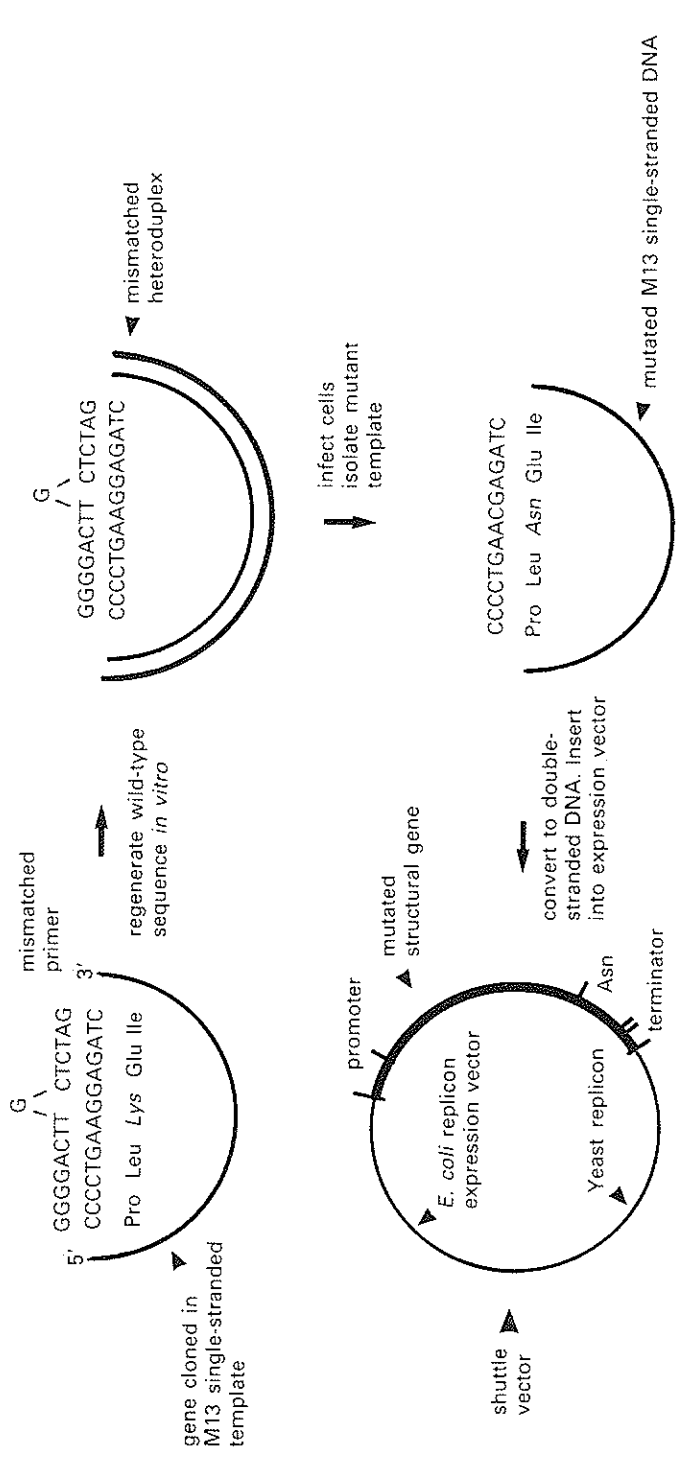

Figure 7. General scheme for oligonucleotide-directed mutagenesis. A hypothetical example is shown using a mismatched primer to direct a G→C change in a gene cloned in an M13 vector. There are several more complicated strategies for the selection of the mutated plus strand of the virus (*see* Leatherbarrow and Fersht, 1986). The mutant gene is converted back into the double-stranded form and cloned into a suitable expression vector. The one shown is a shuttle vector for yeast and *E. coli*.

increases in stability have been relatively small (Hecht *et al.*, 1986) and random mutation usually leads to a decrease in stability. Some form of selection is required to reveal mutations that lead to an increase in stability of a particular enzyme in a population of mutagenized DNA molecules.

Other workers have exploited the known correlation between the sequence structure of mesophilic and thermophilic kanamycin nucleotidyltransferase of *Bacillus* plasmids to isolate mutant enzymes with enhanced temperature stability (Matsumara *et al.*, 1984). Liao, McKenzie and Hageman (1986) introduced the gene coding for the mesophilic enzyme into a shuttle vector and isolated plasmid DNA from an *Escherichia coli* strain carrying the *mutD5* mutation, which increases the frequency of spontaneous mutations. Transformants of *Bacillus stearothermophilus* were selected for kanamycin resistance at 63°C. The base sequences of the mutant genes were all found to differ at position 240, corresponding to an aspartic acid to tyrosine replacement at position 80 in the protein. Second-step isolation of mutants gave enzymes which had threonine changed to lysine at position 130 of the protein. The position of this amino acid change is identical to that seen by Matsumara *et al.* (1984) for the nucleotidyltransferase from thermophilic plasmid pTB913. The advantage of this biological selection procedure for mutants was the ability to achieve increased heat stability without compromising catalytic activity and without a detailed three-dimensional structure for the molecule.

Aiba's group has examined the amino acid replacements in kanamycin nucleotidyltransferase using a combination of mutagenesis techniques and biological selection. The results of these experiments have been described in an earlier section, so only the technical details of the experiments are described below. Matsumara and Aiba (1985) transferred the gene for kanamycin nucleotidyltransferase from the mesophilic *Bacillus* plasmid pUB110 to an M13 single-strand vector by cloning in *Escherichia coli*. Hydroxylamine mutagenesis was carried out on the non-coding strand and the surviving phage were transfected into *E. coli*. Double-stranded DNA was prepared from individual phage particles that were still able to transduce *E. coli* to kanamycin resistance, recloned in the shuttle vector pTB922 and transformed back into *Bacillus stearothermophilus*. This experiment examined the regions which were significant for increased thermal stability by essentially random mutagenesis and reassembly of the cloned fragment (*Figure 6*).

A second set of experiments examined the effects of single amino acid replacements in the carboxy-terminal region of the protein (Matsumara, Kataoka and Aiba, 1986). The coding strand of the carboxy-portion of the gene was isolated and treated with sodium nitrite *in vitro*. The mutated DNA was annealed to an M13 gapped heteroduplex composed of the non-coding strand of the gene cloned in mp10 plus the complementary strand of the bacteriophage vector. The single-stranded region was repaired with DNA-directed DNA polymerase I (EC 2.7.7.7) and T4 DNA ligase (polydeoxyribonucleotide synthase(ATP), EC 6.5.1.1) and transfected into an *E. coli* strain deficient in mismatch repair. The mutated gene was reisolated from the bacteriophage replicative form and the plasmid pUC12 vector was used

as an expression vector, placing the gene under the control of the *lac* promoter. The mutant enzymes were purified from *E. coli* and their heat stability tested.

Chemical techniques of mutagenesis provide single point mutations but double mutants are rare. Double mutants are difficult to produce using oligonucleotide-directed mutagenesis unless the mutant sites are adjacent on the DNA. The combination of *in vitro* mutagenesis and oligonucleotide-directed mutagenesis allows the assembly of mutants altered in more than one position in the protein and at widely separated sites. Matsumara, Yasumura and Aiba (1986) combined a change of aspartic acid to tyrosine at position 80 (mutant Y80) generated by hydroxyalanine with a conversion of threonine to lysine at position 130 (mutant K130) by oligonucleotide-directed mutagenesis of an M13 gapped duplex to give the double mutant (Y80 K130). The triple mutant Y80 K130 L252 was generated by *in vitro* recombination by replacement of a *Bgl*II-*Hin*dIII fragment containing the codon for proline 252 with the same fragment from a mutant gene with an altered carboxyl-terminus coding region generated by sodium nitrite and carrying a leucine codon. As described previously, the effect of each of these mutations on thermostability of kanamycin nucleotidyltransferase was additive.

Some of the original experiments on oligonucleotide-directed mutagenesis utilized the tyrosyl-tRNA aminoacyl synthetase (EC 6.1.1.1) of *Bacillus stearothermophilus* (Winter *et al.*, 1982) and a systematic investigation has revealed the involvement of various residues at the active site of the enzyme in the formation of tyrosyladenylate (reviewed by Leatherbarrow and Fersht, 1986). The gene for tyrosyl tRNA synthetase from the extreme thermophile *Bacillus caldotenax* has been cloned in *E. coli* by complementation and sequenced. Two amino acid changes were found in the active site of the *Bacillus caldotenax* enzyme compared with *B. stearothermophilus*. Each of these changes has been incorporated individually into the *B. stearothermophilus* gene by oligonucleotide-directed mutagenesis. Substitution of asparagine for histidine at position 48 in the *B. stearothermophilus* tyrosyl tRNA synthetase produced an enzyme that cannot be distinguished kinetically from wild type. A threonine→alanine change at position 51 gives an enzyme with decreased K_m for ATP (Jones *et al.*, 1986).

Discussion

Thermophilic organisms have been known for at least a century but most of the extreme thermophilic aerobic and anaerobic bacteria have been isolated only recently. A number of bacteria with optimal growth temperatures above 80°C have now been described, and many of these have been cultured in superheated conditions under greater than atmospheric pressure (reviewed by Deming, 1986). Despite numerous reviews advocating the use of extreme thermophiles in a variety of biotechnological applications, the major usage of thermophiles is still in relatively low-technology applications such as anaerobic fermentation of waste and sewage sludge, aerobic thermophilic composting and ore-pile leaching (Weigel and Ljungdahl, 1986).

There is a general paucity of genetic information available for almost all thermophilic bacteria apart from *Bacillus stearothermophilus* and this fact can be attributed in large part to the technical difficulties of handling bacteria at temperatures where conventional genetic techniques are made difficult as the result of dehydration of support material and a lack of genetic transfer systems that are available to the *E. coli* or *Bacillus subtilis* geneticist. The influence of medical aspects and applications of microbiology on the development of gene transfer techniques for mesophiles cannot be over-estimated in the rapid development of genetic techniques for enteric organisms. We have no doubt that there would be a variety of gene transfer techniques available now for thermophiles if an equivalent effort had been expended on their study.

Two strategies can be employed in the biotechnological applications of enzymes from thermophiles. First, the thermophiles themselves might be used as sources of enzymes, but this application would be problematical because of the difficulties of strain improvement in the absence of gene transfer systems for thermophiles. Knowledge of the physiological characteristics of most thermophiles is at present so limited that it is unlikely that they could be used in tank fermentations, but they might possibly be employed as immobilized cells. Secondly, where a purified enzyme is required to catalyse a specific reaction with a substrate, it may be possible to produce it in, and isolate it from, a mesophile. However, it is necessary to have a suitable assay for the gene of interest. Several methods are available.

1. The enzyme can be attributed to a particular cloned fragment of DNA if it produces an assayable product, or if it can complement a specific host mutation, or react with an antibody to the purified enzyme. Several examples of the successful use of such assays have been described earlier.
2. The gene may be identified by DNA hybridization with a probe constructed from the gene coding for the same enzyme activity in a different host, or using a synthetic probe based on the amino acid sequence of the enzyme isolated from the thermophile or a related bacterium. For example, the amino acid sequence of thermolysin (EC 3.4.24.4) from *Bacillus thermoproteolyticus* is known (Titani *et al.*, 1972) and it is quite simple to construct suitable oligonucleotide probes, based on the nucleotide sequence of the active site of the enzyme, to use in the identification of neutral protease genes from related organisms (our unpublished work).
3. Another means of identifying a gene of interest would be to use insertional inactivation of the activity in the original host with an insertion sequence (IS) as described for *Clostridium thermocellum* (Snedecor, Chen and Gomez, 1982). The position of the insertion sequence and the flanking gene sequence in a particular band of DNA in a restriction digest could be established using the IS as a probe; then the flanking sequences can be used to identify the wild-type gene in a genomic library of the thermophilic organism.

Once the gene of interest has been identified and isolated, it could be cloned in a vector which provides the necessary transcriptional and translational regulatory and secretion sequences for the enzyme to be produced in a mesophilic host. Purification of the thermostable enzyme away from the bulk of the mesophilic proteins can be accomplished by a high-temperature incubation step (Tanaka, Kawano and Oshima, 1981; Iijima, Uozumi and Beppu, 1986; Love and Streiff, 1987).

Conclusions

Genetic studies on thermophilic micro-organisms are at a rudimentary stage but there is every reason to believe that suitable host vector systems can be constructed, and meanwhile, genetic manipulations can be performed in *Escherichia coli* and other genetically well-known organisms. Thermophilic proteins would appear to have natural advantages for industrial processes in terms of their inherent stability, and the availability of their cloned genes means that their physical and kinetic characteristics can be altered and enhanced by contemporary mutagenesis techniques. More efficient conversion of substrates in some individual processes is limited by the temperature optima of key enzymes which might be replaced by thermophilic enzymes. The role of molecular genetics will be to allow the provision of the enzymes in economic quantities after cloning and mutagenic treatments. Some immediate examples of potential products are a thermostable pullulanase and a true glucose isomerase which is thermostable and has superior catalytic characteristics.

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