# PRODUCTION, PURIFICATION, CHARACTERIZATION AND CLONING OF THE CYCLOMALTODEXTRINASE FROM *BACILLUS SUBTILIS* HIGH TEMPERATURE GROWTH TRANSFORMANT H-17: COMPARISON TO THE PARENT ENZYMES FROM *BACILLUS SUBTILIS* 25S AND *BACILLUS CALDOLYTICUS* C2

BY

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# PRODUCTION, PURIFICATION, CHARACTERIZATION AND CLONING OF THE CYCLOMALTODEXTRINASE FROM *BACILLUS SUBTILIS* HIGH TEMPERATURE GROWTH TRANSFORMANT H-17: COMPARISON TO THE PARENT ENZYMES FROM *BACILLUS SUBTILIS* 25S AND *BACILLUS CALDOLYTICUS* C2

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The p-nitrophenyl-α-D-maltoside hydrolyzing cyclomaltodextrinase (EC 3.2.1.54) from the mesophile *Bacillus subtilis* 25S, the obligate thermophile *Bacillus caldolyticus* C2, and the *Bacillus subtilis* high-temperature growth transformant H-17 were purified, characterized and compared. All three cyclomaltodextrinases displayed maximal rates of hydrolysis and identical hydrolysis patterns for linear malto-oligosaccharides and α- and ß- cyclodextrin, with maltose and glucose as the final products. Starch, amylose, and amylopectin were degraded slowly to maltose,

in an exo-fashion by preferential cleavage of maltose units from the nonreducing ends. Neither enzyme showed activity against p-nitrophenyl-α-D-glucopyranoside, maltose, isomaltose, isomaltotriose or panose. The enzymes demonstrated pullulan hydrolase activity due to their hydrolysis of pullulan to glucose, maltose, and (iso)panose.

The 25S, C2, and H-17 enzymes were composed of two identical subunits of $M_r$ 55,000, 60,000, and 55,000, respectively. The 25S, C2, and H-17 enzymes had a pI of 4.85, pH optimum of 7.5, 7.0, and 7.5, and $K_m$ values for the chromogenic substrate p-nitrophenyl-α-D-maltoside of 2.96 mM, 1.31 mM, and 1.46 mM, respectively. The 25S enzyme exhibited optimal activity between 35-37°C, and complete inactivation after 10 min at 45°C. This contrasts with the C2 enzyme which showed optimal activity at 60°C, and retained 100% of initial activity at 60°C for 2 h, and with the H-17 enzyme which showed optimal activity between 65°C and 68°C, and retained 100% of initial activity at 65°C for 1 h. Both the C2 and H-17 enzymes required 2-mercaptoethanol or EDTA for thermostability. A comparison of the amino acid compositions showed an increase in proline, alanine, and leucine residues for the C2 enzyme, and an increase in proline, alanine, and glycine residues for the H-17 enzyme.

The H-17 cyclomaltodextrinase gene was cloned on separate Pst 1, Bam H1, and Eco R1 fragments in the plasmid vector pUC18, but was expressed in an inactive form in the host, *E. coli* DH5α. High level constitutive expression of the

gene product was also detrimental to the *E. coli* host, which led to structural instability of the recombinant plasmid. The cyclomaltodextrinase gene was then cloned on a 3 kb Eco RI fragment in the plasmid vector pPL708, and the fragment was structurally maintained in the host *B. subtilis* YB886. The cloned gene product appeared to be in an enzymatically active form in the *B. subtilis* host; however, expression was at a low level.

# CHAPTER 1
# INTRODUCTION

One aspect in the evolution of biotechnology is the use of enzymes to replace chemical catalysts as the agents of chemical processes. The industrial hydrolytic depolymerisation of starch, once achieved by an acid-catalyzed process, is now achieved by starch-degrading enzymes, the amylases. Ironically, the first enzymes to be recognized as specific biocatalysts were those which could hydrolyze starch. Diastase, the active component of malt, was first isolated in France in 1833, and later shown to be a combination of α- and ß-amylase [24]. In 1894, a mixture of amylases from *Aspergillus oryzae* was used by the Japanese as a digestive aid for the consumption of rice starch [24]. Historically, acid-catalyzed hydrolysis of starch to glucose was first conducted on an industrial scale in Germany, in 1850 [24]. It was not until the late 1960s that the traditional acid-catalyzed production of glucose was redesigned to an enzyme hydrolysis procedure on a commercial scale. This change was prompted by the breakthrough in the development of amyloglucosidase, which enabled starch to be completely degraded enzymatically to glucose. The advantages to an enzyme hydrolysis were higher yield, higher purity, and easier crystallization. The process was further developed in 1973 by introduction of a thermostable α-amylase from *Bacillus licheniformis*. Starch liquefaction could now

be rapidly achieved at 95°C. followed by saccharification with amyloglucosidase, which enabled production of syrups containing up to 98% glucose. Today, the precise end-product composition can be controlled with different amylases, to give corn syrups having various levels of oligosaccharides, maltose, and glucose, with the desired chemical and physical properties. Furthermore, high glucose corn syrup may now be converted to the food system sweetener, high fructose corn syrup, by glucose isomerase [20,24].

The use of the thermostable *B. licheniformis* α-amylase at 95°C in the starch conversion process has several advantages, most notably a greater reaction rate, decreased risk of microbial contamination, lowered viscosity, and increased solubility of substrate and product. While liquefaction rapidly occurs within two hours at pH 6.5, the conversion of maltodextrins to glucose or maltose syrups currently suffers several drawbacks. Saccharification by amyloglucosidase or ß-(maltogenic) amylase requires a drop in temperature to 55-60°C, and pH to 4.0-4.5 or 5.0-5.5, respectively. Furthermore, unacceptably long holding times for saccharification (48-96 h) are required to achieve maximal yields of glucose or maltose [20,24,72]. Therefore a new amyloglucosidase, α-glucosidase, or maltogenic amylase with higher thermostability and pH optimum may have commercial applications following the partial hydrolysis of starch.

Since most commercial enzymes are of microbial origin, the development of industrially important thermostable enzymes has predominantly involved three

approaches. One system employs screening multitudes of bacteria, usually thermophilic, for the production of a naturally occurring enzyme with high thermostability and pertinent biochemical characteristics. A second approach involves mutagenesis of the microorganism that produces minute quantities of a desired enzyme, such that expression of the enzyme's gene is derepressed and the enzyme is overproduced. The third technique is currently the most actively pursued among enzymologists and molecular biologists. The gene for a thermolabile enzyme is cloned, and specific amino acid substitutions are introduced into the enzyme by cassette mutagenesis of the gene. Theoretically, these amino acid alterations should produce additional intramolecular non-covalent or covalent interactions that will increase the enzyme's resistance to denaturation during elevated temperatures. Unfortunately, all three approaches are tedious, time consuming, and cost inefficient. In particular, site-directed mutagenesis requires knowledge of the enzyme's three dimensional structure through x-ray crystallography studies. The strategy then relies upon knowledge of the primary amino acid sequence, and the ability to predict which of 20 possible amino acid substitutions might result in an increase in enzyme thermostability, without sacrifice of catalytic efficiency.

A previously developed, unique procedure [39] may prove to be a viable alternative to achieving thermostability in commercially important, thermolabile enzymes. The approach involves the genetic conversion of a mesophilic strain to a thermophilic strain, by transforming the mesophile with a small fragment of

genomic DNA from a thermophilic donor of the same genus. It should be stressed that only a small, but critical fragment of DNA is transferred, which induces wide pleiomorphic effects. The high temperature growth (HTG) transformants resulting from this genetic conversion are then evaluated for their ability to produce proteins and enzymes that are thermostable *in vivo*. Therefore, this method does not rely upon gene cloning to obtain knowledge of the primary amino acid sequence of the thermolabile enzyme; nor does it rely upon one's ability to predict amino acid substitutions that may result in increased enzyme thermostability. The strategy is to simply allow the intracellular biochemical processes of the HTG transformant to perform the subtle "work" necessary to convert all the cellular components, including enzymes, into thermostable macromolecules.

The overall aim of this dissertation was to screen a wide range of HTG transformants, generated from the mesophile *Bacillus subtilis* 25S, for their ability to produce various thermostable starch degrading enzymes. This evaluation involved searching for a possible glucogenic amylase that may have industrial value. After studying several glucan hydrolases produced by one HTG transformant, *B. subtilis* H-17, an enzyme was chosen based on its ability to degrade maltodextrins. During this investigation, the enzyme was classified as a cyclomaltodextrinase. Within the overall aim was the biochemical, biophysical and genetic characterization of the cyclomaltodextrinase, and its comparison to the analogous enzymes from the mesophilic and thermophilic parents.

The specific objectives to this study were as follows:

(a) To determine optimal growth conditions for maximal production of the cyclomaltodextrinase from *B. subtilis* 25S, *B. caldolyticus* C2, and *B. subtilis* H-17.

(b) To purify, characterize, and compare the thermostable cyclomaltodextrinase from H-17 and the thermolabile cyclomaltodextrinase from 25S.

(c) To determine the possible molecular mechanism(s) which confer the thermostable properties of the H-17 cyclomaltodextrinase, based on structure-stability relationships

(d) To determine if the donor, *B. caldolyticus* C2, also produces a thermostable cyclomaltodextrinase.

(e) To show that the H-17 cyclomaltodexrinase is not a product of a specific thermophilic gene transferred from donor to recipient during transformation of 25S to thermophily.

(f) To determine if the H-17 cyclomaltodextrinase has advantages over currently used fungal amyloglucosidase or ß-amylase for the industrial conversion of starch to glucose or maltose, respectively.

(g) To clone the H-17 cyclomaltodextrinase gene and express its product in a suitable host.

(h) To determine the thermostability of the cloned H-17 cyclomaltodextrinase gene product in a mesophilic host.

The results would hopefully demonstrate the potential for development of thermostable starch-processing enzymes by the generation of HTG transformants from generally recognized as safe (GRAS) status *Bacillus* mesophiles. Furthermore, cloning the H-17 cyclomaltodextrinase gene may allow a determination of its origin, and the molecular mechanism(s) which confer enzyme thermostability within the HTG transformants.

# CHAPTER 2
# REVIEW OF LITERATURE

## Thermophiles and Thermophily

Temperature is one of the most important environmental factors that affects biological processes, the structure and metabolic function of cellular components, and the evolution of life. The majority of living organisms have adapted to a moderate environmental temperature near that of the Earth's surface average of 12°C [116,117]. While the upper temperature limit for all living organisms is still unknown, it is assumed that all forms of higher organisms do not survive above 50°C. In addition, no eukaryotic microorganisms are known to exist above 62°C. Molds and yeasts are generally considered thermophilic if they grow as high as 40-50°C [11,83,116]. However, in high temperature environments, many prokaryotic microorganisms have upper temperature limits greater than 60-70°C. Thus far, only bacteria are able to adapt optimally to elevated temperatures. Bacterial growth in nature has been shown to occur from -5°C to 100°C [83,116]. The bacterial species diversity within a high temperature region is further influenced by other environmental parameters such as pH, available energy sources, osmolarity, mineral content, and toxic metals. Examples of extreme thermal environments include terrestrial hot springs, deepsea hydrothermal vents, continental and submarine

volcanic areas, small bays warmed by the sun, solar heated soil, thermally polluted streams, geothermal power plants, hot water storage tanks, and mechanical heating systems [11,44,88,116]. For bacteria living in regions of high environmental temperatures, molecular mechanisms of thermophily, and how such organisms evolved, have become a focal point of investigation.

Bacteria are usually classified into arbitrary groups based upon their preference to live within a limited temperature optimum for growth. Psychrophilic bacteria are capable of growth between -5-25°C. Mesophiles have temperature optima between 25-45°C. Thermophilic bacteria show optimal growth between 45-100°C [115,116]. Thermophiles may be further divided into (a) facultative thermophiles, which have an optimal growth temperature of 45-55°C but may grow at 25-30°C, (b) obligate thermophiles, which have an optimal growth temperature of approximately 55-65°C but cannot grow below 40°C, and (c) extreme thermophiles, which grow optimally above 70°C but cannot grow below 50°C [28,79,108]. However, one should be cautious when classifying according to growth temperatures since many microorganisms are borderline and could be assigned to either category depending upon growth conditions and the investigator's point of view.

In order to live optimally within their normal environmental temperature range, microorganisms must structurally adapt their proteins, nucleic acids, and lipids to function efficiently [115,116]. Since bacterial cells are essentially aqueous

chemical systems, their viability may only be limited to temperatures at which water exists in a liquid state [81,116]. Indeed, microbial growth is thought to occur at temperatures greater than 100°C, in which water remains in a liquid state under high pressure, for example, near deepsea hydrothermal vents [83]. However, every bacterial species has optimal growth within a relatively limited temperature range that rarely exceeds 30°C. This environmental temperature regulates in the cell, not only the rates of enzyme catalyzed reactions, but also the structural state of water and of active biopolymers such as proteins, nucleic acids, and lipid membranes [115,116]. The structure and function of cellular biopolymers is based on the number and distribution of noncovalent bonds. Since these relatively weak bonds are essential for stability and function, then even slight inputs of thermal energy can disrupt noncovalent interactions which may lead to drastic structural and functional alterations [114]. To achieve thermoadaptation, it is reasonable to assume that the functional molecules of metabolism, and the structural molecules of cellular components have been adapted such that bacteria are designed to attain maximal metabolic efficiency at their optimal growth temperature [115,116]. Not only do cellular components of thermophiles become structurally thermostable, but thermophilic metabolism is based upon enzymes that have adapted to a high temperature optimum for biological activity [114].

## The Origin and Genetics of Thermophiles

From an evolutionary standpoint, arguments regarding the origin of thermophiles have developed along two philosophies. The first, that thermophiles evolved from mesophiles by either adaptation or mutation is based upon the ubiquitous occurrence of thermophilic species in non-thermophilic environments [82]. If the thermostabilities of proteins and enzymes are encoded in their structural genes, then thermostability may be altered if a mutation occurs. Therefore, one could hypothesize that any type of microorganism could develop thermophilic properties if given sufficient time and opportunity. However, the conversion of a mesophile to a thermophile would require so many mutations that the probability of all these occurring in one cell is extremely small. Since the phylogenetic transition from a mesophile to a thermophile cannot be due to a spontaneous mutation of a single protein, it is improbable that acceptable mutations of most, if not all, enzymes and proteins could occur that would render a corresponding variation in thermostability and biological activity [114].

A more credible argument, that mesophiles originated from thermophiles, is based on speculation that the earliest cellular forms evolved in primordial waters considerably warmer than contemporary oceans or lakes [82]. The spontaneous transition between thermophilic and mesophilic bacilli, as observed in recent years, not only supports this second argument, but suggests that thermophilic properties

are encoded in a small number of adaptor genes that control structure genes [17,85,116].

Several attempts to identify the genetic determinant(s) of bacterial growth temperatures have implied that the transfer of a small number of genes may transform a mesophile into a thermophile, or *vice versa*. Cotransformation and cotransduction experiments in *Bacillus subtilis* [47,48] demonstrated that the temperature sensitive locus (*tms*-26) is closely linked to the streptomycin region. The function of the *tms*-26 marker in a high temperature growth *B. subtilis* mutant was to confer growth at 55°C. Since genetic modification of the S-12 protein within the 30s ribosomal subunit confers streptomycin resistance, the study suggests the *tms*-26 gene's involvement with ribosomal structure.

DNA studies [12,18] of several related *Bacillus* species show that the region surrounding the streptomycin marker is highly conserved in base sequence. This conserved core of genetic material also includes genes which encode for ribosomal and transfer RNA's, and resistance to other antibiotics such as erythromycin and micrococcin. While there appears to be a gradient of conservatism, the results suggest the region to be relatively resistant to evolutionary change.

This high base sequence homology may have allowed recombination of a segment(s) of *Bacillus caldolyticus* DNA with the *B. subtilis* chromosome [39]. In a unique study, mesophilic *B. subtilis* (*str*$^{S}$, *pur*A$^{-}$ [*ade* 16], growth optimum 37°C) was transformed with purified genomic DNA from the obligate thermophile

*B. caldolyticus* (*str*$^r$, *pur*A$^+$ [*ade* 16], growth optimum 72°C). *Str*$^r$, *pur*A$^+$ *B. subtilis* transformants were isolated that grew at 70°C and 55°C but not at 37°C. Since the majority of genes encoding for antibiotic resistance, ribosomal proteins, rRNA, tRNA, and protein synthesizing components are located immediately following the *pur*A gene in the early replicating region of the *B. subtilis* chromosome [64], the *str*$^r$ and *pur*A$^+$ genes were assumed to be cotransformed with those encoding for ribosomal and tRNA functions. The study suggested that recombination of these genes into the host would induce wide pleiomorphic effects in which microbial stability is achieved by converting the entire cell of the mesophile into that of a thermophile. Specifically, alteration of the protein-synthesizing machinery at the tRNA or ribosomal level would produce translationally-modified enzymes and proteins with increased thermostability. To support their assertion, the researchers isolated L-histidinol dehydrogenase (HDH) from the thermophilic transformants. Compared to inactivation at 70°C of HDH from the recipient *B. subtilis*, the HDH's from several high temperature growth transformants were thermostable at 100°C, whether assayed as crude extracts or as purified enzymes.

In a similar study [22], *B. subtilis* was transformed with DNA from *Bacillus stearothermophilus* or *B. caldolyticus*. High temperature growth transformants that grew at 65°C were isolated. The ribosomal proteins from the thermostable ribosomes of the transformants were analyzed using two-dimensional polyacrylamide gel electrophoresis. The gel patterns suggested that essentially all the genes that

encode for ribosomal proteins were transferred from donor to recipient. However, the mechanism by which these and any other cotransferred genes exert microbial thermostability in the host has yet to be determined.

An alternative approach [17] screened for spontaneous thermotolerant mutant derivatives of mesophilic bacteria at 10°C above their upper growth limit. When prototrophic strains of *B. subtilis* and *Bacillus pumilis* were plated out in large numbers, thermophilic mutants that were able to grow between 50°C and 70°C were isolated at a frequency of $10^{-10}$. DNA from one thermophilic *B. subtilis* mutant was used to transform a mesophilic *lys*$^-$, *trp*$^-$ *B. subtilis* auxotroph. Doubly-prototrophic (*lys*$^+$, *trp*$^+$) *B. subtilis* transformants were thermophilic, ie. grew between 50°C and 70°C. The transformation frequency of the thermophilic trait was about $10^{-7}$, while the cotransformation frequency of the two unlinked prototrophic markers (*lys*$^+$, *trp*$^+$) was also about $10^{-7}$. Since the transformation frequency of the thermophilic trait is similar to that for the transformation of two unlinked genes, the authors suggest that thermophily is the result of mutations in two unlinked genes. However, they do not speculate as to what those two genes are, and how close they are to the *lys* and *trp* markers. In a follow-up study [23], ribosomes from one of the spontaneous thermophilic *B. subtilis* mutants were thermostable at 60°C for 30 minutes, while ribosomes from the mesophilic parent were completely inactivated. Furthermore, the addition of polyamines to the cell-free extract from the thermophilic mutant stimulated polyphenylalanine synthesis at both 55°C and 65°C,

but inhibited protein synthesis with the cell-free extract from the mesophilic parent. The authors suggest, in the transition from mesophile to thermophile or *vice versa*, global mechanisms are in operation and that prime candidates may include genes encoding for polyamines, protein methylases, and DNA topoisomerases.

## Thermostability of Cell Components

While the origin of thermophiles and their evolutionary relationship to mesophilic species within the same genus is by no means established, thermophilic and mesophilic microorganisms do seem to have a common origin [44,114,115]. Not only are thermophilic species found in most bacterial genera, but they resemble their mesophilic counterparts in that they ferment similar carbohydrates, utilize similar nitrogen sources, and have similar oxidative pathways. Cellular structures and sequence homologies of proteins, enzymes, and nucleic acids of thermophiles are also very much alike if not nearly identical to that of their mesophilic counterparts except that the former usually have much higher thermostabilities [41,82,114].

With respect to mesophiles, several mechanisms have been proposed to explain the optimal growth and reproduction of thermophiles at high temperatures. Early theories attribute heat stability of thermolabile components to a) low cellular water content, b) transport of protective factors such as calcium from the environment into the cell, c) alteration in the nature of cell membranes, and d)

rapid resynthesis of heat-denatured cell components [43,114]. Newer theories include a) synthesis of organic polymers, such as polyamines, that act as protector molecules, b) lipid interaction such that cell membranes stabilize heat labile macromolecules, and c) site-specific biochemical modification of macromolecules [58]. Perhaps there is no single mechanism that confers thermophily. Rather, a combination of molecular alterations allow optimal growth at elevated environmental temperatures. However, contrary to earlier theories, a massive turnover of cell components does not occur. Rather, thermophily is based on the thermostability of individual cellular components [90]. It now appears evident that structural comparisons of thermophilic and mesophilic nucleic acids, proteins, and lipids from closely related organisms can best provide a molecular explanation for the exceptional ability of thermophilic bacteria to live at high temperatures.

Deoxyribonucleic Acid

Studies [79,87,104] concerned with the thermostability of DNA have compared the guanine plus cytosine (G+C) content and melting temperature of DNA isolated from thermophilic strains with that of mesophilic strains of the same genus. The DNA of thermophiles showed a consistently higher G+C content than that of DNA from mesophiles. Thermal melting profiles demonstrated that thermophilic DNA had higher melting temperatures than DNA from mesophiles. The greater stability to thermal-induced strand separation is attributable to a more

extensive hydrogen bonding that occurs with a higher G+C content. Therefore, the G+C content often correlates with maximum growth temperature, although *Clostridium* species are an exception. However, one cannot conclude that the thermostability of DNA, due to a higher G+C content, has any relationship with the ability of a thermophile to grow at high temperatures.

Ribonucleic Acid and Ribosomes

The base composition of messenger RNA (mRNA), isolated from the obligate thermophile *B. stearothermophilus*, was, as expected, nearly identical to the values obtained for DNA from the same organism [77]. As with DNA, it was concluded that the thermostability of mRNA probably does not play an important role in the ability of thermophiles to grow at high temperatures. Since active mRNA exists in a linear single stranded form, elevated growth temperatures should also have little or no effect on the secondary structure of mRNA, regardless of G+C content.

The base compositions of transfer RNA (tRNA) from several strains of *B. stearothermophilus* have been shown to be very similar to that reported for the mesophile *Escherichia coli*. In addition, the thermal melting profiles of tRNA from *B. stearothermophilus* and *E. coli* were nearly identical [19,43,104]. The tRNA(Val) and tRNA(Phe) from *B. stearothermophilus* also reacted with the respective valine and phenylalanine-amino-acyl-tRNA synthetases from *E. coli* and yeast [43].

Consequently, it appears that tRNA structures are very similar, regardless of microbial source. However, the G+C content of tRNA from the extreme thermophile *Thermus aquaticus* (63.5%) was higher than that of either *E. coli* (59.5%) or *B. stearothermophilus* (58%) [113]. Furthermore, tRNA from *T. aquaticus* and *E. coli* had melting points of 86°C and 80°C, respectively. Since *T. aquaticus* has a maximum growth temperature 11-17°C higher than that reported for strains of *B. stearothermophilus*, it is possible that the enhanced thermostability of *T. aquaticus* tRNA is a reflection of the greater G+C content.

Related studies [101-103] have also shown that tRNA from the extreme thermophile *Thermus thermophilus* not only has a melting point higher than that from mesophiles, but has a high G+C content (90%) in the base paired region and a modified base, 5-methyl-2-thiouridine (or 2-thio-ribothymidine), instead of unmodified ribothymidine in the T-loop. Furthermore, when they compared the sequence of tRNA specific for formyl-methionine from *T. thermophilus* with that of *E. coli*, they found not only a 90% G+C content in the base-paired region of the thermophilic tRNA, but that a G-U pair in *E. coli* tRNA was replaced by a G-C pair in the *T. thermophilus* tRNA. The results indicate that thermophilic tRNA(fMet) is thermally stabilized by formation of an extra intramolecular hydrogen bond when a uridyl residue is replaced with a cytydyl residue, and by increased stacking interaction due to thiolation of a ribothymidine residue. Although the changes are quite subtle, they are located in a region quite distant

from the anticodon loop and amino acid accepting terminal, both of which are important for tRNA function.

Reviews [19,43,90,104] indicate a variety of conflicting results regarding the effect of ribosomal RNA (rRNA) G+C content and melting points on the thermostability of ribosomes. The thermal melting profiles of ribosomes have shown that ribosomes from thermophiles undergo thermal denaturation at higher temperatures than ribosomes from mesophiles, perhaps due to the increase in G+C content of rRNA from thermophiles. Elucidation of the molecular basis of ribosomal thermostability remains unclear. Although the rRNA nucleotide composition may play a significant role, the same authors [19,43,90,104] agree that other factors such as primary structure of ribosomal proteins, the stacking arrangement of ribosomal protein and rRNA, and the association of polyamines with ribosomes, are probably more important in the stability of ribosomes in thermophiles. Specific polyamines, such as the tetramine spermine and the triamine spermidine, both synthesized by *B. stearothermophilus*, have been shown to aid in the association of ribosomal subunits at high temperatures [43,56]. More than 12 distinct polyamines, $NH_2(CH_2)_3[NH(CH_2)_3]_xNH(CH_2)_4NH_2$, were also isolated from *T. thermophilus* [56,59]. The tetramines, thermine and thermospermine were the main forms, while the pentamines, caldopentamine and homocaldopentamine, and hexamines, caldohexamine and homocaldohexamine were also present. Thermine or thermospermine were shown to initiate and maintain polyphenylalanine synthesis

directed by polyuridylic acid at high temperatures (50-80°C). When the tRNA(Phe), polyuridylic acid, and ribosomes were incubated at high temperature in the absence of the polyamine, a ribosome-mRNA-amino-acyl-tRNA ternary complex also formed but was inactive. The results indicate that during protein synthesis, thermine and thermospermine may play an in vivo role in the initial formation of the active ternary complex between ribosomes, mRNA, and amino-acyl-tRNA at high temperatures.

Thermine was also found in the extreme thermophiles *T. aquaticus* and *Thermus flavus*, while no detectable amount of thermine occurred in the obligate thermophile *B. stearothermophilus* [56]. It appears that, not only thermine and thermospermine, but other novel polyamines such as caldine and sym-homospermidine occur only in extreme thermophiles, which implies their involvement in extreme thermophily [57]. Although not yet understood, these unusual polyamines may also play important roles in other biochemical reactions such as DNA replication, transcription, and cell division, besides just translation.

Lipids and Cell Membranes

The central structural feature of microbial membranes is the phospholipid bilayer, which consists of peripheral proteins bound to the polar portion of the lipid bilayer, and integral proteins embedded within the nonpolar portion of the lipid bilayer [43,49,105]. A unique feature of the phospholipid bilayer is its ability to

undergo a reversible phase transition from a thermotropic gel (solid) to a liquid-crystalline state [49]. In the gel or solid state, the fatty acyl chains form a close hexagonal packing, which results in a restricted inter and intramolecular motion, and a rigid, somewhat impermeable bilayer structure. During the gel to liquid-crystalline transition, selective melting of the phospholipid hydrocarbon chains in the interior of the bilayer occurs. However, the transition is not sharp but broad due to heterogeneity of fatty acyl chains, which results in simultaneous domains of gel and liquid-crystalline phases. In the liquid-crystalline state, although the hydrocarbon chains are in a partially melted condition, the bilayer structure is maintained by electrostatic interactions between polar head groups and hydrophobic forces, which allows for a loosely packed, fluid, and somewhat permeable bilayer structure [49]. Consequently, cell membranes must be in the liquid-crystalline state such that transport functions, and activity of membrane-associated enzymes ensure cell growth [43].

The temperature range at which the gel to liquid-crystalline transition occurs depends upon the nature of the fatty acyl content of membrane lipids. It is generally accepted that most microorganisms alter their membrane lipid composition in response to a change in environmental temperature. The membranes of thermophiles have shown a higher content of apolar fatty acyl residues with higher melting temperatures than the residues of psychrophilic and mesophilic microorganisms. This would minimize the effect of high environmental temperature

on the physical state of the membrane lipids by raising the temperature of the gel to liquid-crystalline phase transition. Therefore, when thermophilic bacteria adapt to increases in cultivation temperature, their membranes shift to a higher proportion of longer, saturated fatty acids, and monomethyl-branched chains, and to a decreased proportion of unsaturated fatty acids [36,43,49,67,70,104,105]. As a general trend, the *iso-* and *anteiso-* monomethyl-branched chains tend to be the predominant form of apolar fatty acyl residues in thermophilic bacteria. However, in nearly all thermophiles examined, the majority, if not all, of the complex (polar) lipids contain a carbohydrate residue. It appears that lipids enriched in carbohydrates usually form the major lipid class, which implies a significant role in membrane thermostability [36,90]. Although not well understood, analysis of lipids from thermophiles suggest general trends in which there may be a combined variety of strategies for the molecular basis of thermostable membranes.

Proteins and Enzymes

Proteins have a limited temperature range within which structural integrity is maintained. All known proteins (enzymes) unfold, denature and thereby lose biological activity upon a certain intensity of thermoexposure. A thermophilic enzyme is more thermostable at higher temperatures, but less active at lower temperatures than a mesophilic enzyme. Thermostable enzymes from thermophiles, compared to thermolabile enzymes from mesophilic species of the same genus,

resist thermal unfolding at elevated temperatures due to differences in the number, strength, and distribution of their intramolecular noncovalent forces [32,50,99,117]. Therefore, the primary structure of an enzyme ultimately determines the number and type of noncovalent interactions. Amino acid sequence analysis of several mesophilic enzymes and their thermophilic counterparts has shown that a few specific amino acid substitutions at critical regions may account for very subtle structural and conformational differences that lead to large alterations in thermostability [2,32,99,117]. That is, a gain in thermostability of an enzyme usually does not require a drastic or extreme structural rearrangement in its conformation, but does require structural maintenance of its catalytic site. An enzyme from a thermophile consistently shows the same structural characteristics such as polypeptide chain length, secondary structure, catalytic site, globular domains, subunit structure, and modulation of activity by metal ions and effectors, as does the same enzyme isolated from a mesophile of the same genus [52,86].

Probably the most important, if not decisive, mechanism that confers protein thermostability is hydrophobic interaction [3,50,99]. The total hydrophobic amino acid content of a thermophilic enzyme, and that of its mesophilic counterpart, may or may not greatly differ. Rather, thermostability depends upon an increased proportion of hydrophobic residues around the active site. The overall effect is a greater internal and decreased external hydrophobicity. Therefore, strengthening of the internal nonpolarity is achieved by substitution of specific amino acid

residues, characteristic of mesophilic enzymes, with aliphatic amino acids, with retention of the overall structure and catalytic properties. As a result, internal hydrophobic domains contribute to compact packing of amino acid residues. A compact globular structure excludes water from internal cavities, which allows for improved enzyme stability.

A second factor that confers protein thermostability is the formation of additional electrostatic interactions such as divalent cation salt bridges and ion pairs [50,62,63,99]. Since electrostatic interactions are formed between negatively-charged aspartate and glutamate residues and positively-charged lysine, histidine, and arginine residues, then a greater proportion of these residues in a thermophilic enzyme may account for a higher thermostability than that of its mesophilic counterpart. Furthermore, thermophilic enzymes frequently have higher levels of arginine relative to lysine and histidine. Because the pKa's for lysine and histidine are lower than the pKa for arginine, at alkaline pH values, lysine and histidine side chains will dissociate and electrostatic interactions will be disrupted. Greater thermostability results from stronger electrostatic interactions formed by arginine rather than lysine or histidine [32].

A third molecular mechanism that enhances enzyme thermostability is an increase in intramolecular hydrogen bonds [50,99]. A comparison of thermophilic ferredoxins and hemoglobins with mesophilic counterparts concluded that thermostability is increased by the formation of a few new hydrogen bonds and salt

bridges [63]. In addition, 19 additional hydrogen bonds were detected in thermophilic protease, which were absent in the mesophilic enzyme [54]. It appears that an alteration in the number of hydrogen bonds sometimes induces a change in the secondary structure of thermophilic enzymes compared with their mesophilic counterparts. However, some thermophilic proteins lack polar amino acids, mostly serine and threonine [50]. Assuming a high degree of internal hydrophobicity, internal localization of serine and threonine is thermodynamically unfavorable. The substitution of internal serines and threonines with nonpolar residues theoretically increases thermostability. Therefore, in some thermophilic proteins, intramolecular hydrogen-bonding may be assumed to be localized at the surface of the molecule. Similarly, in an enzyme's compact interior, all polar groups are hydrogen-bonded and that alteration of size, shape, or polarity of a single side chain could destabilize enzyme structure [86]. Since water mobility presents little barrier to intramolecular hydrogen bonding alterations between surface residues, an increase in polar and a decrease in nonpolar surface residues could enhance thermostability.

Other factors that enhance protein thermostability include the formation of intramolecular disulfide bonds, substrate binding, and post-translational modification such as glycosylation or chemical modification of key surface groups [50,52,99]. However, one enzyme's mechanism of thermostability may be different from another's. Thermostabilization may also be attributable to the simultaneous contribution of several different mechanisms. Consequently, interpretation often

becomes complex and even contradictory when comparing structural mechanisms of thermostability among different thermostable enzymes. Rather than present a comprehensive review of all comparisons between thermostable enzymes and their mesophilic counterparts, only a few significant studies will be presented in which the factors that confer thermostability are somewhat understood.

The complete lactate dehydrogenase (LDH) primary sequences from the thermophiles *Bacillus caldotenax, B. caldolyticus,* and *B. stearothermophilus*, the mesophiles *Bacillus megaterium,* and *B. subtilis*, and the psychrophile *Bacillus psychrosaccharolyticus* underwent an extensive comparative structural analysis [25,115,117]. The sequence homology between the LDH variants was between 60% and 70%. Specific temperature-related amino acid substitutions occurred in which polar amino acids, particularly serine and threonine, in the mesophilic (psychrophilic) enzymes are exchanged for hydrophobic and charged residues, particularly alanine, arginine, and aspartate in the thermophilic enzymes. These substitutions did not occur among thermophilic or among mesophilic LDH variants, which contrasts to the 12-13 substitutions between thermophilic and mesophilic LDH. Three-dimensional structure analysis of all the LDH variants indicated that most of the substitutions were buried in the same strategically important regions, particularly near the active site and in contact regions of the subunits. This indicates that there are apparent regions of significant importance in temperature adaptation. Furthermore, in the transition of mesophilic (psychrophilic) to

thermophilic LDH, a dynamic hydrogen bond system is converted to hydrophobic interactions or ion pairs in which the free energy of the enzyme is increased. Conversely, in the transition of thermophilic to mesophilic (psychrophilic) LDH, a reduction in energy-yielding hydrophobic interaction and ion pairs occurs in favor of hydrogen bonding. The author concluded that, at high temperatures, the preferred thermophilic residues of thermophilic LDH should increase thermostability, but produce a more rigid and less active structure at low temperatures. However, polar residues, perhaps hydrated, should lead to a more dynamic, flexible, and active structure at low temperatures, but a labile structure at high temperatures. These temperature-related amino acid substitutions are stored in the structure of the genetic code, and hence are based on evolutionary temperature-related base substitutions.

The genes for alanine dehydrogenase were cloned and sequenced from the mesophile *Bacillus sphaericus* and *B. stearothermophilus* [34]. A comparison of the primary amino acid sequences showed a 73% homology, with the non-identical residues clustered in a few regions of relatively short length. The residues involved in catalysis and coenzyme binding were conserved in a sequence of high homology (>80%), while the short sequences of low similarity were believed to contribute to the thermostability of the *B. stearothermophilus* enzyme. An additional cysteine in the *B. stearothermophilus* alanine dehydrogenase may form an interior disulfide bond with one of two nearby cysteines, which may enhance thermostability.

The oligo-1,6-glucosidases from *Bacillus cereus* (mesophile), *Bacillus coagulans* (facultative thermophile), *Bacillus* sp. KP1071 (facultative thermophile), *Bacillus thermoglucosidasius* (obligate thermophile), and *Bacillus flavocaldarius* (extreme thermophile) were compared for amino acid composition, structural parameters, and thermostability [95]. Results showed that the proline content greatly increased in a linear fashion with the increase in thermostability among all five enzymes. The hydrophobic residues, in particular alanine and leucine, also showed upward tendencies in parallel with the increase in thermostability, while polar residues showed downward trends. Although the enzymes were quite similar in terms of structural parameters, the content of α-helix formers decreased with an increase in thermostability, while ß-sheet formers remained nearly constant. The increase in proline, an α-helix breaker, could contribute to improved turn stabilization. While this may increase the disordered regions at the expense of helix formations, it could produce a greater potential for close packing of hydrophobic regions. The strengthened hydrophobic interactions would tighten the molecule as a whole and thereby enhance thermostability.

The thermostable α-amylase from *B. stearothermophilus* was compared to the thermolabile α-amylase from *B. subtilis* [111]. Results indicated that binding of calcium ions to both mesophilic and thermophilic α-amylase considerably enhances the stability of the native conformation of both enzymes. However, calcium-free thermophilic α-amylase was very susceptible to thermal denaturation, but was more

resistant to heat than calcium-free mesophilic α-amylase. This suggests differences in the amino acid sequence between the two proteins. It was concluded that the difference in thermostability of α-amylase from *B. stearothermophilus* and *B. subtilis* is caused by the difference in the enzyme's affinity to the calcium ion at elevated temperatures, which may be a function of amino acid sequence.

The liquefying α-amylases produced by *Bacillus licheniformis, Bacillus amyloliquefaciens,* and *B. stearothermophilus* contained highly homologous amino acid sequences, with a 64% homology between the *B. stearothermophilus* and *B. amyloliquefaciens* enzymes, a 67% homology between the *B. stearothermophilus* and *B. licheniformis* enzymes, and an 80% homology between the *B. licheniformis* and *B. amyloliquefaciens* enzymes [26]. Because the regions of extensive homology include active sites, they probably are required for maintaining protein conformation and enzymatic activity. Furthermore, the thermostable *B. stearothermophilus* α-amylase contained two cysteine residues located near the enzymatically functional region, while the thermolabile *B. amyloliquefaciens* enzyme contained no cysteine residue. The formation of a disulfide bridge between the two cysteine residues could account for thermostability in the *B. stearothermophilus* α-amylase. However, the structural differences in non-homologous regions of the three α-amylases are presumed to be responsible for differences in thermostability [112]. Because the hydropathy profiles of all three enzymes were significantly hydrophilic, the authors

suggested that salt bridges between charged and polar amino acids within non-homologous regions may account for thermostability of the α-amylases.

## Protein Engineering of Industrially Important Enzymes

The current total world market for industrial enzymes is over 500 million dollars per year. About 20 microbial enzymes account for the majority of this market. Microbial enzymes applied to food processing and industrial operations include protease, α-, ß-, and gluco-amylase, pullulanase, glucose isomerase, cellulase, hemicellulase, lipase, pectinase, lactase, and alcohol dehydrogenase [14,52,83]. Where possible, thermostable enzymes are now utilized extensively for industrial processing. Adequate thermostability of commercial enzymes may be defined as retention of enzyme activity upon exposure to temperatures of 50°C or above, for prolonged periods [50,99]. Their industrial application may be advantageous for several reasons [32,52,84,99]: (a) Higher reaction rates can be obtained, since for every 10°C increase in temperature, reaction rates approximately double. Consequently, for each 10°C increase in operating temperature, the holding times can be shortened, or the amount of enzyme required for a given conversion can be theoretically halved. (b) Microbial contamination of enzyme reactions lasting several days is less likely to occur at operation temperatures of 60°C or greater. (c) At elevated temperatures, higher reactor productivity may be achieved due to greater solubility of reactants, reduced viscosity, and improved mass transfer rate.

(d) Enzymes of high thermostability often show increased resistance to chemical denaturation and thus longer shelf lives.

One factor that limits commercial enzyme application is the high cost of isolating and purifying sufficient amounts of the enzyme. A second limitation is that, while enzymes have evolved to function optimally under normal physiological conditions, they may not function under nonphysiological industrial conditions that include extremes of pH, ionic strength, oxidation, and temperature [27,52,97]. Consequently, robust enzymes with longer half-lives under process conditions are required for industrial applications. As a solution, genetic engineering techniques have attempted to the improve production of commercial enzymes by (a) amplification of the production of specific enzymes by mutation of microorganisms, (b) cloning and synthesis of enzymes in Generally Recognized As Safe (GRAS) organisms, and (c) protein engineering by genetic modification (mutagenesis) in which enzyme structure is altered such that one or more functional properties are improved under nonphysiological, extreme conditions, that is, high temperatures.

The major cost savings resulting from the benefits of thermostable enzymes currently prompt the development of thermostable properties to be engineered into enzyme molecules based on structure-stability relationships. Specifically, one goal of protein engineering is to enhance the thermostability by genetically introducing new noncovalent and/or covalent bonds within the enzyme. Genetic modification

may involve several techniques [65,99,107]. One approach relies upon knowledge of the enzyme's three-dimensional structure by high resolution x-ray crystallography and computer analysis, which delineates possible amino acids responsible for thermal sensitivity [1]. Variant amino acid sequences may then be designed by site-directed mutagenesis of the cloned gene encoding for the enzyme, so that specific nucleotide substitutions are created. Alternatively, cassette mutagenesis may achieve the same substitutions utilizing synthetic oligonucleotides. Restriction and ligation enzymes are employed to replace any sequence of the cloned gene with the synthetic fragment carrying one or more specific nucleotide substitutions (or deletions/insertions). The recombinant plasmid is then transformed into the appropriate host and transformants are screened for production of thermostable enzyme. However, either approach may (a) be time consuming due to the substitution of many possible amino acids at each site of interest, and (b) cause destabilization due disruption of pre-existing stabilizing interactions.

At Genex Corp., site-directed mutagenesis has successfully introduced a disulfide bond into subtilisin BPN, the alkaline protease secreted by *B. subtilis* [60]. While this enzyme has no pre-existing cysteine residues in the wild type structure, cysteine substitutions at Thr-22 and Ser-87 positions generated a Cys-22/Cys-87 disulfide subtilisin variant that had catalytic activity essentially equivalent to that of the wild-type enzyme. The disulfide variant, expressed in subtilisin-negative *B. subtilis*, had a melting temperature of 3.1°C higher than that of the wild type

protein and 5.8°C higher than that of the reduced form (-SH HS-) of the variant. Furthermore, under a variety of kinetic conditions, the disulfide variant underwent thermal inactivation at half the rate of that of the wild-type enzyme.

Conversely, a related approach, which employs random mutagenesis of the cloned gene, can unpredictably create new and interesting enzymes that may have novel properties, including increased or decreased thermostability. The key element to this approach is the ability to screen large numbers of variants for increased thermostability. The same researchers at Genex Corp. [69] used chemical mutagenesis to introduce random mutations into the cloned subtilisin gene, and transformed the recombinant plasmids into subtilisin-negative *B. subtilis*. A chromogenic substrate activity stain of a nitrocellulose bacterial colony lift was employed to isolated ten enzyme variants with increased resistance to thermal inactivation. All the variants were the result of a single amino acid substitution due to a point mutation. One variant enzyme had more than a four-fold thermal resistance at 65°C. A single amino acid substitution of serine for asparagine at position 218 was present in the variant protease. The mutation, which occurred at one end of a ß-hairpin structure, caused shortening of hydrogen bonds across the chains of the hairpin [106]. A triple combination mutant was then constructed from this Asn-218 to Ser variant by using oligonucleotide-directed cassette mutagenesis. The addition of Gly-131 to Asp, and Thr-254 to Ala mutations increased the variant's $t_{1/2}$ of thermal inactivation at 65°C to 11.6-fold over that of the wild-type,

without alteration of catalytic properties. Therefore, minor independent alterations in amino acid sequence dramatically increased thermostability without radical changes in the tertiary protein structure.

The genes for the neutral protease from *B. stearothermophilus* and *B. caldolyticus* have been cloned and sequenced [98]. A comparison of the derived primary amino acid sequences showed both enzymes to differ at only three amino acid positions, 4, 59, and 66. Furthermore, the *B. caldolyticus* enzyme had a thermostability and temperature optimum of 7 to 8°C higher than that of the *B. stearothermophilus* enzyme. Using cassette mutagenesis, the substitutions Ala-4 to Thr, Thr-59 to Ala, and Thr-66 to Phe were introduced into the *B. stearothermophilus* enzyme. The mutation Thr-66 to Phe increased thermostability by 6.2°C, while the mutations Ala-4 to Thr and Thr-59 to Ala increased thermostability by 1.75 and 1.5°C, respectively. While the thermostability of the triple mutant theoretically should have been 9.45°C higher, it was only equivalent to that of the wild-type *B. caldolyticus* neutral protease. A three-dimensional model of the variant enzyme showed the substituted residues to be surface located. The results indicated that solvent-exposed residues may be important in conferring thermostability to neutral proteases, even though two hydrophilic residues were replaced with hydrophobic residues.

Oligonucleotide-directed mutagenesis of the α-amylase from *B. amyloliquefaciens* deleted the Arg-176 and Gly-177 residues, and substituted Gln

for Glu-178 and Ala for Lys-269 [109]. Results showed the variant enzyme to be as thermostable as the α-amylase from *B. licheniformis*, that is, more than 80% retention of activity after 30 min at 90°C. However, the variant enzyme demonstrated a kinetic temperature optimum of 65°C, which suggested reversible inactivation at temperatures above 65°C.

Oligonucleotide-directed mutagenesis was also employed to introduce single point mutations in the cloned lactate dehydrogenase (LDH) genes from *B. megaterium* and *B. stearothermophilus* [118]. The substitutions of Thr-29 or Ser-39 to Ala residues in the mesophilic LDH from *B. megaterium* increased the enzyme's thermostability by 15°C. When alanine was simultaneously introduced at both positions, a 20°C increase in thermostability was observed. The authors suggest that the more helix-forming alanine residues stabilize the α-B helix of LDH, and serve to exclude water molecules across the Q-axis between the subunit α-helices. Unfortunately, an increase in $K_m$ for pyruvate resulted, which led to a three-fold reduction in activity when compared to the wild type enzyme. The reverse double substitutions, Ala-29 to Thr and Ala-39 to Ser, in thermophilic LDH from *B. stearothermophilus*, did not alter the high thermostability. However, the LDH activity of this variant was increased two-fold. The results indicate the stability and activity of the *B. stearothermophilus* and *B. megaterium* LDH to be based on a highly cooperative system of noncovalent bonds which is influenced differentially by amino acid substitutions.

A third approach to genetic modification of enzymes involves isolation of enzyme variants without structural information on the wild type protein. A gene encoding an enzyme from a mesophile is cloned, introduced into a thermophilic host, and enzyme activity is selected at the higher growth temperature of the host. The cloned gene for mesophilic kanamycin nucleotidyltransferase (KNTase) was introduced into *B. stearothermophilus*, and kanamycin resistant transformants were selected at 63°C [37]. All the purified KNTase variants were more thermostable than the wild type enzyme, and all had the same single amino acid replacement of Asp-80 to Tyr. Variants even more thermostable were obtained from the first variant by selecting for *B. stearothermophilus* kanamycin resistance at 70°C. All these Tyr-80 variants carried the same additional substitution of Thr-130 to Lys. The authors suggest that the alterations at positions 80 and 130 act independently and additively to thermostabilize KNTase. Furthermore, all the KNTase variants had specific activities equivalent to that of the wild type enzyme. The advantage to this technique is that, by selecting for enzyme activity, thermostable variants are generated in which enhancement of stability was not made at the expense of catalytic efficiency. This biological selection strategy accounts for all variables of activity and structural stability simultaneously. As thermophilic host-vector cloning systems are further developed, the strategy can be readily extended to other mesophilic genes of greater industrial value.

Another approach to achieving enzyme thermostability involves conversion of the entire cell of a mesophile into that of a thermophile [39]. Although less well studied, potentially any mesophile, producing a thermolabile enzyme(s) of commercial interest, could be converted to a thermophile by transforming it with DNA from a related thermophilic species. This method does not rely upon structural knowledge of the mesophilic enzyme, nor does it rely upon one's ability to predict appropriate amino acid substitutions. The high temperature transformant carries out all the necessary changes to convert a thermolabile enzyme to a thermostable form. Another advantage is that it does not rely on gene cloning, mutagenesis, transformation, and screening for expression of the variant cloned gene. In theory, the transfer of a small number of genes to the mesophile would exert wide pleiomorphic effects in which translationally modified proteins are produced with increased thermostability.

## Microbial Amylases in Starch Bioprocessing

Amylases are starch degrading enzymes that have several industrial applications in the production of corn syrups that contain varied amounts of malto-oligosaccharides, maltose, and glucose. Although amylases are widely distributed in nature and are produced by a variety of microorganisms, most commercial amylases are produced from *Bacillus* species. The composition profile of the corn syrup produced from starch hydrolysis depends upon the nature of the amylase, and

the reaction conditions employed. The three basic types of amylolytic enzymes used in starch conversion are (a) endo-amylase (α-amylase) (b) exo-amylase (ß-amylase and glucoamylase) and (c) debranching enzyme (pullulanase and isoamylase). α-Amylases hydrolyze the internal α-1,4-glucosidic bonds in amylose and amylopectin to produce short-chained maltodextrins, but the α-1,6-glucosidic branches in amylopectin are not attacked. Depending on the length of malto-oligosaccharides produced and the source of enzyme, α-amylases are further classified as saccharifying or liquefying. For example, the *B. subtilis* saccharifying enzyme produces large quantities of maltotriose, a slow decrease in starch viscosity, and a rapid increase in reducing power, while the *B. amyloliquefaciens* liquefying enzyme produces mainly maltohexaose, with a slower increase in reducing power but a rapid decrease in starch viscosity. Because of their extremely high thermostability (85°C-100°C), the α-amylases from *B. licheniformis* and *B. amyloliquefaciens* are the most commercially significant and most widely employed [9,20,21,72].

Glucoamylase and ß-amylase act in exo-fashion by consecutively cleaving glucose and maltose units, respectively, from the non-reducing ends of amylose, amylopectin, and maltodextrins. Although all commercial glucoamylases are of fungal origin and have low thermostability (40°C-60°C), they are used for the production of high glucose syrups subsequent to starch liquefaction. ß-amylase, which produces maltose in the ß-anomeric form, is found widely in higher plants. However, several bacteria, including *B. polymyxa*, *B. megaterium*, and *B. circulans*,

produce ß-amylases similar to those of plant origin. Although bacterial ß-amylases are also of low thermostability (40°C-60°C), their industrial application is in the production of high maltose syrups in excess of 80% maltose [9,20].

The industrially-employed *Klebsiella pneumoniae* or *Bacillus acidopullulyticus* pullulanase catalyses the hydrolysis of α-1,6-glucosidic linkages in amylopectin and pullulan to produce linear maltodextrins and maltotriose, respectively. Because it also has low thermostability (45°C-60°C), it generally is used in combination with glucoamylase or ß-amylase to improve the yields of syrups high in glucose or maltose, respectively [9,20,72]. Recently, novel highly-thermostable (>90°C) pullulanases have been isolated from thermophilic anaerobic bacteria. Although these enzymes cleave the α-1,4-glucosidic linkages of starch, they are classified as either isopullulanase or neopullulanase based on their hydrolysis of α-1,4 linkages of pullulan to produce isopanose or panose, respectively [72,81].

The industrial conversion of starch to glucose or maltose syrups currently suffers several drawbacks [20,21,72]. While liquefaction rapidly occurs within 2 hours at 95°C, pH 6.5, saccharification requires a drop in temperature to 55°C-60°C, and pH to 4.0-4.5 (glucoamylase) or 5.0-5.5 (ß-amylase). Furthermore, the saccharification step requires a 48-96 hour holding time in order to achieve maximal levels of glucose or maltose. Other problems include the formation of reversion products and the possibility of microbial growth at the lower saccharification temperature [20,72]. Consequently, the development of glucogenic,

maltogenic, and debranching enzymes with exceptionally high thermostabilities and more neutral pH optima would make industrial starch processing more cost-efficient. Ideally, the production of corn syrups would be a single step process in which liquefaction, debranching, and saccharification occur simultaneously. Furthermore, these enzymes must be produced from microorganisms that have GRAS status by the Food and Drug Administration. To this end, protein engineering techniques of starch degrading enzymes may eventually produce variant enzymes with the desired catalytic properties that will optimize any type of starch conversion process.

## Bacterial Cyclomaltodextrinases

Many amylolytic microorganisms capable of catalyzing the hydrolysis of starch by the production of α-amylases, also produce intracellular and extracellular maltodextrinases and α-glucosidases. These latter enzymes play an essential role in the microbial conversion of starch to glucose, by hydrolyzing maltodextrins and maltose produced from amylolytic hydrolysis of starch [4,20,91,96]. Several bacterial species also produce cyclomaltodextrinases (CDase) (EC 3.2.1.54), which rapidly cleave cyclodextrins and linear maltodextrins, but hydrolyze starch at significantly slower rates [30,31,53]. Cyclodextrins (CDs) are cyclic oligosaccharides composed of six (α-CD), seven (ß-CD), eight (γ-CD), or more α-linked glucose units. Because CDs lack terminal non-reducing glucose residues, they resist the hydrolytic action

of exo-amylases. They may competitively inhibit many ß-amylases and pullulanases. Due to their cyclic nature, they are very slowly cleaved, if at all, by endo-amylases [20,51,71,89]. Consequently, CDases appear to be a separate, special amylase class in which they often share common biochemical characteristics, substrate specificities, and end-product profiles.

The purified cyclodextrinase from *B. coagulans* had a molecular weight, as determined by SDS-PAGE, of 62.000, and an isoelectric point of 5.0 [31]. The enzyme optimal activity at pH 6.2 and 50°C, and was thermostable at 45°C, pH 7.0 for two hours. The enzyme hydrolyzed maltotetraose, maltopentaose, maltohexaose, and α-, ß-, and γ, CDs faster than maltotriose and short chain amylose, but did not cleave maltose. The enzyme recognized and cleaved the α-maltosyl group in the non-reducing end [30]. The hydrolysis products had the α-configuration and were mainly maltose. Starch, amylose, and amylopectin were hydrolyzed at rates 1% that for ß-CD.

The intracellular cyclodextrin-hydrolyzing enzyme from *B. sphaericus* was purified and estimated to be a homodimer having a native molecular weight of 144,000, and subunit molecular weight of 72,000 [53]. The enzyme had a pH optimum of 8.0, was stable at 25°C, pH 5.5-9.5 for 24 hours, and was inactivated at 50°C for 10 minutes. The enzyme most rapidly hydrolyzed ß-CD, followed by maltoheptaose, maltopentaose, α-CD, and maltohexaose. Starch, amylopectin, amylose, and pullulan were degraded at less than 4% the rate of ß-CD cleavage.

The purified cyclodextrinase from *B. macerans* had a pH and temperature optimum of 6.2-6.4 and 30-40°C, respectively [15,16]. The enzyme cleaved α-, ß-, and γ CDs, maltoheptaose, maltohexaose, maltoheptaose, maltotetraose, and maltotriose to mainly maltose. Amylose, amylopectin, glycogen, or starch were negligibly degraded. For CDs, the enzyme initially opened the ring to give a linear molecule with the corresponding number of glucose units. Linear malto-oligosaccharides were then degraded by removal of maltose units from the non-reducing end.

A pullulan hydrolase from *B. stearothermophilus* KP1064 rapidly cleaved α- and ß-CD, α-limit dextrins, amylose, and the maltotriose analogue, p-phenyl-α-D-maltoside [92]. Amylopectin, starch, and ß-limit dextrins were hydrolyzed significantly slower. Maltose was the main product from these substrates, while pullulan was slowly cleaved to mainly panose. The ability to split CD rings and cleave pullulan indicated that hydrolytic action could be of the endo type. The enzyme had a native molecular weight of 115,000 and consisted of two identical subunits. The enzyme had an isoelectric point of 4.4, pH optimum of 5.8, temperature optimum of 55°C, and was thermostable at 65°C for ten minutes. Sulfhydryl reagents (p-chloromercuribenzoate, 5,5'-dithio-bis(2-nitrobenzoate) strongly inhibited activity, which indicated that cysteine was required for catalysis.

The authors suggested assignment of the pullulan hydrolase to a unique type of maltogenic α-amylase.

The intracellular cyclomaltodextrinase was purified from an alkalophilic *Bacillus* species that was identified as closely relating *B. circulans* [110]. The enzyme had an isoelectric point of 4.2, a native molecular weight of 126,000, and consisted of two subunits of 67,000. The pH and temperature optima were 6.0 and 50°C, respectively, but heating at 60°C for 10 min led to inactivation. The enzyme rapidly hydrolyzed α-CD, maltotriose, and maltotetraose, while ß- and γ-CD, maltopentaose, maltohexaose, and maltoheptaose were cleaved 2-3 times slower. For all substrates, maltose was the main product. Maltose and starch were not degraded. Thiol reagents inhibited the enzyme, which suggested that sulfhydryl groups may exist in the active site.

The purified maltogenic α-amylase from *Bacillus thermoamyloliquefaciens* KP 1071 had a native and subunit molecular weight of 115,000 and 67,000, respectively [94]. The pI, pH optimum, and temperature optimum were 4.7, 6.2, and 63°C, respectively. Maltogenic α-amylase was thermostable for 10 minutes at 65°C, pH 6.8, in the presence of 5 mM EDTA. However, a decrease in thermostability resulted when EDTA was absent or calcium was present. The enzyme rapidly hydrolyzed α- and ß-CD, maltotriose, maltotetraose, maltopentaose, p-phenyl-α-maltoside, α-limit dextrin, and short chained amylose, while amylopectin, starch, and glycogen were slowly degraded. Maltose was the major product for all substrates,

except maltotriose. Amylopectin was degraded in exo-fashion by preferential cleavage of maltose units from the nonreducing ends, and hydrolysis of it's α-1,6-branch points. While the enzyme slowly cleaved pullulan at the α-1,4-bonds to give mainly panose, small amounts of glucose and maltose indicated the α-1,6-bonds were cleaved at a lower frequency. Although activity was completely inhibited by p-chloromercuribenzoate, the enzyme did not contain a cysteine or a cystine residue. Comparison of amino acid compositions indicated the maltogenic α-amylase to be homologous to the *B. stearothermophilus* KP 1064 maltogenic α-amylase [92]. The authors suggest the *B. thermoamyloliquefaciens* to be the first maltogenic exo-acting α-amylase able to cleave α-1,6-bonds in amylopectin.

The thermostable cyclodextrinase from *Clostridium thermohydrosulfuricum* 39E [73] is the first and, thus far, only CDase to be isolated from a thermophilic anaerobe. The enzyme had optimal activity at pH 6.0 and 65°C, and had a half-life of three hours at 65°C. α-CD was rapidly hydrolyzed, while ß-CD, starch, and amylose were degraded at rates 67%, 50%, and 53%, respectively, to that of α-CD. p-Chloromercuribenzoate inhibited activity, which suggested sulfhydryl groups are involved in activity.

An intracellular amylase from *Pseudomonas* MSI had a molecular weight of 96,000, and optimal activity at pH 5.5 and 50°C [29]. The enzyme rapidly hydrolyzed α-, ß-, and γ-CD, linear maltodextrins, and amylose, while amylopectin, ß-limit dextrin, and glycogen were hydrolyzed approximately 20 times slower. The

amylase had endo-type activity in which equimolar amounts of glucose and maltose were produced as final products for all substrates.

A CD-degrading, cell-bound glucoamylase was isolated from a *Flavobacterium* species [6]. α-, ß-, and γ-CD, maltotriose, and amylose were rapidly degraded, with the final product exclusively glucose. Amylopectin and glycogen were poor substrates. The enzyme had a pH optimum of 5.5-6.5, required calcium for activity, and was inactive at 55°C. The enzyme resembled human intestinal glucoamylase.

Although there is a limited amount of published research on microbial cyclomaltodextrinases, the characterization of the CDases from the various *Bacillus* species indicates several common features shared among the enzymes: (a) They are dimers that have identical subunits. (b) They rapidly cleave CDs and linear malto-oligosaccharides at varying rates, while starch polymers are degraded much slower. (c) They produce mainly maltose with small quantities of glucose as the final degradation products, by apparent exo-cleavage of maltose units from the nonreducing ends. (d) In most cases, thiol reagents inhibit activity. Due to the high relatedness among *Bacillus* species, it is very probable that other species of bacilli also produce their own forms of CDase. To this date, there are no reports of purifications and characterizations of a CDase from either *B. subtilis* or *B. caldolyticus*. Consequently, the information presented in the following chapters should be considered new and unique with respect to bacterial CDases.

# CHAPTER 3
# PURIFICATION, CHARACTERIZATION, AND COMPARISON OF THE CYCLOMALTODEXTRINASE FROM *B. SUBTILIS* 25S, *B. CALDOLYTICUS* C2, AND *B. SUBTILIS* HIGH TEMPERATURE GROWTH TRANSFORMANT H-17

Enzymology is currently experiencing unprecedented growth and expansion in the development of thermostable industrial enzymes, particularly amylolytic enzymes for commercial starch processing [100]. Comparisons between thermolabile and thermostable enzymes may help to elucidate the molecular basis of enzyme thermostability, thus facilitating future protein engineering. The ideal enzymes for comparison should be derived from the same genus, and have similar physical, chemical, and structural properties but differ only in thermostability.

*B. subtilis* high temperature growth (HTG) transformants were previously generated by transformation of mesophilic, amylolytic *B. subtilis* 25S with DNA from the obligate thermophile *B. caldolyticus* C2 [39]. This chapter describes the purification, characterization, and comparison of a p-nitrophenyl-α-D-maltoside-hydrolyzing cyclomaltodextrinase (CDase) from *B. subtilis* 25S, *B. caldolyticus* C2, and *B. subtilis* HTG transformant H-17.

## Materials and Methods

### Organisms and Growth Conditions

The methodology for generation of *B. subtilis* 25S, *B. caldolyticus* C2, and *B. subtilis* H-17 has been described previously [38,39]. All strains were grown aerobically, to late log phase, in 4 L batches (250 rpm, airflow 0.5 L/min) at 37°C (25S) and 60°C (C2, H-17) in a Queue Systems Mouse Fermenter (Parkersburg, WV). The growth medium consisted of 0.5% maltose (autoclaved separately), 0.5% starch, 2.5% Bacto peptone, 0.3% Bacto yeast extract, 0.2% Bacto meat extract, 0.3% dipotassium hydrogen phosphate, and 0.1% potassium dihydrogen phosphate, pH 7.2.

### Enzyme Purification

Four liters of 25S, C2, or H-17 cells were harvested by centrifugation at 9,000 x g in a Sorvall RC-5B GSA rotor, at 4°C for 20 min. The pellet was suspended in 20 mM, pH 8.0 potassium phosphate buffer, and lysozyme was added to a final concentration of 100 μg/ml. The suspension was stirred at 37°C for 3 h, after which the cell debris was removed by centrifugation at 27,000 x g in a Sorvall RC-5B SS-34 rotor, at 4°C for 15 min. Nucleic acids were precipitated by slow addition of a neutralized 10% (wt/vol) solution of streptomycin sulfate (1/10 volume of the supernatant) [74], and after 1 h of stirring at 4°C, the suspension was

centrifuged at 27,000 x g at 4°C for 15 min. Solid ammonium sulfate was added to the supernatant and the 40-80% fraction was retained, and redissolved in 20 mM, pH 7.0 potassium phosphate buffer containing 0.05% 2-mercaptoethanol (2-Me) (buffer A). Phenylmethylsulfonyl fluoride was added to a final concentration of 2mM, and the solution was dialyzed for 24 h against 4 x 2 L volumes of buffer A. Precipitated protein was removed from the dialysate by centrifugation at 27,000 x g at 4°C for 15 min. The dialysate was applied to a 2.5 x 35 cm DEAE-cellulose (Pharmacia, Uppsala, Sweden) column previously equilibrated with buffer A, after which the column was washed with 500 ml of buffer A at 30 ml/h (25°C) to remove unretained protein. The enzyme was then eluted at 15 ml/h with a 1 L linear 0-0.25 M, pH 7.0 NaCl gradient, in buffer A. Peak active fractions were pooled, concentrated (to approximately 10 ml) by ultrafiltration with a YM30 Amicon membrane, and applied to a 2.75 x 75 cm Sephadex G-75 (Pharmacia) column previously equilibrated with buffer A. The enzyme was eluted at a rate of 10 ml/h (25°C) with buffer A. Peak active fractions were pooled, concentrated (to approximately 6 ml) by ultrafiltration with a YM30 membrane, and then equilibrated with 5 mM, pH 7.0 phosphate buffer containing 0.05% (2-Me) (buffer B) by three ultra-filtrations with the same YM30 membrane. The retentate was applied to a 2.5 x 20 cm hydroxyapatite (HA-Ultrogel) column previously equilibrated with buffer B. The column was then washed with 200 ml of buffer B at 16 ml/h (25°C) to remove unretained protein. The enzyme eluted

immediately behind the void volume which eliminated the need for a phosphate gradient. Peak active fractions were pooled, concentrated (to approximately 6 ml) by ultrafiltration with a YM30 membrane, and applied to a 2.75 x 50 cm Sephacryl S-200 (Pharmacia) column previously equilibrated with buffer A. The enzyme was eluted at a rate of 12 ml/h (25°C) with buffer A, and peak active fractions were pooled and concentrated (to approximately 2 ml) by ultrafiltration with an Amicon Centriprep-30. The enzyme sample was sterile-filtered, and stored aseptically with 0.05% 2-Me or 0.01 mM EDTA.

Enzyme Assay

Cyclomaltodextrinase (CDase) activity was determined by the release of p-nitrophenol from p-nitrophenyl-α-D-maltoside (PNM) using a Beckman DU-7 spectrophotometer with an electrically-heated (pelltier) temperature control (0-99°C +/- 0.02°C). The standard reaction mixture (1.0 ml) contained 33.3 mM, potassium phosphate buffer, 1.98 μmol PNM, and 0-0.1 ml of enzyme preparation. 25S and H-17 CDase were assayed at pH 7.5 in the presence of 0.02% 2-Me, while C2 CDase was assayed at pH 7.0 in the presence of 0.01 mM EDTA. The reaction mixture was incubated at 35°C, 60°C, or 65°C for the 25S, C2, or H-17 enzyme, respectively. The complete mixture (0.9 ml) without substrate was allowed to thermally equilibrate for 1 min after which 0.1 ml of warmed substrate was added. The increase in absorbance/min at 400 nm was automatically calculated at 25 sec

intervals for approximately 5 min. A molar extinction coefficient of 9,600 $M^{-1}$ $cm^{-1}$ was used to calculate the amount of p-nitrophenol released/min [93]. One unit of enzyme activity was defined as the amount of enzyme required for the release of 1 μmol of p-nitrophenol/ ml/min. Protein was estimated by the method of Lowry et al. [45], with bovine serum albumin as the standard.

To perform activity stains on polyacrylamide and isoelectric focusing gels, the gel was soaked in 2 x 200 ml volumes of 100 mM, pH 7.5 potassium phosphate buffer for 10 min, and then incubated at 35°C or 60°C for 10-20 min in 33 mM, pH 7.5 potassium phosphate buffer containing 3 mg/ml PNM and 0.02% 2-Me. Bands that displayed enzymatic activity stained yellow, while the gel remained clear. The gel was then rinsed in deionized water and stained for protein with Coomassie Brilliant Blue R-250 and Amidoschwartz 10B.

Relative Molecular Mass ($M_r$)

The $M_r$ of the enzymes was determined by polyacrylamide gel electrophoresis (PAGE) with and without sodium dodecyl sulfate (SDS) and 2-Me, using Bio-Rad Mini-Protean II slab gels and the buffer system of Laemmli [35]. Spacing and separating gels were 4.0% and 7.5% polyacrylamide, respectively. Molecular weight standards (26,600-180,000) were obtained from Sigma Chemical Co., St. Louis, Mo.

Gels were stained with 0.15% Coomassie Brilliant Blue R-250 and 0.05% Amidoschwartz 10B in 40% ethanol/10% glacial acetic acid for 2 h.

Isoelectric Point (pI)

The pI values were determined according to manufacture's instructions, using Isogel Agarose IEF Plates (FMC BioProducts) over a pH range of 3-10. IEF standards (pI 3.55-9.9) were purchased from Sigma. Isoelectric focusing was performed with a Hoeffer Isobox cooling chamber at 12°C. After staining for activity (see Enzyme Assay), the gel was then rinsed in deionized water and stained for protein with Coomassie Brilliant Blue R-250 as described by the manufacturer.

Effect of Temperature and pH

Temperature profiles for the 25S, C2, and H-17 enzymes were determined under standard conditions over the range 10-52°C (25S) and 25-80°C (C2, H-17). The pH profiles were determined under standard assay conditions in 33 mM phosphate, over the pH range 4-10 for the 25S, C2, and H-17 enzymes. Initial velocities within the first 1.5 min were used to calculate relative activities (% of maximum).

Effect of Chemicals

To determine the effect of various cations, all solutions used were prepared from triple-deionized water. The 25S, C2, or H-17 enzyme was dialyzed against 2 x 0.5 L volumes of pH 7.5, 7.0, or 7.5, respectively, 50 mM sodium barbital/ 10 mM EDTA/0.05% 2-Me, for 8 h at 4°C. The enzymes were then dialyzed against 3 x 0.5 L volumes of the same buffer without EDTA, for 16 h at 4°C. Activity was determined under standard conditions, except that the assay buffer was 50 mM sodium barbital/0.02% 2-Me, and contained a final concentration of 5 mM cation (chloride form). The reaction mixture without substrate was pre-incubated with the selected cation for 10 min at 35°C (25S) or 45°C (C2, H-17). Residual activity was then determined at standard incubation temperatures. The effect of 2-Me, EDTA or Tris-HCl on 25S, C2, and H-17 activity was examined under standard assay conditions. The final concentration ranges were 0.005-0.5% (2-Me), 0.005-1.0 mM (EDTA) or 5mM (Tris HCl).

Thermal Stability

The effect of 2-Me (0.005-0.5%) or EDTA (0.005-1.0 mM) on thermostability was examined. Under standard assay conditions, the enzyme was incubated in closed cuvettes for 1 h, at a single temperature maintained by the spectrophotometer heating unit. Incubation temperatures ranged from 35-45°C (25S) and 60-75°C (C2, H-17). Assays were performed at 5-15 min intervals during

the incubation period, by the addition of PNM. Initial velocities were calculated and related to those at time zero.

$K_m$ Value Determination

The initial rates of hydrolysis of PNM were performed under standard assay conditions. The $K_m$ values were determined by plotting 1/V vs 1/S according to the Lineweaver-Burk method [42].

Amino Acid Analysis

Amino acid analyses were performed by the Protein Chemistry Core Facility in the Department of Biochemistry, University of Florida. Protein samples were prepared by hydrolysis in 6N HCl, or DMSO/HCl hydrolysis for cysteic acid determination. Amino acid analyses were performed in duplicate on a Beckman 6300 instrument, using a cationic exchange resin and ninhydrin-based quantification. Standards were run before and after each analysis, and internal standards were included for every analysis.

## Results and Discussion

The purifications of the 25S, C2, and H-17 CDase are summarized in Tables 1-3, respectively. Initial studies indicated that the 25S enzyme required a minimum of 0.02% 2-Me in order to maintain activity, while the C2 or H-17 enzyme required a minimum of 0.02% 2-Me or 0.01 mM EDTA for thermostability. Therefore, 0.05% 2-Me was included in all column chromatography buffers.

All three enzymes migrated as single bands during SDS-PAGE (Figures 1-3). The $M_r$ of the 25S, C2, and H-17 CDase was approximately 55,000, 60,000, and 55,000 as determined by SDS-PAGE, and, 110,000, 120,000, and 110,000 as determined by PAGE, respectively. This indicates that the native structure of all three enzymes is a dimer composed of two subunits of equivalent $M_r$. Table 4 summarizes the biochemical and biophysical characterization of the 25S, C2, and H-17 CDase. All three enzymes had the same pI (Figure 4), and similar pH optima (Figure 5), $K_m$ values for PNM, and Tris-HCl inhibition. Neither enzyme had a cation requirement for activity or thermostability, while each enzyme exhibited similar cation inhibition (Table 5). However, the 25S, C2, and H-17 CDase exhibited strikingly different thermostabilities. The purified 25S enzyme showed optimal activity between 35-37°C (Figure 6), and complete inactivation after incubation at 45°C for 10 min, when assayed at pH 7.5 (Figure 7). Although 0.02%

Table 1. Purification of *B. subtilis* 25S cyclomaltodextrinase.

| Purification Step | Total Protein (mg) | Enzyme Activity: Total Activity (Units) | Enzyme Activity: Specific Activity (U/mg Prot.) | Enzyme Activity: Purification (Fold) | Enzyme Activity: Yield (%) |
|---|---|---|---|---|---|
| Cell-free extract | 650 | 42.0 | 0.065 | 1.0 | 100 |
| 40-80% $(NH_4)_2SO_4$ fraction | 217 | 28.6 | 0.12 | 1.8 | 64 |
| DEAE-cellulose chromatography | 28.5 | 9.8 | 0.34 | 5.2 | 23 |
| Sephadex G-75 chromatography | 15.4 | 6.7 | 0.44 | 6.8 | 16 |
| Hydroxyapatite chromatography | 2.5 | 6.3 | 2.52 | 38.8 | 15 |
| Sephacryl S-200 chromatography | 0.5 | 4.9 | 10.0 | 154 | 12 |

Table 2. Purification of *B. caldolyticus* C2 cyclomaltodextrinase.

| Purification Step | Total Protein (mg) | Enzyme Activity: Total Activity (Units) | Enzyme Activity: Specific Activity (U/mg Prot.) | Enzyme Activity: Purification (Fold) | Enzyme Activity: Yield (%) |
|---|---|---|---|---|---|
| Cell-free extract | 550 | 28.0 | 0.051 | 1.0 | 100 |
| 40-80% $(NH_4)_2SO_4$ fraction | 160 | 15.0 | 0.09 | 1.8 | 54 |
| DEAE-cellulose chromatography | 22.1 | 8.2 | 0.37 | 7.4 | 29 |
| Sephadex G-75 chromatography | 11.4 | 8.0 | 0.70 | 14.0 | 29 |
| Hydroxyapatite chromatography | 2.0 | 7.3 | 3.7 | 74.0 | 26 |
| Sephacryl S-200 chromatography | 0.9 | 5.3 | 6.0 | 120 | 19 |

Table 3. Purification of *B. subtilis* H-17 cyclomaltodextrinase.

| Purification Step | Total Protein (mg) | Enzyme Activity: Total Activity (Units) | Enzyme Activity: Specific Activity (U/mg Prot.) | Enzyme Activity: Purification (Fold) | Enzyme Activity: Yield (%) |
|---|---|---|---|---|---|
| Cell-free extract | 520 | 31.7 | 0.061 | 1.0 | 100 |
| 40-80% $(NH_4)_2SO_4$ fraction | 136 | 15.6 | 0.11 | 1.8 | 49 |
| DEAE-cellulose chromatography | 34.0 | 9.2 | 0.27 | 4.4 | 29 |
| Sephadex G-75 chromatography | 14.4 | 7.7 | 0.53 | 8.7 | 24 |
| Hydroxyapatite chromatography | 3.3 | 6.6 | 1.8 | 29.5 | 19 |
| Sephacryl S-200 chromatography | 0.9 | 5.7 | 6.3 | 103 | 18 |

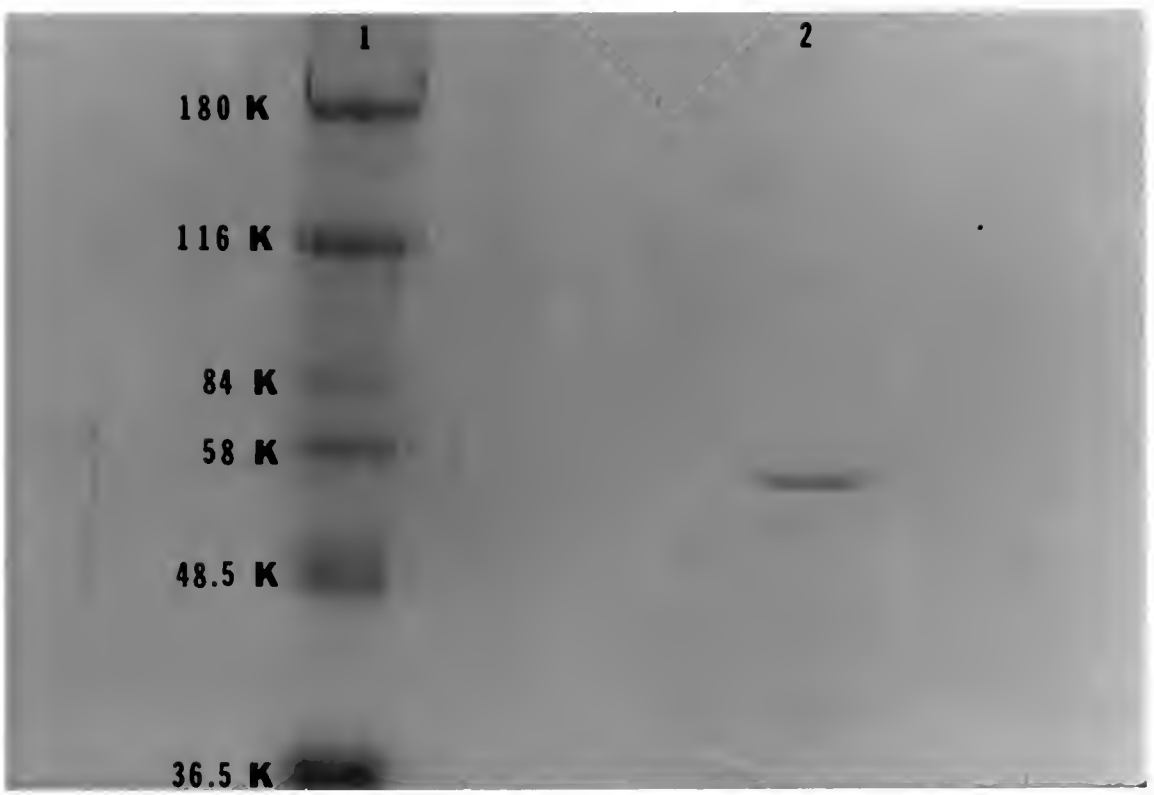



Figure 1. SDS-PAGE of purified *B. subtilis* 25S cyclomaltodextrinase on a 7.5% polyacrylamide gel. Lane 1: SDS molecular weight standards (1) $\alpha_2$-macroglobulin; (2) ß-galactosidase; (3) fructose-6-phosphate kinase; (4) pyruvate kinase; (5) fumarase; (6) lactate dehydrogenase. Lane 2: 10-20 $\mu$g of purified enzyme.

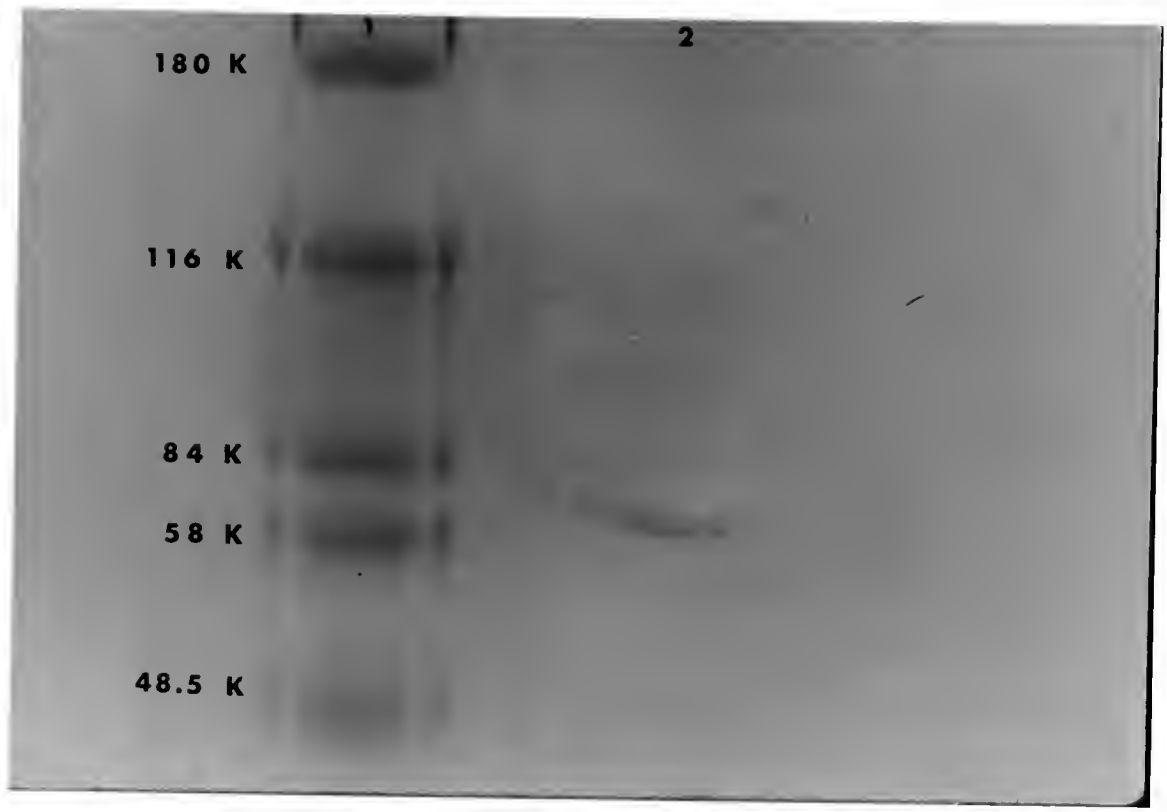



Figure 2. SDS-PAGE of purified *B. caldolyticus* C2 cyclomaltodextrinase on a 7.5% polyacrylamide gel. Lane 1: SDS molecular weight standards (1) $\alpha_2$-macroglobulin; (2) ß-galactosidase; (3) fructose-6-phosphate kinase; (4) pyruvate kinase; (5) fumarase. Lane 2: 10-20 $\mu$g of purified enzyme.

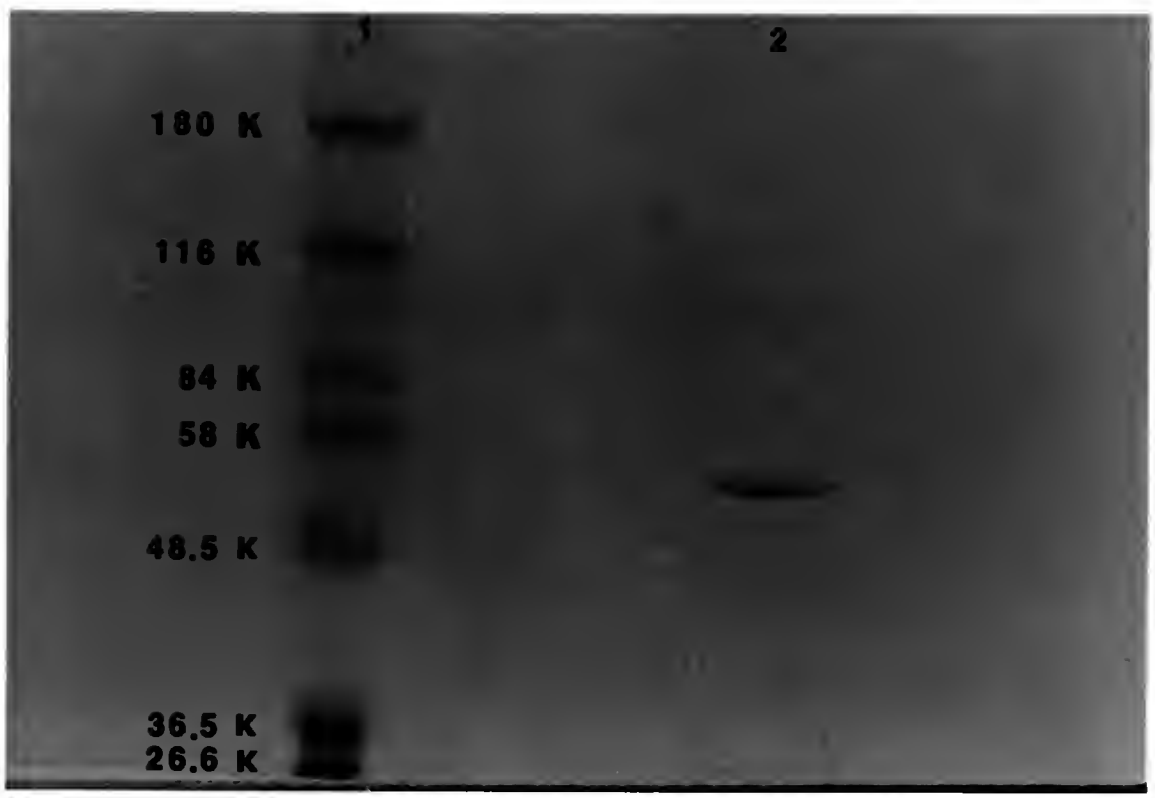



Figure 3. SDS-PAGE of purified *B. subtilis* H-17 cyclomaltodextrinase on a 7.5% polyacrylamide gel. Lane 1: SDS molecular weight standards (1) $\alpha_2$-macroglobulin; (2) ß-galactosidase; (3) fructose-6-phosphate kinase; (4) pyruvate kinase; (5) fumarase; (6) lactate dehydrogenase; (7) triosephosphate isomerase. Lane 2: 10-20 $\mu$g of purified enzyme.

Table 4. Biochemical and biophysical comparison of *B. subtilis* 25S, *B. caldolyticus* C2, and *B. subtilis* H-17 cyclomaltodextrinase.

| | H-17 | 25S | C2 |
|---|---|---|---|
| Temperature Optimum | 65-68°C | 35°C | 60°C |
| % Activity Remaining After 1 hr at 65°C | 100 | 0 | 66 |
| pH Optimum | 7.5 | 7.5 | 7.0 |
| Isoelectric Point | 4.8 | 4.8 | 4.8 |
| $K_m$ for PNM (mM) | 1.46 | 2.96 | 1.31 |
| Relative Molecular Mass ($M_r$) (SDS-PAGE) | 55,000 | 55,000 | 60,000 |
| 0.02% 2-Mercaptoethanol Requirement | + | + | + |
| 0.005 mM EDTA Requirement | + | - | + |
| Inhibition by 5 mM Tris | + | + | + |

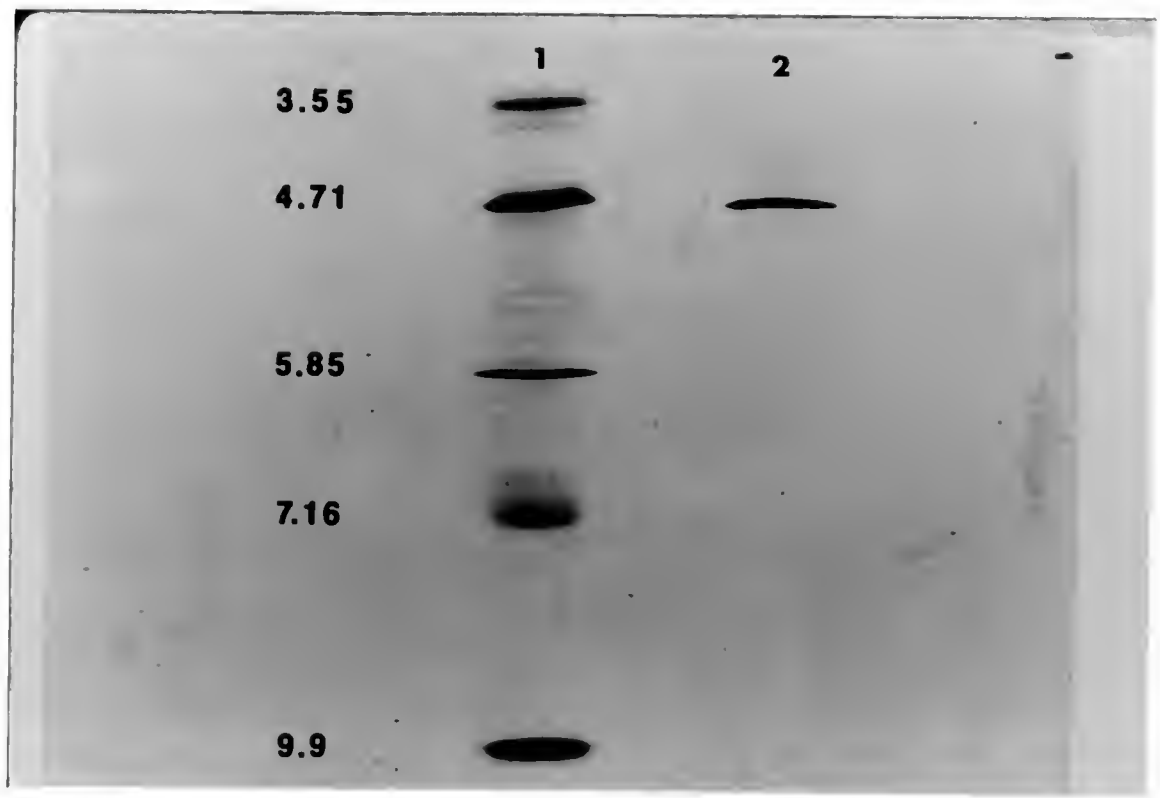



Figure 4. Isoelectric focusing gel of purified *B. subtilis* 25S, *B. caldolyticus* C2, or *B. subtilis* H-17 cyclomaltodextrinase. Lane 1: pI standards (1) amyloglucosidase; (2) ovalbumin; (3) carbonic anhydrase; (4) horse myoglobin (major and minor); (5) cytochrome C. Lane 2: 10-20 μg purified enzyme.

Figure 5. Effect of pH on the activity of (A) *B. subtilis* 25S cyclomaltodextrinase, (B) *B. caldolyticus* C2 cyclomaltodextrinase, and (C) *B. subtilis* H-17 cyclomaltodextrinase. The pH profiles were determined under standard assay conditions in 33 mM phosphate, over the pH range 4-10 for each enzyme. Initial velocities within the first 1.5 min were used to calculate relative activities (% of maximum).

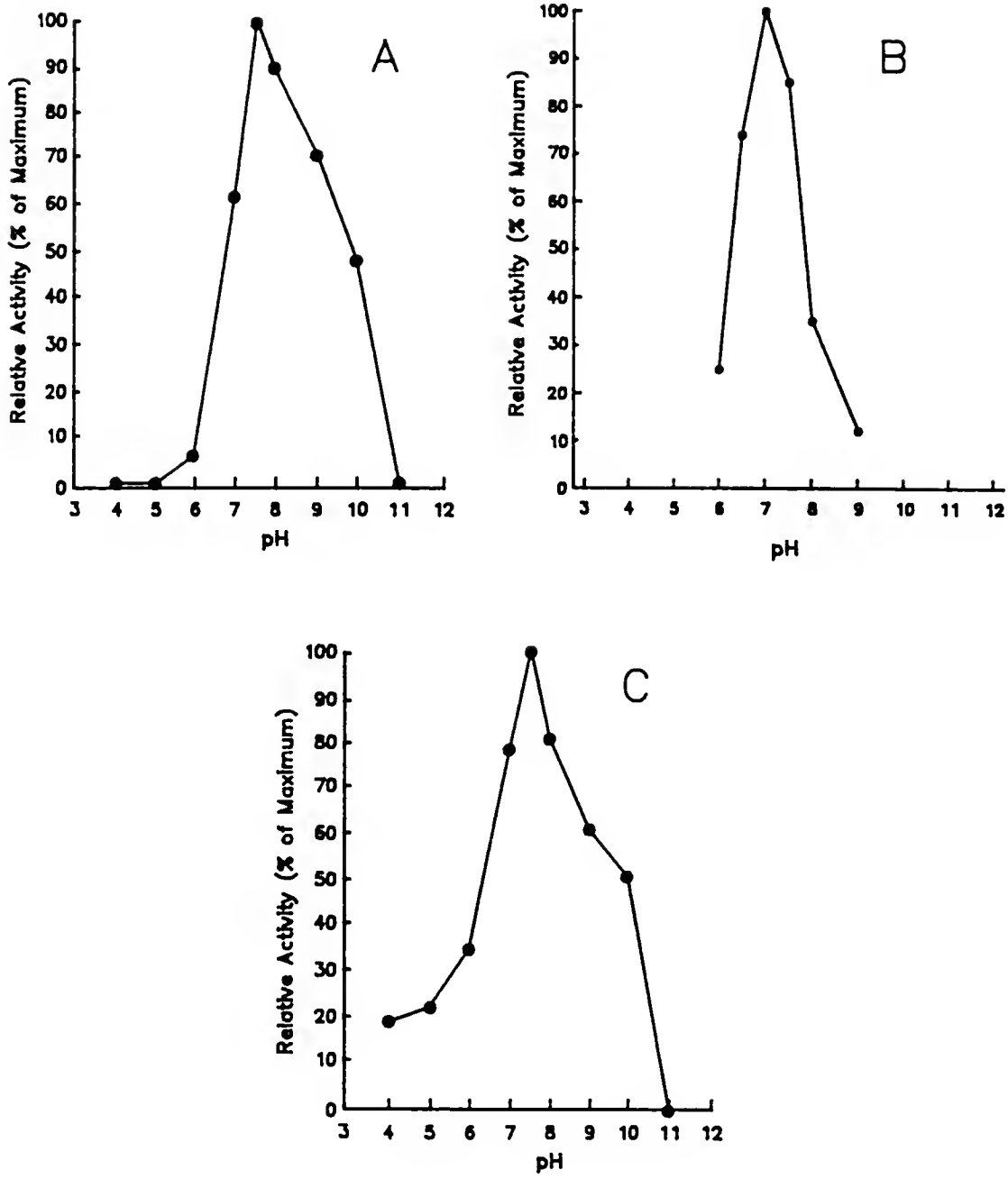

A
B
C
Relative Activity (% of Maximum)
pH
100
90
80
70
60
50
40
30
20
10
0
3
4
5
6
7
8
9
10
11
12

Table 5. Cationic inhibition of *B. subtilis* 25S, *B. caldolyticus* C2, and *B. subtilis* H-17 cyclomaltodextrinase.

| (5 mM final conc.) | % Inhibition 25S | % Inhibition H-17 | % Inhibition C2 |
|---|---|---|---|
| $MgCl_2$ | 69 | 42 | 41 |
| $CaCl_2$ | 90 | 72 | 66 |
| $SrCl_2$ | 78 | 38 | 47 |
| $BaCl_2$ | 87 | 50 | 40 |
| $MnCl_2$ | 100 | 100 | 100 |
| $CoCl_2$ | 100 | 100 | 100 |
| $ZnCl_2$ | 100 | 82 | 100 |
| LiCl | 14 | 18 | 23 |
| NaCl | 0 | 0 | 0 |
| KCl | 0 | 0 | 0 |
| CsCl | 0 | 0 | 0 |

Figure 6. Effect of temperature on the activity of (A) *B. subtilis* 25S cyclomaltodextrinase, (B) *B. caldolyticus* C2 cyclomaltodextrinase, and (C) *B. subtilis* H-17 cyclomaltodextrinase. Temperature profiles were determined under standard conditions over the range 10-52°C (25S) and 25-80°C (C2, H-17).

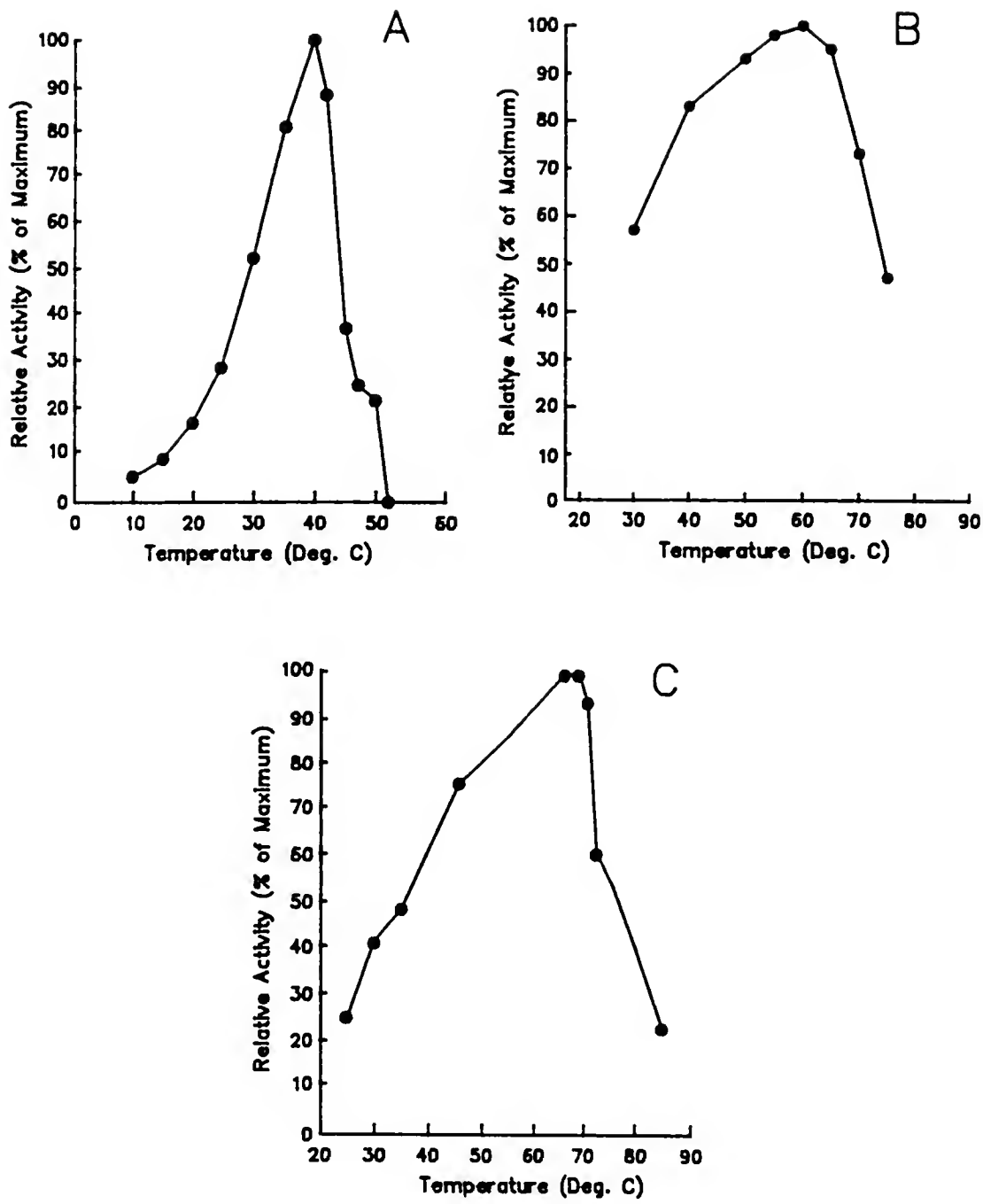
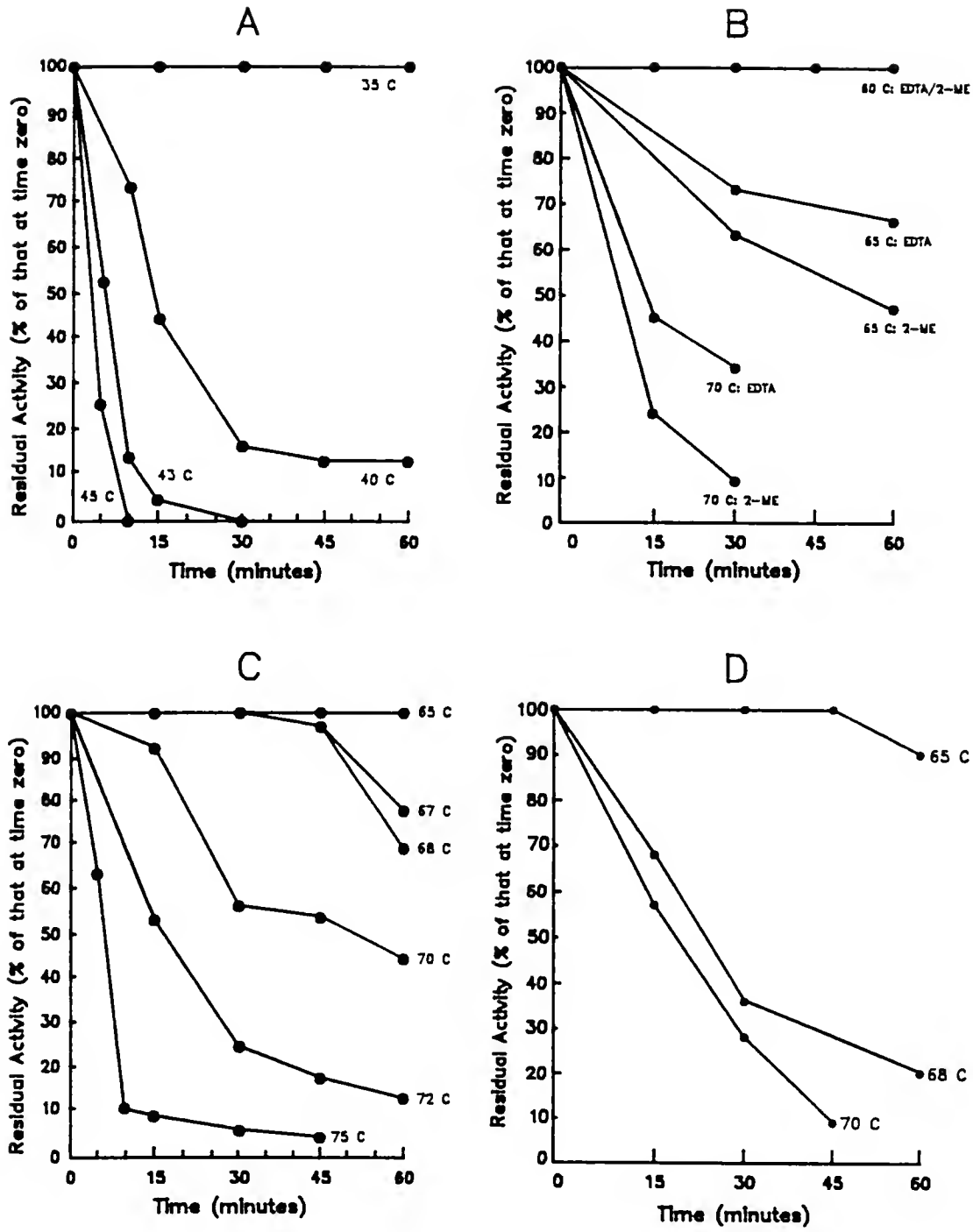

A
Relative Activity (% of Maximum)
Temperature (Deg. C)
B
Relative Activity (% of Maximum)
Temperature (Deg. C)
C
Relative Activity (% of Maximum)
Temperature (Deg. C)

Figure 7. (A) Effect of temperature and incubation time on the thermostability of *B. subtilis* 25S cyclomaltodextrinase. (B) Effect of temperature, incubation time, and 0.02% 2-Me or 0.01 mM EDTA on the thermostability of *B. caldolyticus* C2 cyclomaltodextrinase. (C) Effect of temperature, incubation time, and 0.02% 2-Me on the thermostability of the *B. subtilis* H-17 cyclomaltodextrinase. (D) Effect of temperature, incubation time, and 0.005 mM EDTA on the thermostability of *B. subtilis* H-17 cyclomaltodextrinase. Under standard assay conditions, the enzyme was incubated in closed cuvettes for 1 h, at a single temperature maintained by the spectrophotometer heating unit. Incubation temperatures ranged from 35-45°C (25S) and 60-75°C (C2, H-17). Assays were performed at 5-15 min intervals during the incubation period, by the addition of PNM. Initial velocities within the first 0.5 min were calculated and related to those at time zero.


A
Residual Activity (% of that at time zero)
Time (minutes)
35 C
40 C
43 C
45 C
B
Residual Activity (% of that at time zero)
Time (minutes)
60 C: EDTA/2-ME
65 C: EDTA
65 C: 2-ME
70 C: EDTA
70 C: 2-ME
C
Residual Activity (% of that at time zero)
Time (minutes)
65 C
67 C
68 C
70 C
72 C
75 C
D
Residual Activity (% of that at time zero)
Time (minutes)
65 C
68 C
70 C

2-Me was required to maintain 25S enzyme activity, neither 2-Me or EDTA, at all levels tested, enhanced the thermostability of 25S enzyme.

This contrasts with the purified C2 enzyme which showed optimal activity at 60°C (Figure 6), and retained 100% of initial activity after incubation at 60°C for 2 h (Figure 7), when assayed at pH 7.0. However, a minimum concentration of 0.02% 2-Me or 0.01 mM EDTA was required for thermostability of C2 CDase, although EDTA more effectively stabilized the C2 enzyme, than did 2-Me. Furthermore, the H-17 enzyme showed optimal activity between 65-68°C (Figure 6), and retained 100% of initial activity after incubation at 65°C for 1 h (Figure 7), when assayed at pH 7.5. A minimum concentration of 0.02% 2-Me or 0.005 mM EDTA was required for thermostability of the H-17 enzyme. In contrast with the C2 enzyme, 2-Me more effectively stabilized the H-17 enzyme, than did EDTA. For both the C2 and H-17 CDase, higher levels of either compound did not enhance thermostability, and combinations of 2-Me and EDTA did not produce a synergistic effect whereby thermostability was increased.

The maintenance of 25S enzyme activity by 2-Me probably occurs by reduction of an oxidized sulfhydryl residue(s) (cysteine) at the active site, or by reduction of a disulfide bond that forms at the active site due to oxidation. However, the mechanism by which 2-Me confers C2 and H-17 enzyme thermostability is unknown. The low level of 2-Me may be sufficient to reduce oxidized sulfhydryl groups to allow them to undergo disulfide bond formation.

Another, but less likely, mechanism could be 2-Me's intramolecular bifunctional hydrogen bonding with polar side groups. However, the use of an extrinsic factor such as 2-Me for stabilization may simply reflect the nature of the reducing atmosphere and presence of other sulfhydryl-containing molecules inside the cell which protect cysteine sulfhydryl groups. It is possible that EDTA confers H-17 enzyme thermostability by either forming an ion pair bridge between its negatively charged carboxyl groups and positively charged amino side groups, or by chelating cations that interfere with intramolecular ion-pair formation between charged amino acid side groups.

Table 6 shows the amino acid compositions of the 25S, C2, and H-17 CDase. The most significant difference between the 25S and C2 enzyme is the increase in the hydrophobic residues alanine, leucine, and proline. One of the most important mechanisms that confers enzyme thermostability is hydrophobic interaction [3,50,99]. Our results concur with a previous observation which reported a strong correlation between an increase in alanine, leucine and particularly proline, and the rise in thermostability of five *Bacillus* oligo-1,6-glucosidases [95]. Proline, due to its α-helix breaking ability, can contribute to improved turn stabilization and a greater potential for close packing of protein domains. Thus, by increasing the number of proline residues in conjunction with other hydrophobic residues such as leucine and alanine, thermostability may be enhanced by strengthened hydrophobic interactions, thereby tightening the molecule as a whole.

Table 6. Amino acid compositions of *B. subtilis* 25S, *B. caldolyticus* C2, and *B. subtilis* H-17 cyclomaltodextrinase.

| | 25S | | H-17 | | C2 | |
|---|---|---|---|---|---|---|
| Amino Acid | Mol% | Res/Subunit | Mol% | Res/Subunit | Mol% | Res/Subunit |
| Cys | 1.51 | 8 | 1.60 | 8 | 2.05 | 11 |
| Asx | 11.94 | 60 | 7.30 | 37 | 9.36 | 51 |
| Thr | 3.82 | 19 | 3.27 | 16 | 5.00 | 27 |
| Ser | 4.20 | 21 | 4.33 | 22 | 4.72 | 26 |
| Glx | 10.16 | 51 | 9.66 | 48 | 11.05 | 60 |
| Pro | 3.79 | 19 | 4.53 | 23 | 4.86 | 27 |
| Gly | 18.31 | 92 | 22.65 | 113 | 9.63 | 53 |
| Ala | 6.58 | 33 | 8.75 | 44 | 12.02 | 66 |
| Val | 5.32 | 27 | 5.96 | 30 | 5.68 | 31 |
| Met | 0.22 | 1 | 0.23 | 1 | 0.15 | 1 |
| Ile | 4.68 | 23 | 4.15 | 21 | 4.75 | 26 |
| Leu | 7.13 | 36 | 7.59 | 38 | 9.73 | 53 |
| Tyr | 3.34 | 17 | 2.19 | 11 | 3.28 | 18 |
| Phe | 5.63 | 28 | 3.41 | 17 | 4.11 | 22 |
| His | 3.12 | 16 | 2.55 | 13 | 2.72 | 15 |
| Lys | 5.02 | 25 | 6.58 | 33 | 5.55 | 30 |
| Arg | 5.23 | 26 | 5.36 | 27 | 5.34 | 29 |

Similarly, the H-17 enzyme shows a significant decrease in (aspartate/asparagine), tyrosine and phenylalanine residues, accompanied by significant increases in glycine, alanine, proline and lysine residues, when compared to the thermolabile 25S enzyme. The increase in alanine, glycine and proline may increase the H-17 enzyme's internal hydrophobicity, which in turn might influence the enzyme's thermostability. Interestingly, although there were changes in the amino acid composition between the two enzymes, there was no change in the pI value. For the H-17 enzyme, the increase in lysine residues may be offset by the decrease in aspartate/asparagine, especially if the decrease was in asparagine residues.

The results indicate that protein thermostability may be achieved via a unique approach in which the entire cell of a mesophile is converted into that of a thermophile. Previous studies have proposed that transformation to thermophily involves a limited number of genes which exert wide pleiomorphic effects [39]. These effects manifest themselves in the production of thermostable cellular components and products, possibly by specific mistranslation of messenger RNA [39,40]. Thus far, none of the enzymes purified from the *B. subtilis* 25S HTG transformants have been shown to be products of specific thermophilic genes transferred from donor to recipient during the transformation of 25S to thermophily [13]. Because the donor, *B. caldolyticus* C2, also produces a thermostable CDase, it was necessary to exclude the possibility that the thermostable H-17 enzyme was

actually encoded by a cointegrated gene from *B. caldolyticus* C2. This enzyme has a different $M_r$, pH optimum, temperature optimum, and effect of 2-Me or EDTA on thermostability, when compared to the H-17 enzyme. The C2 enzyme appears to achieve thermostability by a mechanism different than H-17. The H-17 enzyme may, in part, derive its greatest thermostability through disulfide bond formation, whereas C2 may, in part, derive its greatest thermostability by undergoing an altered conformation in the presence of EDTA. It is therefore believed that the H-17 and C2 enzymes are not products of the same gene, and that the *B. caldolyticus* C2 enzyme is not produced by the HTG transformant H-17.

In conclusion, amino acid sequence analyses of several mesophilic enzymes and their thermophilic counterparts have suggested that a few specific amino acid substitutions at critical regions may account for very subtle structural and conformational differences, that lead to large alterations in thermostability [32,50,99,115]. Cloning and nucleic acid sequencing of the 25S, C2, and H-17 CDase genes will allow determination of the primary amino acid sequences. This will aid in elucidating the structural mechanisms for increased thermostability in these enzymes, which is a fundamental requirement for future protein engineering.

# CHAPTER 4
# SUBSTRATE SPECIFITIES, AFFINITIES, AND CLEAVAGE PATTERNS OF THE CYCLOMALTODEXTRINASE FROM *B. SUBTILIS* 25S, *B. CALDOLYTICUS* C2, AND THE *B. SUBTILIS* HIGH TEMPERATURE GROWTH TRANSFORMANT H-17

Cyclomaltodextrinase (EC 3.2.1.54) (CDase) hydrolyses cyclodextrins (CDs) and linear maltodextrins much more rapidly than starch and other related polymers [30,31,53]. This chapter describes how the 25S, C2, and H-17 enzymes described in Chapter 1, were further characterized by their classification as CDases. Hydrolytic activity was also determined to indicate whether the H-17 CDase has commercial application as a saccharifying and/or debranching enzyme.

## Materials and Methods

### Organism and Growth Conditions

*B. subtilis* 25S, *B. caldolyticus* C2, and *B. subtilis* H-17 were grown aerobically to late log phase as described previously.

Enzyme Purification and Standard Assay

The 25S, C2 and H-17 enzymes were purified as described previously. To eliminate any possible low levels of contaminating enzyme activity during substrate hydrolysis assays, the 25S, C2, and H-17 enzymes were further purified by preparative native PAGE. Two Bio-Rad Mini-Protean II gels were stained for activity, as described previously, and the active band was precisely cut from each gel with a razor blade. The polyacrylamide strips were pulverized in 2-3 ml of standard assay buffer in a hand-held, glass tissue homogenizer. The polyacrylamide suspension was centrifuged at 6,000 x g for 5 min at 25°C, and the supernatant removed. The polyacrylamide was washed with 2 ml of the same buffer and recentrifuged. The supernatants were combined, sterile-filtered, and concentrated and equilibrated in standard assay buffer by ultrafiltration with an Amicon Centricon-30. Enzyme activity was determined by following the release of p-nitrophenol from p-nitrophenyl-α-D-maltoside under previously described standard assay conditions.

Substrate Specificity

Enzyme activity was determined for p-nitrophenyl-α-D-glucopyranoside (PNG), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), maltoheptaose (G7), isomaltose (IG2), isomaltotriose (IG3), panose (Pan), α-cyclodextrin (α-CD), ß-cyclodextrin ß-(CD), pullulan, soluble potato

starch, potato amylose, and potato amylopectin. All malto-oligosaccharide and CD solutions were sterile-filtered and stored at 4°C. All substrates were purchased from Sigma, and all substrate solutions were screened for purity using thin-layer chromatography.

Hydrolysis assays employed 10 mM malto-oligosaccharides, 10 mM CDs, or 2% polysaccharides. The reaction mixture (500 μl) contained 470 μl of substrate in 0.02% 2-mercaptoethanol, 33 mM phosphate buffer (pH 7.5), plus 30 μl (0.05-0.1 units) of purified enzyme. Samples were incubated at 37°C, 60°C, or 65°C for 25S, C2, or H-17, respectively. The reaction was terminated at various intervals (5-120 min) by boiling 100 μl aliquots of the mixture for 3 min. and then assayed for glucose and reducing power. Reducing sugars were determined by the dinitrosalicylic acid method [7], and glucose was determined with a peroxidase/ glucose oxidase colorimetric diagnostic kit (Sigma No. 510-A). Initial rates of hydrolysis were used to determine relative substrate specificities. Enzyme activity for PNG was determined under standard assay conditions.

$\underline{K_m \text{ and } V_{max} \text{ Determination}}$

Initial velocities were determined under the above assay conditions and were expressed as μmoles of reducing groups/min/mg protein, with glucose as the standard. Protein was determined by the method of Lowry et al. [45] with bovine serum albumin as the standard. $K_m$ and $V_{max}$ values were determined according

to the Lineweaver-Burk method [42]. Soluble potato starch and potato amylopectin concentrations ranged over 0.2-4.0%, while maltoheptaose and α-cyclodextrin ranged over 0.2-10 mM.

Thin-Layer Chromatography (TLC)

The products of malto-oligosaccharide, CD, and polysaccharide hydrolysis were analyzed by TLC using precoated Silica Gel G Redi/Plates (Fisher Scientific, Orlando). The plates were developed by a single ascent at 25°C, with the solvent system n-butanol-ethanol-water (3:4:1.25, vol/vol) in a pre-equilibrated 10 x 25 x 29 cm developing tank. Dried plates were stained for carbohydrate by spraying with a 6.5 mM solution of N-(1-naphthyl) ethylenediamine dihydrochloride in methanol containing 3% sulfuric acid [8], followed by heating at 110°C for 15 min. Reference standards were commercial malto-oligosaccharides obtained from Sigma.

## Results and Discussion

The 25S, C2, or H-17 CDase hydrolyzed p-nitrophenol-α-D-maltoside in a linear fashion, regardless of enzyme or substrate concentration. This suggested that maltose and p-nitrophenol were released from PNM. If glucose and PNG were initially released from PNM, then the assay would show a delayed color formation due to the eventual hydrolysis of PNG. Neither enzyme was active against p-nitrophenyl-α-D-glucopyranoside, maltose, isomaltose, isomaltotriose, or panose.

Table 7 shows the experimental initial relative rates of hydrolysis of linear malto-oligosaccharides, α- and ß-CD, and polysaccharides, as determined by reducing power and glucose liberation. The 25S, C2, and H-17 enzymes demonstrated similar substrate specificities as the greatest increase in reducing power was produced with maltohexaose, maltoheptaose, and α- and ß- CD, while starch, amylose, amylopectin, and pullulan were hydrolyzed significantly less (some data for C2 not shown). For either enzyme, hydrolysis of G3 produced the greatest quantity of glucose, while G7 hydrolysis produced the greatest increase in reducing power. It appeared that the enzyme preferred to sequentially cleave a maltose unit from the nonreducing end of the oligosaccharide, since less glucose was produced from the longer-chained substrates. However, there was, in part, random endo-activity since G4 and G6 hydrolysis also produced glucose, instead of only G2. Figure 8 shows the TLC analyses of the 10 min, 1 h, and 3 h products of linear malto-oligosaccharide hydrolysis by either the 25S, C2, or H-17 enzyme. The TLC results confirmed that the endo-hydrolysis by either enzyme was also, in part, nonspecific, since G4 was a low level intermediate produced from both G5 and G7 hydrolysis. Furthermore, in addition to G2 and G4, G3 was an intermediate produced from G6 hydrolysis. The subsequent hydrolysis of the G3 intermediate would account for the production of glucose from G6 cleavage. The enzymes also exhibited slight transglucosylase activity as seen with G4 hydrolysis.

Table 7. Experimental relative rates of hydrolysis of malto-oligosaccharides, cyclodextrins, and polysaccharides by *B. subtilis* 25S, *B. caldolyticus* C2, and *B. subtilis* H-17 cyclomaltodextrinase.

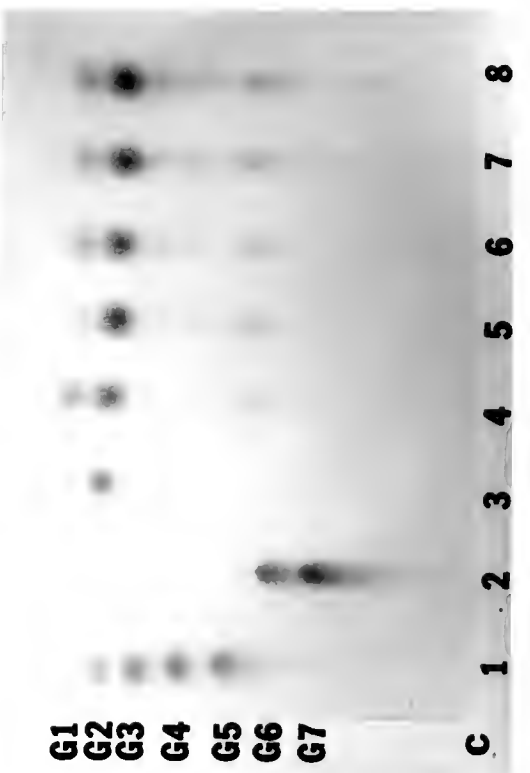
(expressed as % maximum)

| Substrate | Glucose Assay | | | Reducing Sugar Assay | | |
|---|---|---|---|---|---|---|
| | 25S | H-17 | C2 | 25S | H-17 | C2 |
| G2 | 0 | 0 | 0 | 0 | 0 | 0 |
| G3 | 100 | 100 | 100 | 20 | 16 | 24 |
| G4 | 11 | 36 | 41 | 35 | 43 | 45 |
| G5 | 45 | 52 | 71 | 67 | 63 | 85 |
| G6 | 29 | 39 | 64 | 81 | 68 | 98 |
| G7 | 32 | 45 | 65 | 100 | 100 | 100 |
| IG2, IG3, Panose | 0 | 0 | 0 | 0 | 0 | 0 |
| α-Cyclodextrin | | | | 85 | 90 | |
| ß-Cyclodextrin | | | | 95 | 70 | |
| Starch | | | | 10 | 11 | |
| Amylopectin | | | | 22 | 25 | |
| Amylose | | | | 12 | 10 | |
| Pullulan | | | | 7 | 7 | |

Figure 8. TLC of 10 min (A), 1 h (B), and 3 h (C) products of linear malto-oligosaccharide hydrolysis by *B. subtilis* 25S, *B. caldolyticus* C2, or *B. subtilis* H-17 cyclomaltodextrinase. Lane 1: Reference standards G1 (glucose), G2 (maltose), G3 (maltotriose), G4 (maltotetraose), and G5 (maltopentaose). Lane 2: Reference standards G6 (maltohexaose) and G7 (maltoheptaose). Lane 3: G2 + CDase. Lane 4: G3 + CDase. Lane 5: G4 + CDase. Lane 6: G5 + CDase. Lane 7: G6 + CDase. Lane 8: G7 + CDase.


G1
G2
G3
G4
G5
G6
G7
1
2
3
4
5
6
7
8
B


G1
G2
G3
G4
G5
G6
G7
1
2
3
4
5
6
7
8
A


G1
G2
G3
G4
G5
G6
G7
1
2
3
4
5
6
7
8
C

The 25S and H-17 $K_m$ and $V_{max}$ values for maltoheptaose, α-CD, starch, and amylopectin are presented in Table 8. Both enzymes showed greater affinities for maltoheptaose and α-CD than for starch and amylopectin. Consequently, the 25S and H-17 enzymes were classified as cyclomaltodextrinases, based on substrate specificity and affinity. It may be inferred that the C2 enzyme is also a cyclomaltodextrinase based upon the enzyme's similar characteristics. One may note that the H-17 CDase attacked α-CD faster than ß-CD, while the 25S CDase cleaved ß-CD faster than α-CD. Perhaps the increase in the H-17 enzyme's internal hydrophobicity over its 25S counterpart tightens the molecule as a whole through strengthened hydrophobic interaction. While this may enhance thermostability, the larger ring structure of ß-CD may become less accessible than α-CD to the active site of the H-17 enzyme.

Figure 9 shows the TLC analysis of the 20 min hydrolysis products of α- and ß-CD, and 1.5 h cleavage products of amylose, amylopectin, and starch by either the 25S or H-17 enzyme. TLC indicates that the enzymes initially open the ring of α- or ß-CD forming a linear molecule with the corresponding number of glucose units. Degradation of the linear molecule to shorter malto-oligosaccharides follows, with the accumulation of maltose as the final main product. However, it is unclear whether the hydrolysis of CDs by 25S or H-17 CDase proceeds via a multiple attack, or a multiple site attack model [20]. The enzymes appear to exhibit a preferred attack model [20] for starch, amylopectin, and amylose, in which maltose

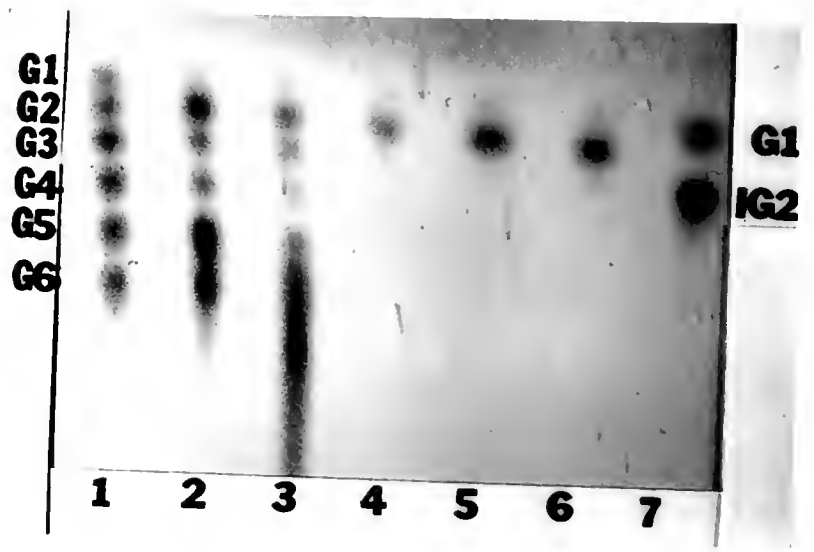
Table 8. $K_m$ and $V_{max}$ values of *B. subtilis* 25S and *B. subtilis* H-17 cyclomaltodextrinase.

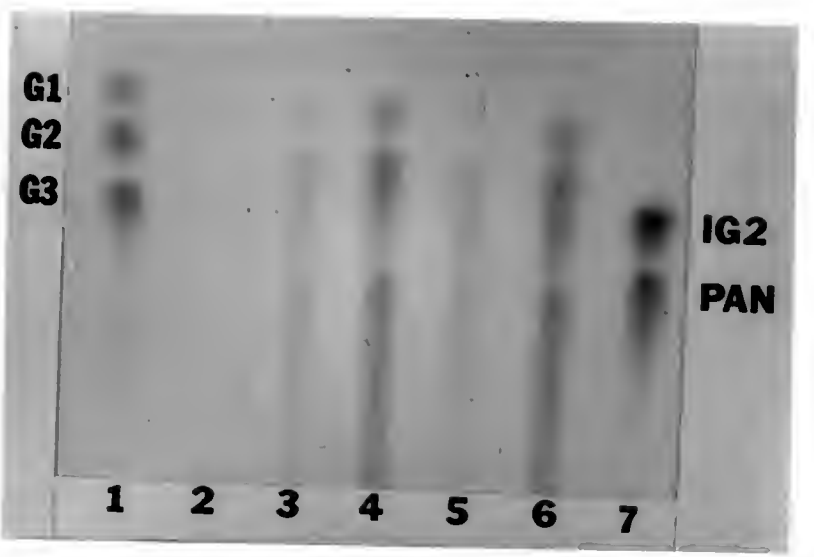
| | 25S | | H-17 | |
|---|---|---|---|---|
| Substrate | $K_m$ | $V_{max}$[a] | $K_m$ | $V_{max}$[a] |
| Maltoheptaose | 1.1 mg/ml (0.96 mM) | 42 | 1.0 mg/ml (0.91 mM) | 45 |
| α-Cyclodextrin | 1.1 mg/ml (1.10 mM) | 36 | 0.9 mg/ml (0.93 mM) | 33 |
| Starch | 20 mg/ml | 15 | 22 mg/ml | 16 |
| Amylopectin | 20 mg/ml | 20 | 20 mg/ml | 17 |

[a] μmoles of reducing groups/min/mg of protein

Figure 9. TLC of 20 min hydrolysis products of CDs and 1.5 h hydrolysis products of polysaccharides by *B. subtilis* 25S or *B. subtilis* H-17 cyclomaltodextrinase. Lane 1: reference standards G1 (glucose), G2 (maltose), G3 (maltotriose), G4 (maltotetraose), G5 (maltopentaose), G6 (maltohexaose). Lane 2: α-cyclodextrin + CDase. Lane 3: ß-cyclodextrin + CDase. Lane 4: amylose + CDase. Lane 5: amylopectin + CDase. Lane 6: starch + CDase. Lane 7: reference standards G1 and IG2 (isomaltose).

Figure 10. TLC of products of pullulan hydrolysis by *B. subtilis* 25S and *B. subtilis* H-17 cyclomaltodextrinase. Lane 1: Reference standards G1 (glucose), G2 (maltose), and G3 (maltotriose). Lane 2: Pullulan (no enzyme). Lanes 3 and 4: 30 min and 2 h hydrolysis of pullulan by 25S CDase, respectively. Lanes 5 and 6: 30 min and 2 h hydrolysis of pullulan by H-17 CDase, respectively. Lane 7: Reference standards IG2 (isomaltose) and PAN (panose).


G1
G2
G3
G4
G5
G6
G1
IG2
1
2
3
4
5
6
7


G1
G2
G3
IG2
PAN
1
2
3
4
5
6
7

is the main end product. TLC indicates these polysaccharides were degraded in exo-fashion by preferential cleavage of maltose units from non-reducing ends, although small amounts of glucose were detectable.

Both 25S and H-17 CDase's ability to cleave α-1,4 bonds of pullulan, together with their rapid attack of α- and ß-CD rings indicate that the enzymes do not necessarily require a non-reducing terminal end and that their action can be of the endo-type. Although the hydrolysis of pullulan was slow, it's 1,6-branch structure suggests that the presence of amylopectin's 1,6-branch points, may be a factor in both 25S and H-17 CDase's slightly faster cleavage of amylopectin over starch or amylose. Figure 10 shows TLC analysis of the 30 min and 2 h products of pullulan hydrolysis by 25S and H-17 CDase. It appears both enzymes produced glucose, maltose, and panose from pullulan. Identical cleavage patterns were observed for the C2 CDase. Traditional pullulanases hydrolyze only the α-1,6-glycosidic linkages of pullulan, releasing maltotriose. Consequently, the 25S, H-17, and C2 CDases demonstrated either isopullulanase (hydrolysis of α-1,4 linkages to produce isopanose), or neopullulanase (hydrolysis of α-1,4 linkages to produce panose) activity [72].

The C2 and H-17 thermostable CDase are very similar to the *B. thermoamyloliquefaciens* KP 1071 thermostable α-amylase II [94], and the *B. stearothermophilus* KP 1064 thermostable maltogenic α-amylase [92] in that they all, (a) behave as low active, exo-acting, maltogenic enzymes against starch, amylose,

and amylopectin, (b) demonstrate pullulan hydrolase activity with panose as a final product, and (c) exhibit a highly-active endo-type attack on CDs, in addition to other similar physiochemical characteristics. The 25S, H-17, and C2 CDase also resemble the *B. coagulans* cyclodextrinase [30,31], which hydrolyses CDs and linear malto-oligosaccharides much faster than starch polysaccharides, and the *B. stearothermophilus* TRS40 [33] neopullulanase which produces panose, maltose and glucose from pullulan, and maltose from starch. However, the H-17 CDase is different from the *B. subtilis* saccharifying α-amylase [20] in that the latter (a) removes maltotriosyl units from the non-reducing end of malto-oligosaccharides [55], (b) hydrolyses maltopentaose and maltohexaose at a very slow rate [61], (c) hydrolyses maltose [80], and (d) produces significant amounts of glucose from starch [20].

Current problems in the industrial production of glucose syrups include the large pH and temperature drop required for the transition from starch liquefaction to maltodextrin saccharification, and the unacceptably long holding times (48-96 h) required for saccharification by fungal glucoamylase [20,24,72]. Unfortunately the H-17 CDase does not appear to fulfill the need for a neutrophilic, highly thermostable glucoamylase. However, the enzyme does have potential applications in beer brewing and in the production of high maltose (high conversion) syrups following α-amylase liquefaction, although the latter would require the removal of calcium ions subsequent to liquefaction. High maltose syrups in food system

applications have several desirable properties such as resistance to color formation and crystallization, low hygroscopicity, mild sweetness, no aftertaste, low viscosity, good heat stability, and easy fermentability. Applications of high maltose syrups include confectioneries, jellies, jams, dessert formulations, baking and brewing, and intravenous feeding (no glucose shock) [20,24]. However, major factors which would limit the commercial application of the H-17 CDase include the low amounts of CDase produced by *B. subtilis*, and sufficient purification of the CDase from the multitude of other *B. subtilis* intracellular and cell wall-bound enzymes.

# CHAPTER 5
# CLONING OF THE CYCLOMALTODEXTRINASE GENE FROM *B. SUBTILIS* HIGH TEMPERATURE GROWTH TRANSFORMANT H-17

The CDase from *B. subtilis* H-17 is a novel type of α-1,4 glucan hydrolase. Unfortunately, the limitations of low yields and tedious purification may hinder the commercial application of the H-17 CDase. However, the enzyme's potential could be improved by cloning the CDase gene and studying its expression in order to develop genetic strategies to amplify production of the enzyme. Subsequent development of a host-vector system, which would direct extracellular secretion of the cloned CDase, could simplify enzyme purification. Additional investigations would (a) determine if the cloned H-17 CDase maintains its thermostability in a mesophilic host, and (b) may determine the origin of the H-17 CDase gene by its hybridization to genomic DNA from *B. subtilis* 25S or *B. caldolyticus* C2. No reports to date have genetically characterized a bacterial CDase gene by cloning and sequencing. This chapter describes the approaches taken to clone the H-17 CDase gene and the problems encountered in its expression in the hosts *E. coli* and *B. subtilis*.

## Materials and Methods

### Bacterial Strains and Growth Media

For preparation of genomic DNA, *B. subtilis* H-17 was grown aerobically to late log phase as previously described. *E. coli* DH5α and *B. subtilis* YB886 were maintained on Luria-Broth (LB) [46] agar. *E. coli* XL1-Blue carrying the *lacI*$^q$ gene on the F' episome was maintained on LB agar supplemented with 12.5 μg/ml of tetracycline. *E. coli* transformants carrying the pUC18 plasmid were grown on LB agar supplemented with 50 μg/ml of ampicillin (LA agar). LA broth was the same medium without agar. *B. subtilis* YB886 transformants carrying the pPL708 plasmid were grown on LB agar supplemented with 10 μg/ml of kanamycin (LK agar). LK broth was the same medium without agar. *E. coli* XL1-Blue transformants carrying the pUC18 plasmid were grown on LB supplemented with 12.5 μg/ml of tetracycline plus 50 μg/ml of ampicillin (LAT agar). All antibiotics were sterile-filtered and added to media cooled to 50°C.

### Agarose Gel Electrophoresis and Southern Blot Procedure

All restriction enzyme digests of chromosomal or plasmid DNA preparations were analyzed by agarose gel electrophoresis for 2.5 h at 55 V using a Bio-Rad Mini Sub Cell. All gels were 0.8% agarose in running buffer (TAE buffer), which was 0.04 M Tris-acetate, 0.001 M EDTA (pH 8.0), containing 1μg/ml of ethidium bromide. Preparative gels were in TAE buffer and consisted of 0.8% high purity

Seakem LE agarose (FMC BioProducts, Rockland, ME). The molecular weight standards for all gels were fragments from Hind III digested lambda phage DNA (Promega Corp., Madison, WI).

Southern Blots were carried out by the capillary transfer method as previously described [75], with the following modifications. The DNA was denatured by soaking the agarose gel, with gentle shaking, in several volumes of 1.5 M NaCl, 0.5 M NaOH for at least 1 h, or until the tracking dye disappeared. The gel was neutralized by soaking, with gentle shaking, in several volumes of 1 M Tris-HCl, 1.5 M NaCl (pH 8.0) for 1 h. Two or three Scotch-Brite scour pads, stacked approximately 0.5-0.75 inches, high were immersed to the top edge in 20 x SSC (3 M sodium chloride, 0.3 M trisodium citrate, pH 7.0). The neutralized gel was sandwiched on top of the scour pads and DNA was transferred overnight onto a Magnagraph nylon membrane (Micron Separations, Inc., Westboro, MA). After transfer, the nylon membrane was not washed, but immediately baked for 1 h at >80°C. The membrane was then used for DNA hybridization analysis.

Preparation and Digestion of Chromosomal DNA

Two liters of *B. subtilis* H-17 cells were centrifuged at 9,000 x g in a Sorvall RC-5B GSA rotor, for 15 min at 4°C. The pellet (approx. 2 g) was washed in 33 mM phosphate-buffered saline solution (pH 7.2) (PBS) and recentrifuged. In a sterile 50 ml centrifuge tube, the pellet was resuspended in 10 mM EDTA,

25 mM Tris-HCl, 10% glycerol (pH 8.0) to give a concentration of 100 mg wet wt cells/ml. Lysozyme was added to give a final concentration of 1 mg/ml and the suspension was incubated overnight at 37°C. DNase-free Proteinase K was added to give a final concentration of 200 μg/ml and the solution was incubated for 12 h at 50°C. A 10% solution of N-laurylsarcosine-sodium salt was added at a level of 100 μl/ml of reaction mixture, and the solution was incubated overnight at 50°C. Two volumes of 95% ethanol were added, and the precipitated DNA was spooled out, rinsed with 70% ethanol, and dried under vacuum in a sterile 50 ml screw cap centrifuge tube. The DNA was dissolved in 33 ml of 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) (TE buffer), and cesium chloride was added to a final concentration of 1 g/ml. The solution was centrifuged at 45,000 x g, for 22 h at 20°C, with a T865 rotor in a Sorval OTD-2 ultracentrifuge. The centrifuge tube was punctured at top and bottom with a 16 gauge needle, and drops of DNA were collected based upon a rapid increase in viscosity/stringiness. The DNA solution (approx. 3 ml) was dialyzed for 24 h against 4 x 1 liter volumes of TE buffer. DNA concentration was determined by its absorbance at 260 nm, and purity by the absorbance ratio of 260nm/280nm. The DNA solution was stored at -20°C.

The H-17 chromosomal DNA was partially digested with the restriction enzyme Sau 3A (Promega) as previously described [5], with the following modifications. Approximately 50 μg of DNA in 500 μl of 1 x reaction buffer was dispensed in 50 μl aliquots among eight microfuge tubes. To the remaining

100 μl in tube one, 2 μl of Sau 3A was added. Enzyme activity was sequentially diluted by removing 50 μl from tube one to tube two, and from tube two to tube three, etc. The tubes were incubated at 37°C for 20 min, and the reactions terminated by the addition of 5μl of 0.5 M EDTA and heating at 65°C for 10 min. H-17 DNA was completely digested with the restriction enzymes Pst I, Bam HI, Eco RI, Hind III, or Sal I (Promega) as previously described [75]. The extent of each digestion was analyzed by agarose gel electrophoresis.

Construction and Amplification of Gene Libraries

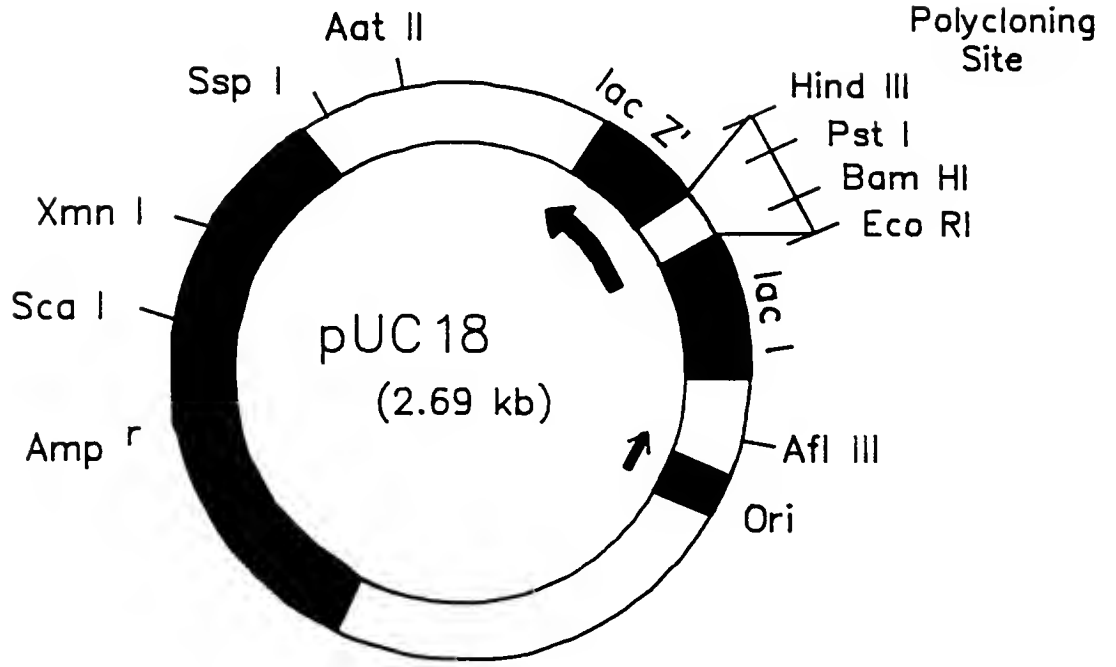
The Sau 3A digestion that gave maximal size distribution at the 4-9 kilobase (kb) level was electrophoresed on a preparative agarose gel. The portion of gel containing the lambda Hind III standards in the outside lane was cut from the gel with a razor blade, visualized under UV light, and the 4.4 kb, 6.6 kb, and 9.4 kb standards were marked. This strip of agarose was placed adjacent to the rest of the gel in order to cut out agarose sections that contained 4-6 kb fragments and 6-9 kb fragments. A similar procedure was employed to isolate fragments from either Eco RI, Bam HI, or Pst I complete digests from preparative agarose gels. The portion of gel containing the lambda Hind III standards in the outside lane, plus one lane-equivalent of the complete DNA digest was Southern blotted. The CDase gene was identified by Southern blot hybridization (see Construction and Detection of DNA Probe), and the nylon membrane was placed adjacent to the

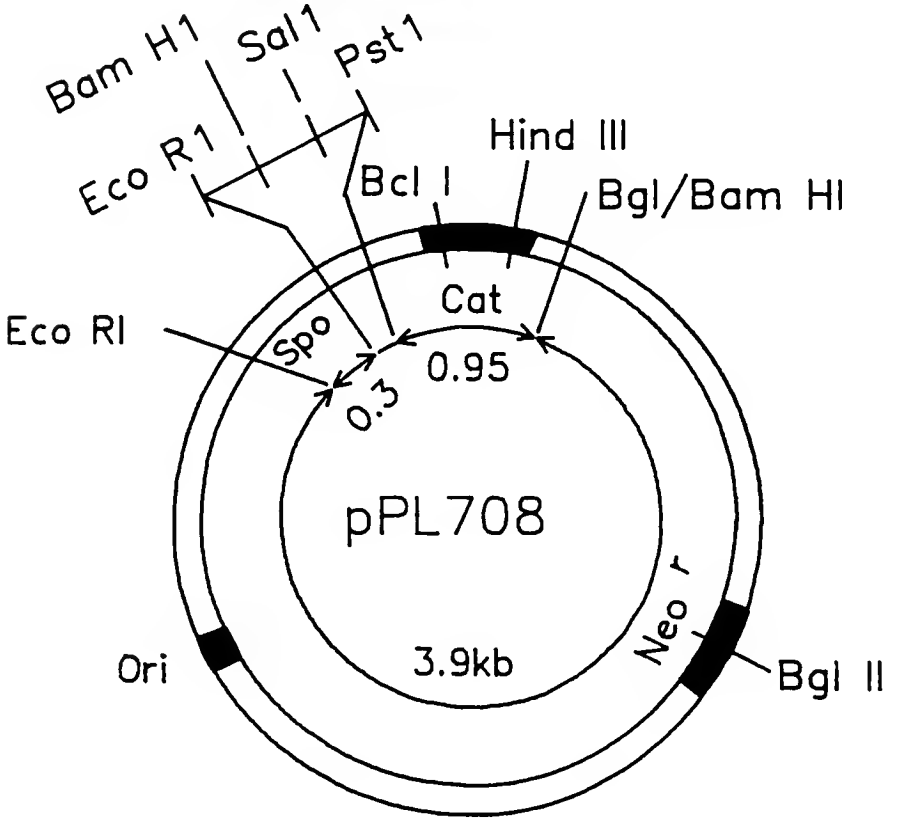
remaining preparative gel. The agarose section that contained fragments which hybridized to the DNA probe was precisely cut from the remainder of the gel. The narrow strips of agarose that contained 4-6 kb, 6-9 kb, or DNA probe-positive fragments were placed in microfilterfuge tubes with 0.45 μm nylon-66 membranes (Rainin Instrument Co., Woburn, MA). The gel segments were gently macerated in the microfilterfuge tubes with a sterile wooden applicator stick, and the tubes were frozen at -20°C. Upon thawing, the tubes were centrifuged at 12,000 x g for 3 h. Filtrates containing the same DNA fragments were pooled and concentrated to 100 μl by extraction with n-butanol [75]. The DNA solutions were extracted with an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1), and then with an equal volume of chloroform/ isoamyl alcohol (24:1) [75]. The DNA was precipitated with 2.5 volumes (250 μl) of 95% ethanol, and pelleted by centrifugation at 12,000 x g for 15 min. The pellet was washed with 70% ethanol, centrifuged at 12,000 x g for 5 min, and the pelleted DNA was dissolved in 50 μl of TE buffer [75]. The DNA fragment solutions were stored at -20°C until subsequent ligations.

The plasmid cloning vectors (Figure 11) for *E. coli* and *B. subtilis* were pUC18 (Boehringer Mannheim, Indianapolis, IN) and pPL708 (Dr. Paul Lovett, University of Maryland), respectively. Approximately 5 μg of pUC18 or pPL708 was linearized by either a Pst I, Bam HI, or Eco RI digest as previously described

Figure 11. Genetic map of *E. coli* plasmid cloning vector pUC18 (top) and *B. subtilis* plasmid cloning vector pPL708 (bottom).

# Cloning Vectors



[75]. Linearized pUC18 was dephosphorylated with calf intestinal alkaline phosphatase (Promega) according to manufacturer's recommendations. Linearized pPL708 was not dephosphorylated. Linearized, dephosphorylated pUC18, and linearized pPL708 were each phenol/chloroform extracted, precipitated with 2.5 volumes of 95% ethanol, and redissolved in 50 μl of TE buffer. For all ligations, pUC18 (0.5 μg) and insert fragments were mixed 2:1, while pPL708 (0.2 μg) and insert fragments were mixed 1:3. The 4-6 kb or 6-9 kb Sau 3A fragments were ligated into the Bam HI site of pUC18, by T4 DNA ligase (Promega), as previously described [75]. In individual ligations, the Pst I, Bam HI, or Eco RI probe-positive fragments were ligated into their respective restriction sites in either pUC18 or pPL708, by T4 DNA ligase. Each gene library was stored at -20°C until host transformation.

*E. coli* DH5α was made competent for transformation by the calcium chloride procedure [75]. Competent *E. coli* cells were transformed with separate pUC18 Sau 3A, Pst I, Bam HI, or Eco RI gene libraries as previously described [75], with the following modifications. After competent cells, mixed with plasmid DNA, were incubated on ice for 30 min, the cells were incubated in a 37°C water bath for 2 min followed by incubation at room temperature for 10 min. Luria broth was then added to the transformed cells and recovery was for 1 h at 37°C. After recovery, transformants were selected by plating the cells on LA agar and incubating at 37°C for 20 h. Colonies that were ampicillin resistant carried the

pUC18 vector. Each gene library was now amplified, with approximately 10,000 colonies/library. The colonies were scraped from the LA plates with Luria broth, pooled by centrifugation at 5,000 x g for 10 min, and plasmid DNA was isolated from the pelleted cells by the alkaline lysis method [75]. Each amplified plasmid library was stored at -20°C, during which aliquots were removed to screen for the CDase gene by retransformation of *E. coli*. The amplified Pst I, Bam HI, and Eco RI libraries were analyzed for the presence of the CDase gene, by digestion with the respective enzymes to liberate the inserts from pUC18, followed by Southern blot hybridization.

*B. subtilis* YB886 was made competent for transformation (Dr. M. Barbosa, Univerity of Florida, personal communication) by growing cells to late log phase in Spizizen's minimal salts medium supplemented with casamino acids, yeast extract, glucose, calcium chloride, and magnesium chloride [10]. Competent *B. subtilis* cells were transformed with separate pPL708 Pst I, Bam HI, and Eco RI libraries by incubating cells with DNA at 37°C for 40 min. Yeast extract and glucose (1% an 2% final conc., respectively) were added to the transformed cells and recovery was for 1.5 h, with shaking at 37°C. Transformants were selected by plating the cells on LK agar and incubating at 37°C for 20 h. Colonies that were kanamycin resistant carried the vector pPL708. The colonies were directly screened for the CDase gene without amplification of each gene library.

Screening Gene Libraries with Substrate Media

All the pUC18 libraries were screened for the H-17 CDase gene by plating *E. coli* DH5α transformants onto LA agar plates supplemented with various carbohydrate substrates. If no colonies initially demonstrated enzyme activity, colonies were made "leaky" by exposing plates to chloroform. Plates were uncovered, inverted, and placed over a wire mesh screen in a wide pan filled with 40 ml of chloroform. The pan was tightly covered with aluminum foil, and plates were removed approximately 15 min later, or until the plastic just started to become soft. The plates were then reincubated at 37°C in a humid environment for 24-48 h. The pPL708 libraries were not screened for H-17 CDase activity using substrate media because *B. subtilis* YB886 produces its own CDase.

Pullulan-Reactive Red (PRR)-LA and Maltrin-Reactive Red (MRR)-LA agarose plates were made as previously described [68], with the following modifications. Pullulan (Hayashibara, Okayama, Japan) or Maltrin M050 (Grain Processing Corp, Muscatine, IA), a maltodextrin solid with average degree of polymerization of 22, were covalently labelled with the dye Reactive Red 120 (Sigma). To remove excess salts and dye, the PRR was exhaustively dialyzed against distilled water, while MRR was precipitated with 95% ethanol (50% final conc.), centrifuged at 5,000 x g for 10 min, and redissolved in distilled water. The procedure was repeated several times until the supernatant was clear. The PRR or MRR solution was diluted to 1% to make Luria broth. Agar was substituted

with 1% agarose and the solution was autoclaved for 15 min before adding ampicillin. Hydrolysis of pullulan or maltrin would produce a clear zone around the colony while the surrounding media remained red.

Cyclodextrin-Phenol Red (CPR)-LA agar plates contained 1% α- or ß-CD (American Maize-Products Co., Hammond, IN) and 0.01% phenol red. Luria broth plus phenol red and 1.5% agar was adjusted to pH 7.7, autoclaved for 15 min, and sterile-filtered CD added. If CDase-producing *E. coli* transformants could degrade the CD to maltose and glucose, then the host would then ferment the available sugars to acid, which would cause phenol red to turn yellow around the colony. The system was developed using 1% glucose as a sole carbohydrate. CPR-LA broth was the same medium without agar. A change in broth color from red to yellow would also indicate acid production from utilization of CDs.

Starch-LA and p-nitrophenyl-α-D-maltoside (PNM)-LA agar plates contained 1% soluble starch and 1 mg/ml PNM, respectively. Starch-LA plates were inverted, a few crystals of iodine were placed on the cover, and the plate was closed to allow iodine vapors to diffuse into the agar. Hydrolysis of starch would produce a clear halo around the colony while the surrounding agar turns purple due to the starch-iodine complex. Hydrolysis of PNM would produce a yellow colony with a yellow halo due to the release of p-nitrophenol.

Construction and Detection of DNA Probe

Highly purified H-17 CDase from preparative native PAGE underwent Tris-Tricine-SDS PAGE with the previously described buffer system [78]. Protein was Western blotted onto a polyvinylidene difluoride (PVDF) membrane at 90 V, for 2.5 h at 4°C, with a Bio-Rad Trans-Blot Electrophoretic Transfer Cell. The transfer buffer was 10 mM MES, pH 6, 10% methanol. The PVDF membrane was stained for 5 min in 0.1% Coomassie Blue R-250 in 50% methanol, destained in 50% methanol, 10% acetic acid for 3-5 min, rinsed in distilled water 3 x 5 min, air dried, and stored at -20°C. The 55,000 $M_r$ band from four separate lanes was excised from the membrane and sequenced by Edman degradation on an Applied Biosystems Model 470A gas phase protein microsequencer, by the Protein Chemistry Core Facility, University of Florida. Amino acids were identified by reverse phase HPLC using a Novapak C-18 column. Based on the N-terminal amino acid sequence, a degenerate DNA oligonucleotide was synthesized on an Applied Biosystems 380B Synthesizer, by the DNA Synthesis Core Laboratory, University of Florida.

The oligonucleotide (6 $\mu$g) was nonradioactively end-labelled with digoxigenin-11-dUTP (Boehringer Mannheim) by terminal transferase (Boehringer Mannheim), as recommended by the manufacturer. The labelled probe was ethanol precipitated, dissolved in 100 $\mu$l of TE buffer containing 0.1% SDS, and stored at -20°C.

Southern blot hybridizations were performed as recommended by the Genius™ Nonradioactive DNA Detection Kit (Boehringer Mannheim). The oven-baked Southern blot was prehybridized in 100 ml of hybridization solution without probe at 68°C for 1 h. The membrane was sealed in a Dow Heavy Duty Ziploc Freezer Bag and submerged in a shaking water bath (50 rpm). Labelled probe was diluted in 10 ml of hybridization solution to give a final concentration of about 600 ng/ml. This hybridization solution was reused several times and was stored at -20°C between use. The prehybridized membrane plus 10 ml of hybridization solution with probe were heat-sealed without air in the same bag and submerged. Hybridization and washing conditions were nonstringent. Hybridization was at 60°C overnight with shaking (50 rpm), while the final wash was 0.5 x SSC. Hybridized probe DNA was detected using the Genius™ Nonradioactive DNA Detection Kit, according to manufacturer's instructions. Detection employed an anti-digoxigenin antibody conjugated to alkaline phosphatase. Membrane location of this antibody conjugate was visualized using the substrates 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium chloride.

Gene libraries were screened for the presence of the H-17 CDase gene by hybridization of the DNA probe to colony lifts of *E. coli* DH5α transformants. Colony lifts were performed as recommended by the Genius™ Nonradioactive DNA Detection Kit, with the following modifications. After Magnagraph nylon membranes were placed on LA agar plates with 1 mm colonies, the membranes

were lifted, inverted, placed colony side up on fresh LA plates, and incubated at 37°C for 4 h. The master plates were also incubated at 37°C until transformant colonies regrew (5-6 h). The membranes were then removed from the agar and laid, colony side up, on Whatman #3 filter paper saturated with 10% SDS for 5 min. The membranes were placed briefly on dry Whatman #3 filter paper and the process was repeated for #3 filter paper saturated with 0.5 M NaOH, 1.5 M NaCl, followed by #3 filter paper saturated with 1 M Tris-HCl, pH 8.0, 1.5 NaCl. The lysed colonies were thoroughly rinsed from the membranes using a squirt bottle containing 5 x SSC, and the membranes were baked at >80°C for 1 h. Hybridization and detection of hybridized probe DNA were essentially as that described for Southern blots, with the exception that hybridization was at 64°C in order to decrease colony background.

Construction and Detection of Antibody Probe

The purified H-17 CDase underwent preparative native PAGE, which yielded a 1 mg sample of enzyme in 0.5 ml 20 mM, pH 7.2, phosphate buffer, to be used as the antigen. Polyclonal antiserum to the H-17 CDase was prepared by Kelfarms, Alachua, FL, using a 4-5 Kg New Zealand white rabbit. Five days prior to antigen injection a volume of preimmune serum was collected from an ear vein as a negative control. One half mg of antigen (250 $\mu$l) was emulsified with 1 ml of Freunds Complete adjuvant, and a total of 5 injections (3 intradermal, 1

subcutaneous, 1 intramuscular) were given. After 30 days, these injections were repeated using 0.5 mg of antigen (250 μl) emulsified with 1 ml of Freunds Incomplete adjuvant. Test bleeds were taken from the ear vein at 7 day intervals after each injection series, and the titer followed. An adequate titer was reached after the second series of injections. At that time, the rabbit was anesthetized with a combination of Ketamine and Rompun, and bled out by cardiac puncture. The serum was separated by centrifugation, and stored in 25 ml volumes at -20°C.

Serum proteins were precipitated from the polyclonal antisera by the addition of caprylic acid. Two volumes (10 ml) of 60 mM sodium acetate buffer (pH 4.0) were added to 5 ml of serum in a centrifuge tube with stir bar. The pH was adjusted to 4.8, and 0.375 ml (0.75 ml per 10 ml original volume serum) of caprylic acid was added slowly (dropwise) with stirring. After continued stirring for 30 min at room temperature, the solution was centrifuged at 5,000 x g for 10 min. The supernatant was decanted, brought to 50% ammonium sulfate, and stirred overnight at 4°C. The precipitate was centrifuged at 3,000 x g for 30 min, dissolved in 2-3 ml of PBS, and dialyzed overnight against 4 x 1 L volumes of PBS.

To eliminate low levels of anti-bacterial antibodies, the dialysate was treated with an *E. coli* DH5α whole cell acetone powder, prepared as follows. One liter of late log phase *E. coli* cells was centrifuged at 5,000 x g for 15 min, and the pellet was resuspended 2 ml of PBS. The cells were chilled on ice, and 8 ml of acetone (-20°C) was added. The suspension was incubated at 0°C for 30 min with

occasional vigorous mixing, and then centrifuged at 10,000 x g for 10 min. The pellet was resuspended in fresh acetone (-20°C), mixed vigorously, allowed to sit at 0°C for 10 min, and recentrifuged at 10,000 x g for 10 min. The pellet was air-dried on filter paper, broken into a fine powder, and stored at -20°C. The acetone powder was added to the antiserum dialysate to a final concentration of 1%, followed by incubation at 4°C for 30 min. The suspension was centrifuged at 10,000 x g for 10 min, and the purified antiserum supernatant was stored at -20°C until immunological screening and Ochterlony gel diffusion.

Gene libraries were immunologically screened for presence of the H-17 CDase gene product by binding of the purified antiserum to colony lifts of *E. coli* DH5α, *E. coli* XL1-Blue, and *B. subtilis* YB886 transformants. Nitrocellulose membranes (Micron Separations, Inc., Westboro, MA) were placed on LA, LAT, or LK agar plates with 1 mm colonies for 5 min. The membranes were lifted, inverted, placed colony side up on fresh LA, LAT, or LK plates, and incubated at 37°C for 5 h. The master plates were also incubated at 37°C until transformant colonies regrew (5-6 h). The membranes were then lifted, exposed to chloroform vapors for 15 min as previously described, and the colonies lysed overnight in lysis buffer as previously described [75]. The membranes were washed, blocked, and hybridized with primary and secondary antibody as previously described [75], with the following modifications. (a) All washing, blocking, and hybridization volumes were approximately doubled per membrane. (b) The blocking buffer included 5%

nonfat dried milk. (c) The purified antiserum, which was the primary antibody, was diluted 1:500 in blocking buffer. (d) The antigen-bound antiserum was detected with an anti-rabbit IgG alkaline phosphatase conjugate (Sigma). This secondary antibody was diluted 1:1,000 in blocking buffer. The antigen-antibody-antibody-alkaline phosphatase complex was visualized using the substrates 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium chloride.

Plasmid Analysis of Probe-Positive *E. coli* DH5α and *B. subtilis* YB886 Transformants

All DNA probe-positive and immuno-positive *E. coli* and *B. subtilis* transformants were grown overnight in 5 ml of LA or LK broth, respectively, at 37°C, with shaking (100 rpm). Plasmid DNA from 1.5 ml of culture was prepared by the alkaline lysis mini-prep procedures as previously described [10,75]. *B. subtilis* YB886 carrying the DNA probe-positive Eco RI fragment in pPL708 was grown overnight in 100 ml of LK broth at 37°C, with shaking (250 rpm). A large quantity of plasmid DNA from 100 ml was prepared by the previously described alkaline lysis procedure [10], followed by cesium chloride density gradient centrifugation, as previously described [75]. Ethidium bromide was extracted from the density gradient-purified plasmid with isoamyl alcohol, as previously described [175]. All plasmid DNA preparations were digested with Pst I, Bam HI, or Eco RI restriction enzymes as previously described. Cloned inserts were analyzed for the presence of

the H-17 CDase gene by agarose gel electrophoresis and Southern blot hybridization as previously described.

Determination of CDase Activity in Probe-Positive *E. coli* DH5α and *B. subtilis* YB886 Transformants

All DNA probe-positive and immuno-positive *E coli* transformants were streaked onto PRR-LA, MRR-LA, CPR-LA, starch-LA, and PNM-LA agar plates and incubated at 37°C for 24-48 h. The plates were then exposed to chloroform vapors as described above. The same transformants were also inoculated into 5 ml of CPR-LA broth and incubated at 37°C for 48 h. To determine if H-17 CDase activity was intracellular in *E. coli*, the same transformants were each grown at 37°C overnight with shaking (100 rpm) in 50 ml of LA broth supplemented with 1% maltrin M050. Forty ml of a culture was centrifuged at 5,000 x g for 10 min, and the pellet was resuspended in 1.5 ml 20 mM phosphate buffer, pH 8.0, and frozen at -20°C. The thawed cells were treated with one-half ml of lysozyme (20 mg/ml) in the same buffer, and the suspension was incubated at 37°C for 30 min. The suspension was sonicated (Fisher Sonic Dismembrator) at 60% relative output, for 20 sec, with a 1 cm probe tip, and centrifuged at 12,000 x g for 15 min. The supernatant (cell-free extract) was assayed for CDase activity at 37°C as previously described.

Preparation of *B. subtilis* YB886 intracellular cell-free extracts was the same as that described under Ochterlony Gel Diffusion. These cell-free extracts were also used to determine specific activities at 37°C for CDase from *B. subtilis* YB886, and from the same host carrying the probe-positive Eco RI fragment cloned in pPL708. The substrates were α- and ß-CD as previously described using the dinitrosalicylic acid method. Protein was determined by the method of Lowry [45].

Ochterlony Gel Diffusion

Ochterlony gels were 0.9% purified agar in PBS. Antiserum, purified as above, was undiluted. *B. subtilis* 25S, *B. caldolyticus* C2, *B. subtilis* H-17, *B. subtilis* YB886, and *B. subtilis* YB886 carrying probe-positive Eco RI fragment in pPL708, were each grown to the same optical density in the previously described starch-maltose based broth. Kanamycin was included at 10 μg/ml for *B. subtilis* YB886 transformants. A colony from an overnight culture was inoculated into 50 ml and grown at optimal growth temperature (37 or 60°C) for 8-10 h, with shaking (250 rpm). Cells were centrifuged at 5,000 x g for 10 min, resuspended in 1.5 ml of 20 mM, pH 8.0, phosphate buffer, and frozen at -20°C. Thawed cells were treated with 0.5 ml of lysozyme (8 mg/ml) in the same buffer, incubated at 37°C for 30 min, and centrifuged at 12,000 x g for 15 min. All supernatants (cell-free extracts) were verified for significant quantities of CDase by performing enzyme assays as previously described. Each cell-free extract was stored at -20°C. For gel diffusions,

each cell-free extract was diluted 1:5 in PBS. Volumes of cell-free extracts and antiserum used in Ochterlony wells were 50 µl. The wells were chased with 50 µl of PBS 2-3 h after the initial sample application. The gels were covered, wrapped in parafilm, and incubated at 37°C for 24-48 h.

Expression of Clones in *lacI*$^q$ Host

The DNA probe-positive Eco RI insert was liberated from pPL708, by digestion of the cesium chloride density gradient-purified plasmid with Eco RI, and the fragment was purified from a preparative agarose gel as described above. The fragment was ligated into the dephosphorylated Eco RI site of pUC18 and the recombinant plasmid was transformed into *E. coli* XL1-Blue, as previously described. In this host, pUC18 ß-galactosidase (*lac* Z) gene expression is repressed by the *lacI*$^q$ gene product, which is encoded on the host's F' episome (plasmid). Therefore, expression of cloned genes that are under control of the pUC18 ß-galactosidase promoter is repressed in XL1-Blue. If isopropyl-thiogalactoside (IPTG) is present, it induces ß-galactosidase expression by preventing the *lacI*$^q$ protein from binding to the *lacZ* operator.

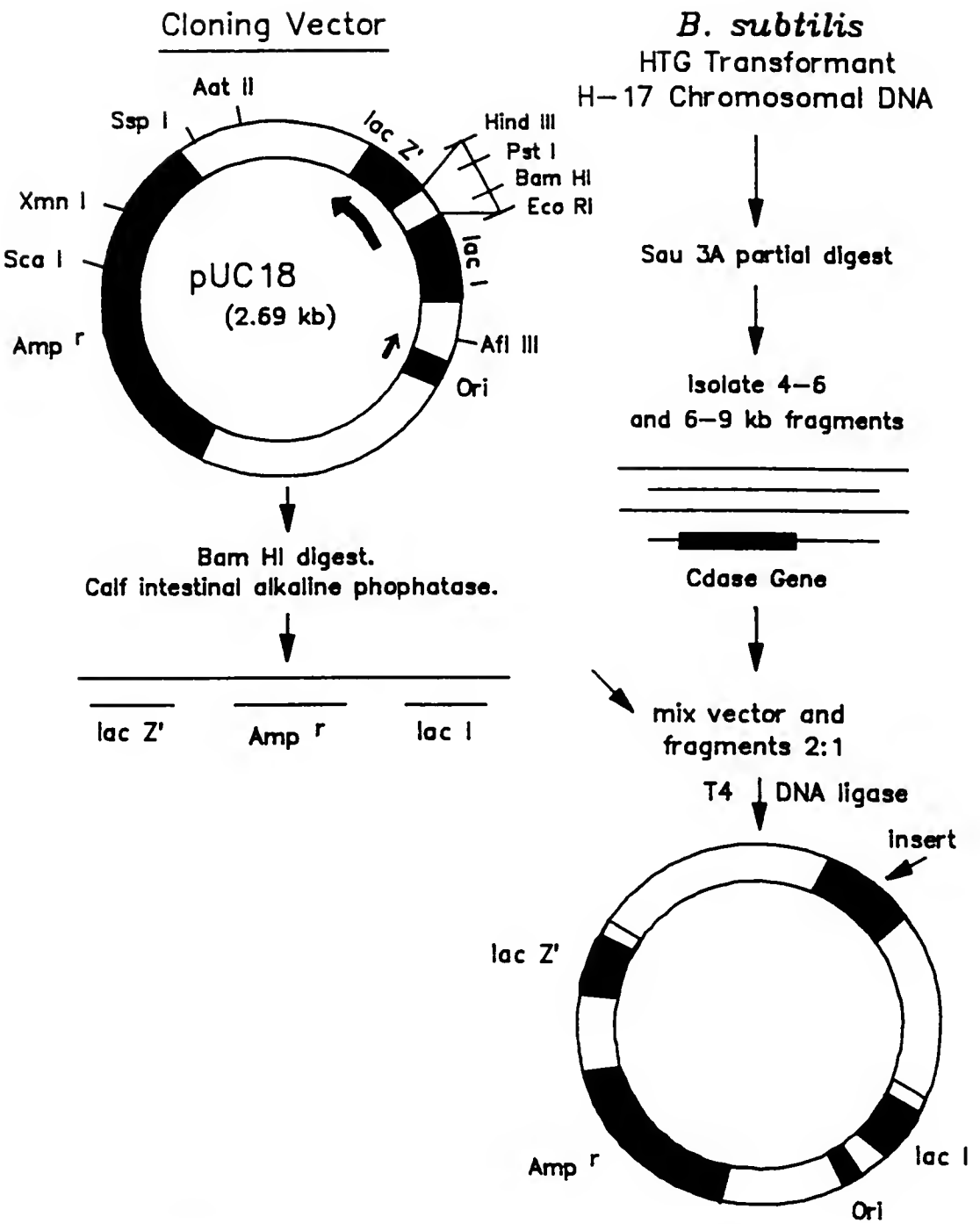
*E. coli* XL1-Blue transformants were plated onto LAT agar and the plates were incubated at 37°C overnight, until colonies were 0.5 mm in diameter. Colonies that were ampicillin and tetracycline resistant carried the pUC18 vector and the F' episome. The colonies were lifted onto nitrocellulose membranes as previously

described, and the membranes were incubated colony side up on LAT plates supplemented with 20 mM IPTG, at 37°C for 7 h. In order to determine if expression of the H-17 CDase gene is actually repressed in this host, some membranes were incubated on LAT plates without IPTG, under the same conditions. Immunological screening of nitrocellulose membranes, as previously described, was used to detect transformants carrying the Eco RI fragment.

## Results and Discussion

The first gene libraries constructed were from the 4-6 kb and 6-9 kb fragments isolated from a partial Sau 3A digest of H-17 DNA. Figure 12 illustrates the general strategy for cloning the Sau 3A fragments in pUC18. Two different 4-6 kb libraries were actually made. Each amplified library was repetitively screened on all the substrate agar media (30,000 colonies/screening). Several transformations of *E.coli* DH5α consistently produced several colonies (1/3,000) with enzyme activity on the starch-LA agar, PRR-LA agar, and the MRR-LA agar. It was determined that the starch-hydrolyzing clones (Figure 13) actually produced liquefying α-amylase that had no CDase activity. It was also determined that the pullulan-hydrolyzing clones (Figure 14) produced pullulanase that could rapidly cleave amylose but had no CDase activity. Although not shown, several clones on MRR-LA agar plates also produced liquefying α-amylase but no CDase. The starch-hydrolyzing clones secreted α-amylase extracellularly, but the pullulan-

Figure 12. General strategy for cloning Sau 3A fragments into the Bam HI site of plasmid vector pUC18.




Transform competent *E.coli* DH5-$\alpha$ cells with recombinant plasmids and plate onto LB-ampicillin agar.

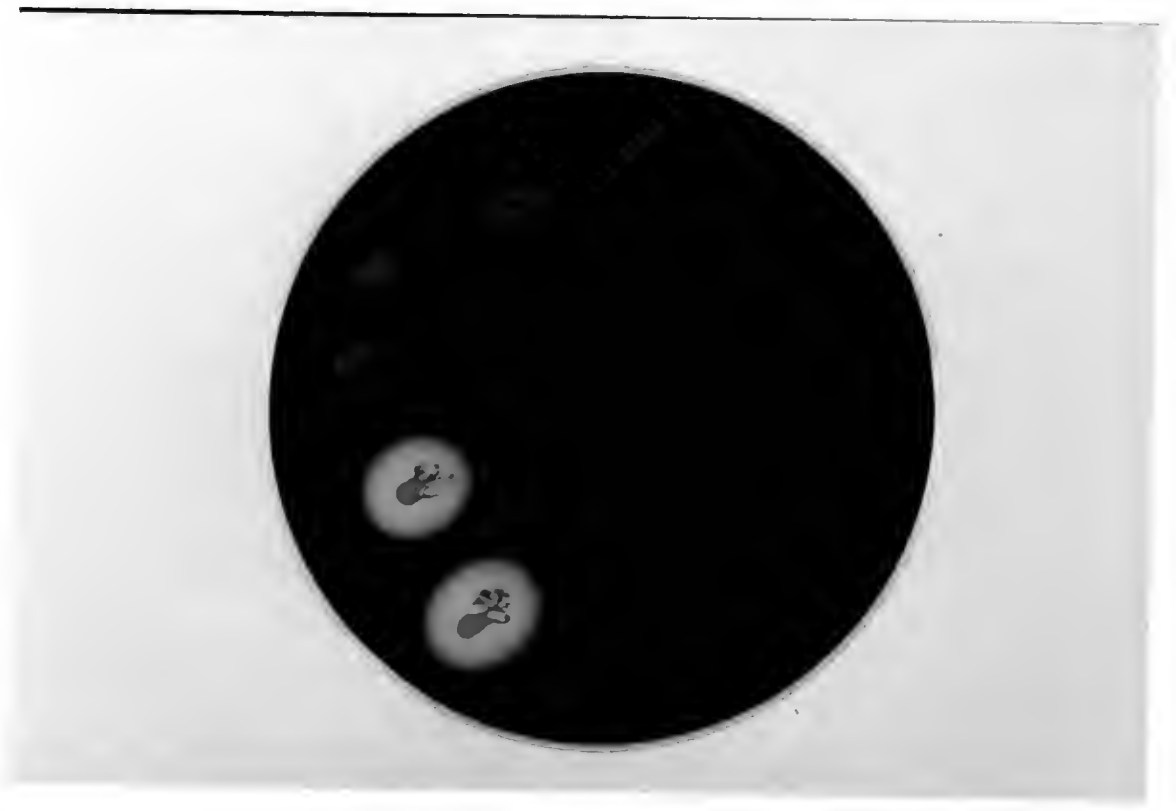


Figure 13. Starch-LA agar plate stained with iodine crystal vapors. Colonies with clear halos are starch-hydrolyzing *E. coli* transformants that carry the cloned α-amylase gene.

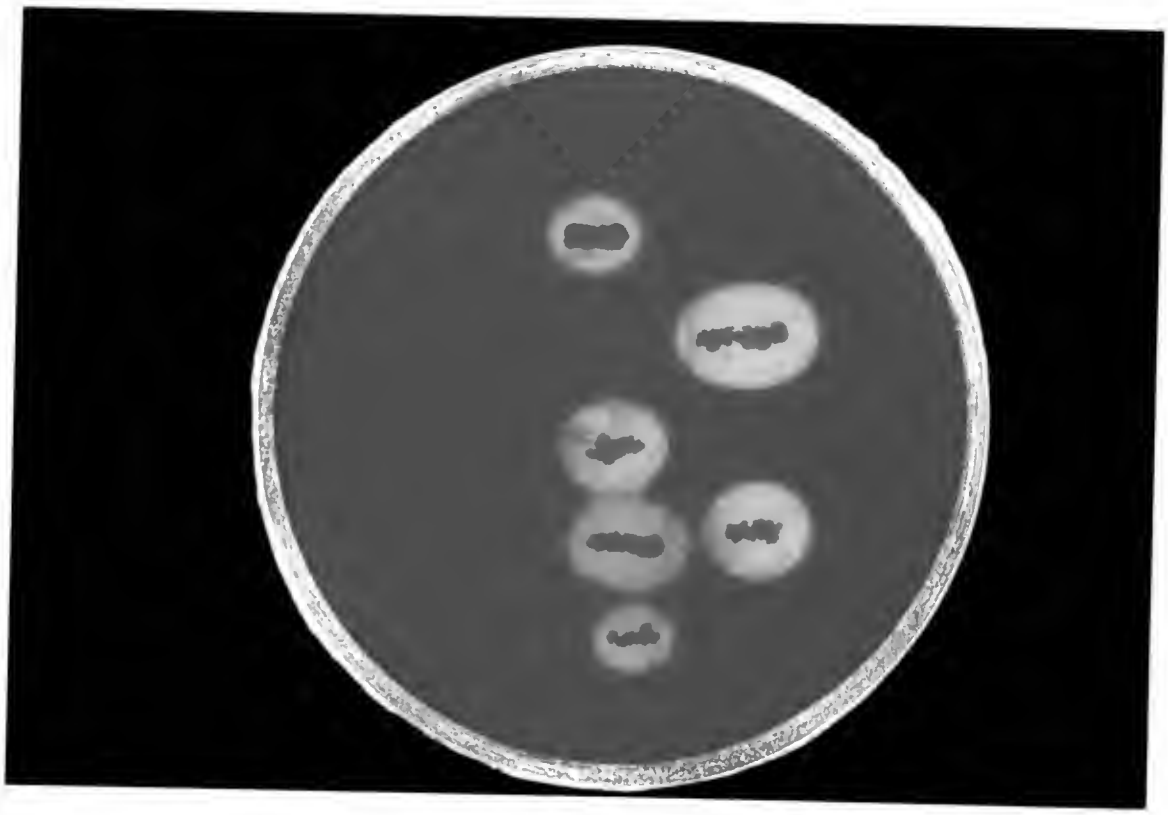


Figure 14. Pullulan-Reactive Red-LA agar plate which had been previously exposed to chloroform vapors. Colonies with clear halos are pullulan-hydrolyzing *E. coli* transformants that carry the cloned pullulanase gene.

hydrolyzing clones required exposure to chloroform vapors to demonstrate pullulanase activity. Unfortunately no CDase clones were isolated after several screenings, partly due to lack of an adequate substrate medium. Ideally, α- or ß-CD covalently bound to Reactive Red may have been the best screening medium. However, attempts to label α- or ß-CD with Reactive Red were unsuccessful, probably due to the carbohydrate ring structure.

The next approach to isolate the H-17 CDase gene involved construction of a DNA-probe based upon the N-terminal amino acid sequence of the H-17 CDase. Figure 15 shows positive identification of 47 out of 48 residues from the N-terminus, in which most of the amino acids had two or more possible codons. The lower lines in bold show the complementary nucleotide sequences chosen, based upon codon usage of several *Bacillus* α-amylases, and an extensive analysis of *B. subtilis* codon usage [64]. The oligomer consisted of 42 nucleotides and had a degeneracy of 64. The N-terminal residue was valine, which implies that a signal sequence was probably cleaved during membrane transport.

The Southern blot in Figure 16 shows positive hybridization of the labelled oligonucleotide to complete Pst I, Bam HI, Eco RI, Hind III, and Sal I digests of H-17 chromosomal DNA. The probe-positive Pst I, Bam HI, and Eco RI fragments were approximately 8 kb, 7 kb, and 3 kb, respectively. Since the H-17 CDase gene was estimated to be 1.5 kb, then there was a fairly good chance that the entire CDase gene was on one or more of these three fragments.

| | | | | | |
|---|---|---|---|---|---|
| 1 | | | | | |
| VAL | VAL | GLY | ASP | PHE | ALA |
| **GTT/A** | **GTT/A** | **GGC/A** | **GAT** | **TTT** | **GCA** |
| 7 | | | | | |
| ILE | GLU | THR | GLU | THR | LEU |
| **ATC/T** | **GAA** | **ACA** | **GAA** | **ACA** | **CTG** |
| 13 | | | | | |
| VAL | VAL | GLY | ALA | GLY | PRO |
| **GTT/A** | **GTT/A** | | | | |
| 20 | | | | | |
| TYR | VAL | ALA | ALA | ILE | ARG |
| 27 | | | | | |
| ALA | ALA | GLN | LEU | GLY | GLN |
| 33 | | | | | |
| LYS | VAL | THR | ILE | VAL | *** |
| 39 | | | | | |
| LYS | GLN | ASN | LEU | GLY | GLY |
| 45 | | | | | |
| VAL | THR | LEU | ASN | | |

Figure 15. N-terminal amino acid sequence of the *B. subtilis* H-17 thermostable cyclomaltodextrinase. The DNA bases chosen to make the synthetic degenerate oligonucleotide are in bold. The complementary DNA sequence was based on *B. subtilis* codon usage [64].

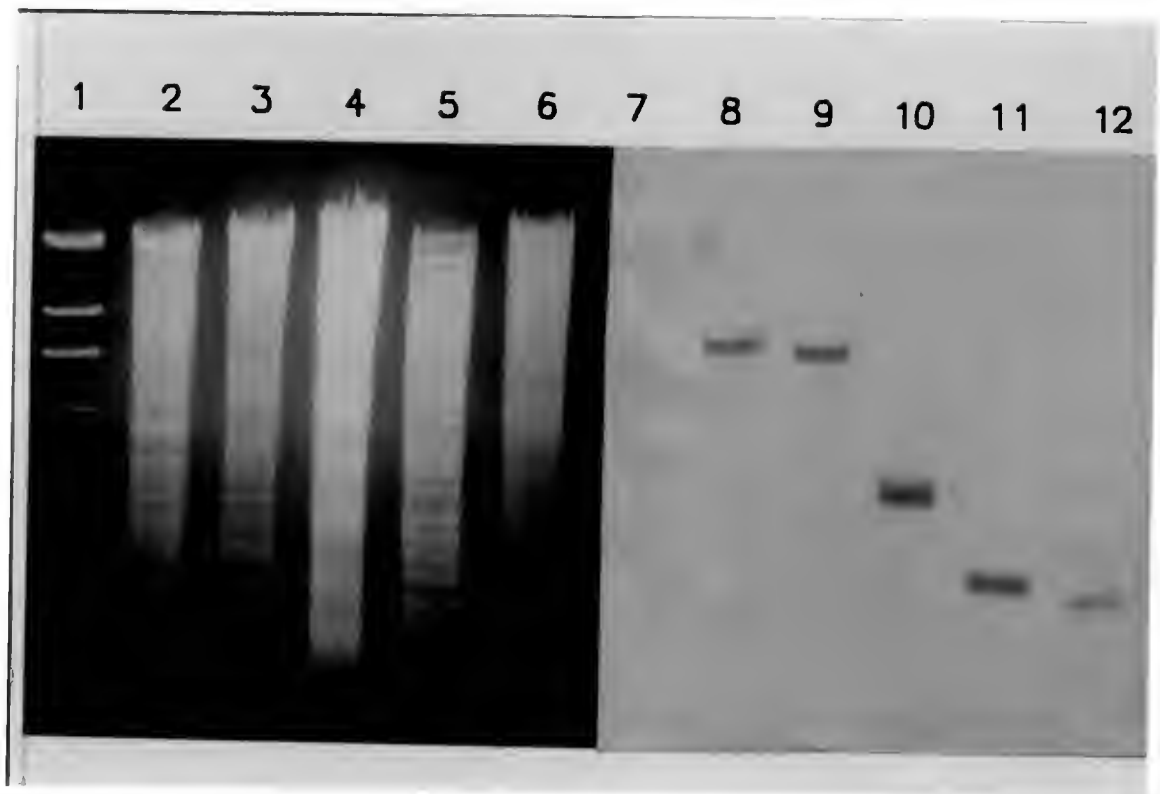



Figure 16. Southern blot hybridization of DNA probe to complete restriction enzyme digests of *B. subtilis* H-17 DNA. Lane 1: Lambda Hind III molecular weight standards (23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb). Lane 2: Pst I digest. Lane 3: Bam HI digest. Lane 4: Eco RI digest. Lane 5: Hind III digest. Lane 6: Sal I digest. Lanes 7-12 represent DNA blotted from lanes 1-6, respectively.

The amplified gene libraries constructed from the Pst I, Bam HI, or Eco RI fragments were digested, respectively, with Pst I, Bam HI, or Eco RI in order to liberate the inserts from pUC18. The Southern blot in Figure 17 shows that the DNA probe hybridized to all three digests, which indicated that the H-17 CDase gene was present in each amplified library. Colony lifts of *E. coli* DH5α transformed with either library (Figure 18) showed that the labelled oligonucleotide hybridized to a significant percentage (10-30%) of transformants. Interestingly, only 10-20% of the DNA probe-positive transformants regrew on the master plates while the probe-negative transformants rapidly regenerated. The probe-positive transformants were subcultured and colony lifts were verified for DNA probe hybridization. When these probe-positive transformants were streaked onto each type of substrate agar plate or grown in CPR-LA broth, no enzyme activity was detected, with or without plate exposure to chloroform vapors. Cell-free extracts of overnight cultures did not show CDase activity.

Although a part of, if not the entire, H-17 CDase structural gene was present in each library, it was unknown whether the gene was transcribed and translated in *E. coli* DH5α to give an active or inactive gene product. Polyclonal antiserum, raised against purified H-17 CDase, was purified and used to screen *E. coli* DH5α transformants for expression of the H-17 CDase gene. Colony lifts of *E. coli* DH5α transformed with Pst I or Bam HI libraries (Figure 19) indicate binding of the polyclonal antiserum (primary antibody) to the cloned CDase gene

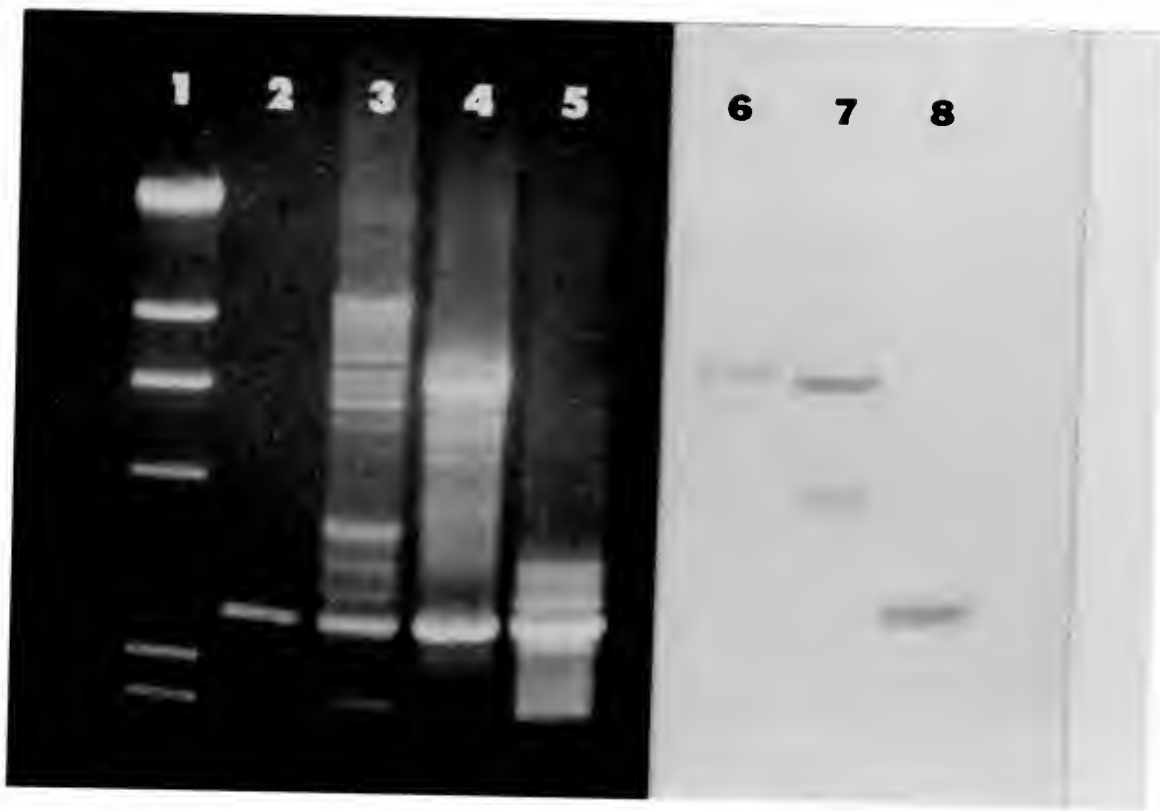



Figure 17. Southern blot hybridization of DNA probe to amplified gene libraries constructed in pUC18. Lane 1: Lambda Hind III standards. Lane 2: pUC18 linearized by an Eco RI digest. Lane 3: Complete Pst I digest of Pst I library. Lane 4: Complete Bam HI digest of Bam HI library. Lane 5: Complete Eco RI digest of Eco RI library. Lanes 6-8 represent DNA blotted from lanes 3-5, respectively.

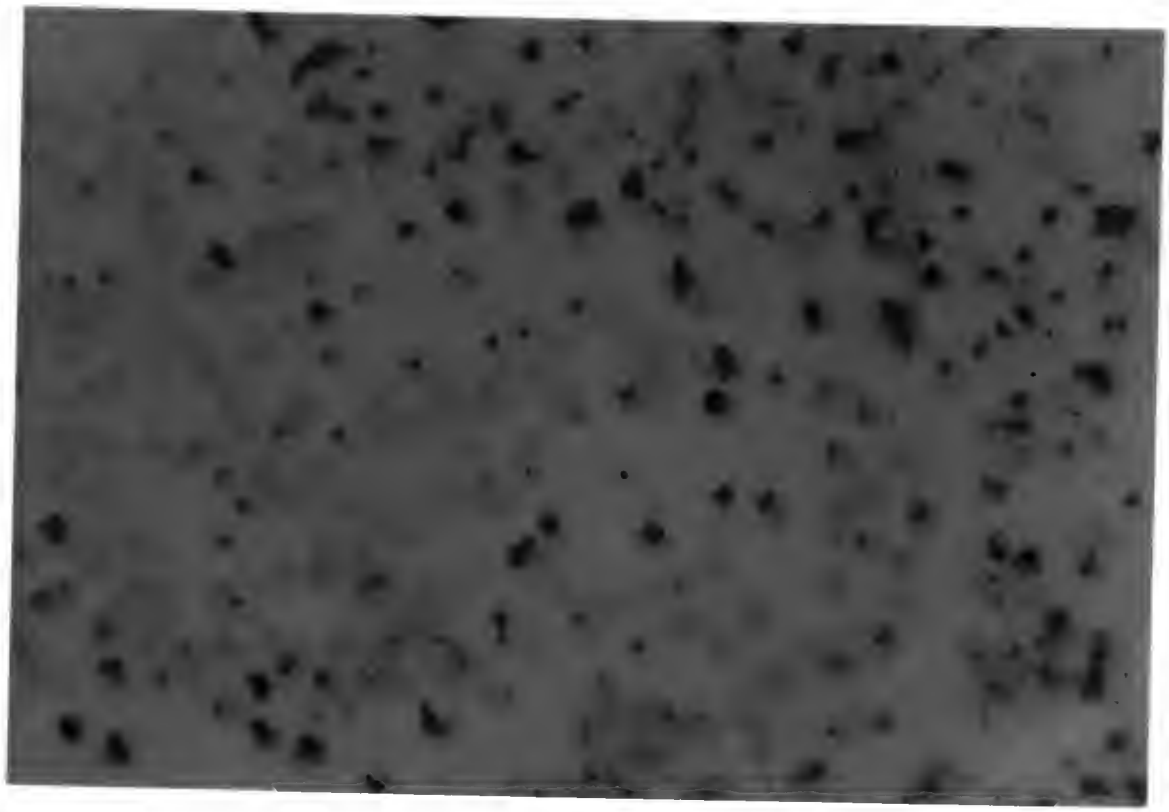
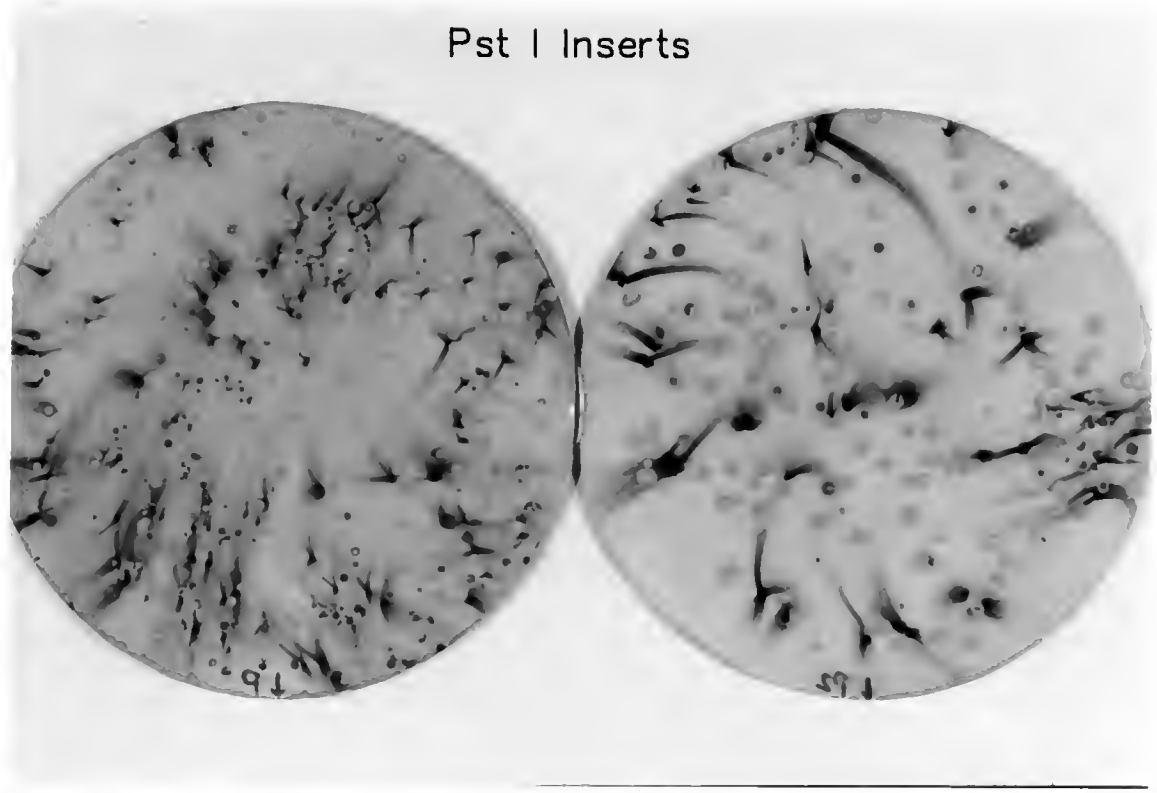
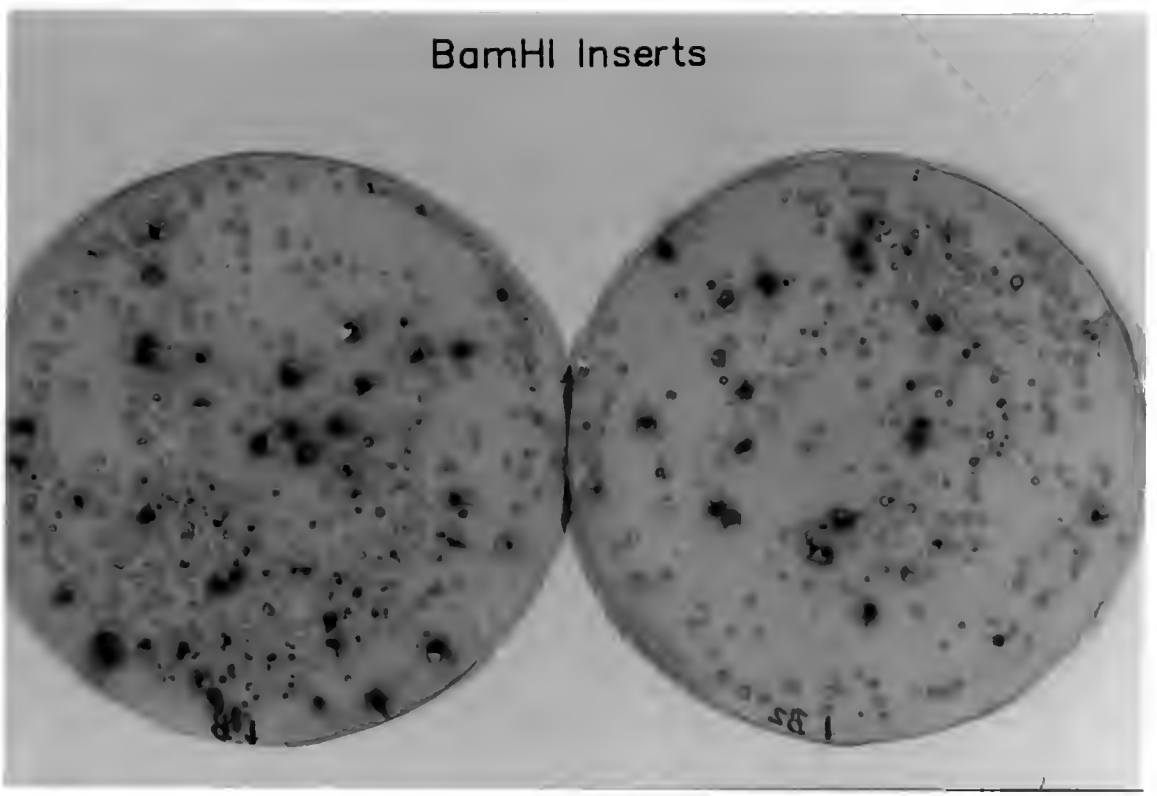


Figure 18. Hybridization of DNA probe to colony lift of *E. coli* DH5α transformed with Pst I, Bam HI, or Eco RI library. Dark colonies are DNA probe-positive.

Figure 19. Immunologically-screened colony lifts of *E. coli* DH5α transformed with gene libraries constructed in pUC18. Top membranes: *E. coli* transformed with amplified Pst I library. Bottom membranes: *E. coli* transformed with amplified Bam HI library.


Pst I Inserts


BamHI Inserts

product. Although not shown, the same results were obtained when *E. coli* DH5α was transformed with the Eco RI library. The frequency of immuno-positive transformants from each library was similar to, or slightly less than, that obtained for DNA probe-positive transformants. The extremely strong immunological signals observed with the colony lifts imply that the H-17 CDase gene is over-expressed. The cloned genes may have been actively transcribed due to the upstream *lac*Z promoter on pUC18. Once again, only 10-20% of immuno-positive transformants regrew on master plates. When these immuno-positive transformants were analyzed for CDase activity with each type of substrate media, no enzyme activity could be detected. Cell-free extracts from overnight cultures also produced no CDase activity. Plasmid DNA from several immuno-positive transformants was analyzed by liberating the Pst I, Bam HI, or Eco RI fragment from pUC18 with the respective restriction enzyme. Figure 20 shows agarose gel electrophoresis of Pst I, Bam HI, and Eco RI inserts, and linearized pUC18. Southern blot analyses (Figure 21) of these, plus other digests did not show DNA probe hybridization to any of the cloned inserts from immuno-positive transformants. Furthermore, when the immuno-positive transformants carrying Pst I and Bam HI inserts were subcultured, their colony lifts (Figure 22) demonstrated a gradual loss of immunological detection of the H-17 CDase gene product. Immuno-positive transformants carrying an Eco RI insert did not become immuno-negative upon subculturing, although Southern blots of the inserts were DNA-probe negative.

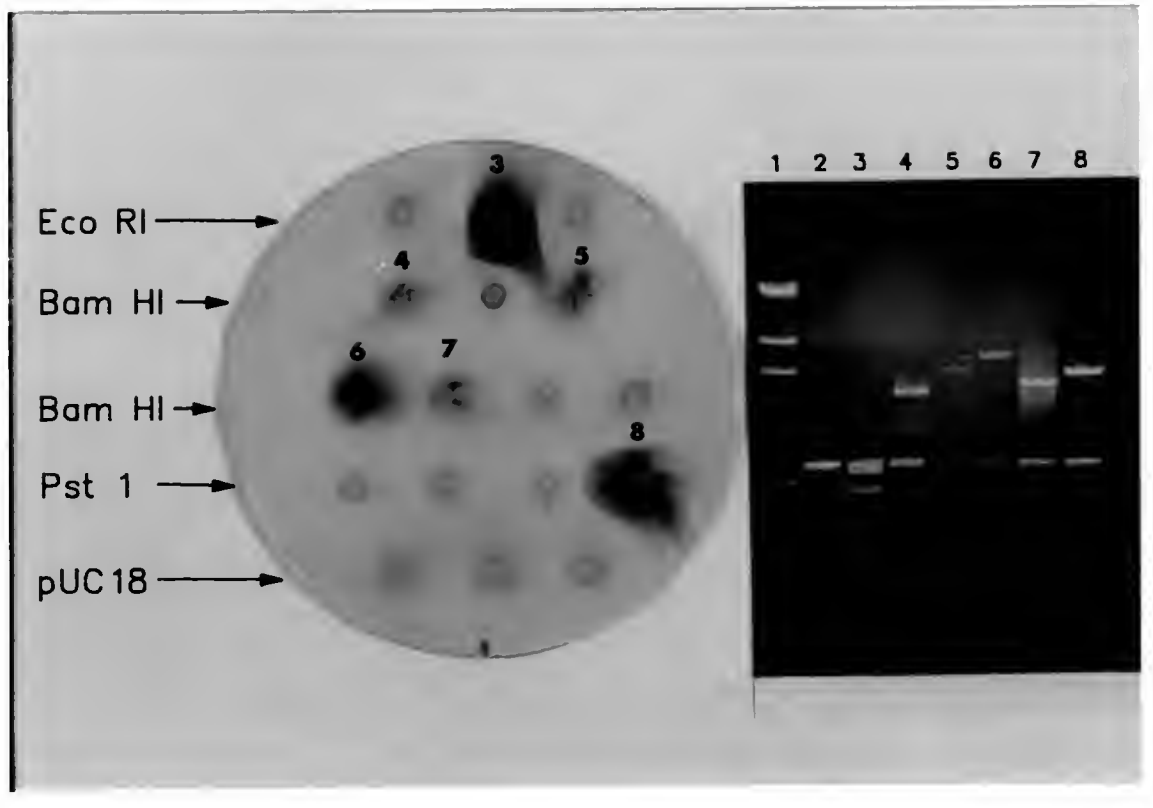
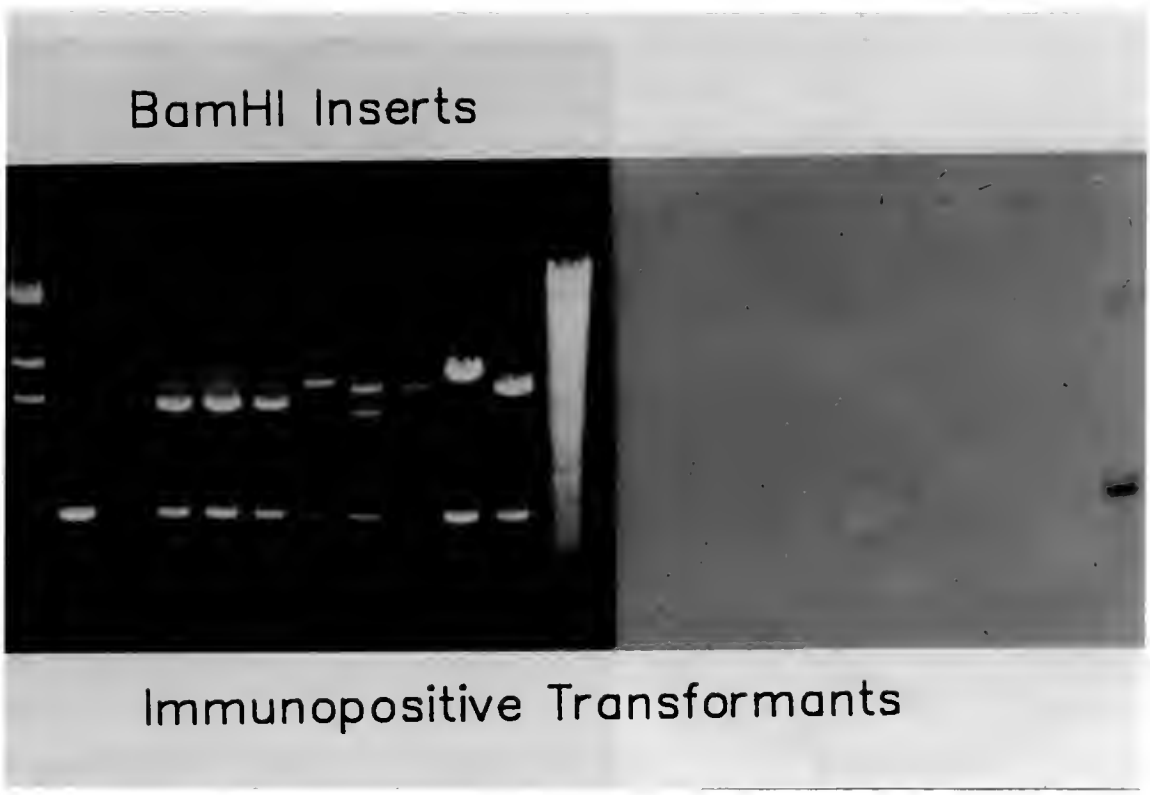



Figure 20. Agarose gel electrophoresis of digested plasmid DNA from several immuno-positive *E. coli* DH5α transformants identified on the corresponding colony lift shown at left. Lane 1: Lambda Hind III standards. Lane 2: pUC18 linearized by an Eco RI digest. Lane 3: Eco RI digest of pUC18 carrying Eco RI inserts. Lanes 4-7: Bam HI digests of pUC18 carrying a Bam HI insert. Lane 8: Pst I digest of pUC18 carrying a Pst I insert.

Figure 21. Top: Southern blot analysis of Pst I digested plasmid DNA from several immuno-positive *E. coli* DH5α transformants carrying pUC18 with a Pst I insert. Far right lane is a complete Eco RI digest of H-17 chromosomal DNA as a positive control.

Bottom: Southern blot analysis of Bam HI digested plasmid DNA from several immuno-positive *E. coli* DH5α transformants carrying pUC18 with a Bam HI insert. Far right lane is a complete Eco RI digest of H-17 chromosomal DNA as a positive control.


Pst I Inserts
Immunopositive Transformants


BamHI Inserts
Immunopositive Transformants

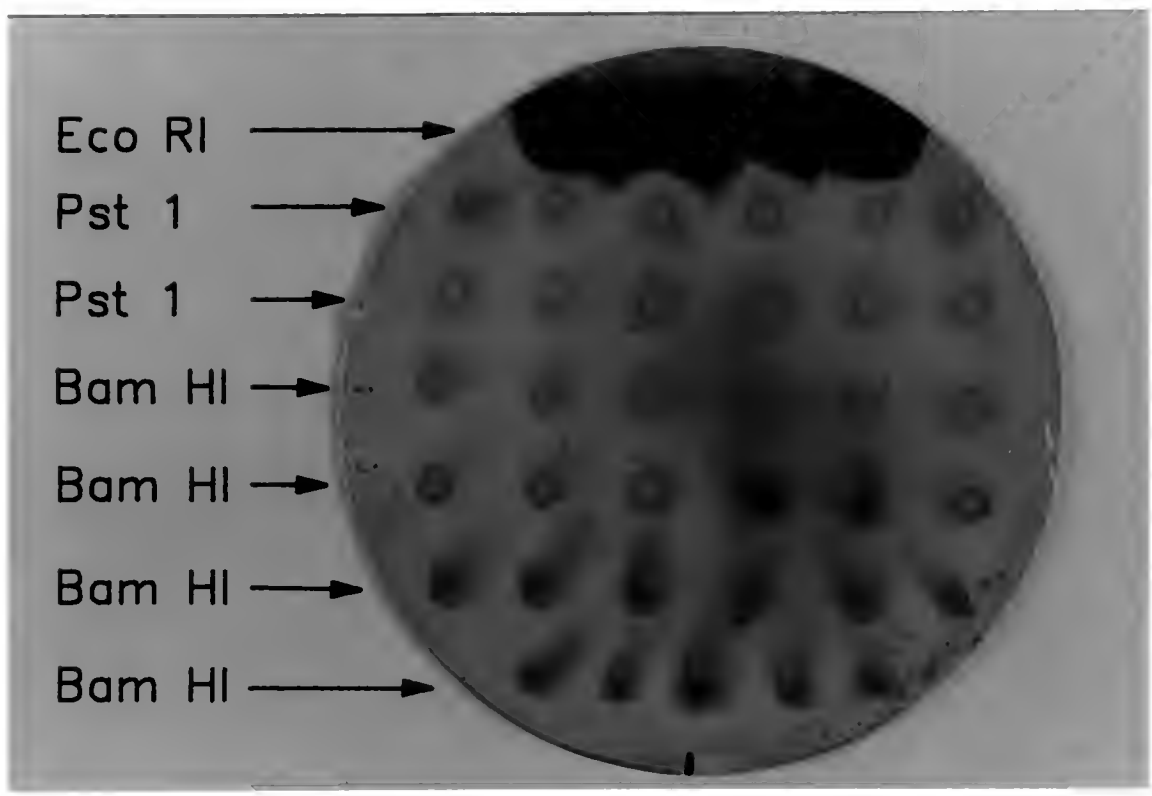



Figure 22. Colony lift of subcultured immuno-positive *E. coli* DH5α transformants. Colonies carrying pUC18 with a Pst I or Bam HI insert show loss of immunological signal.

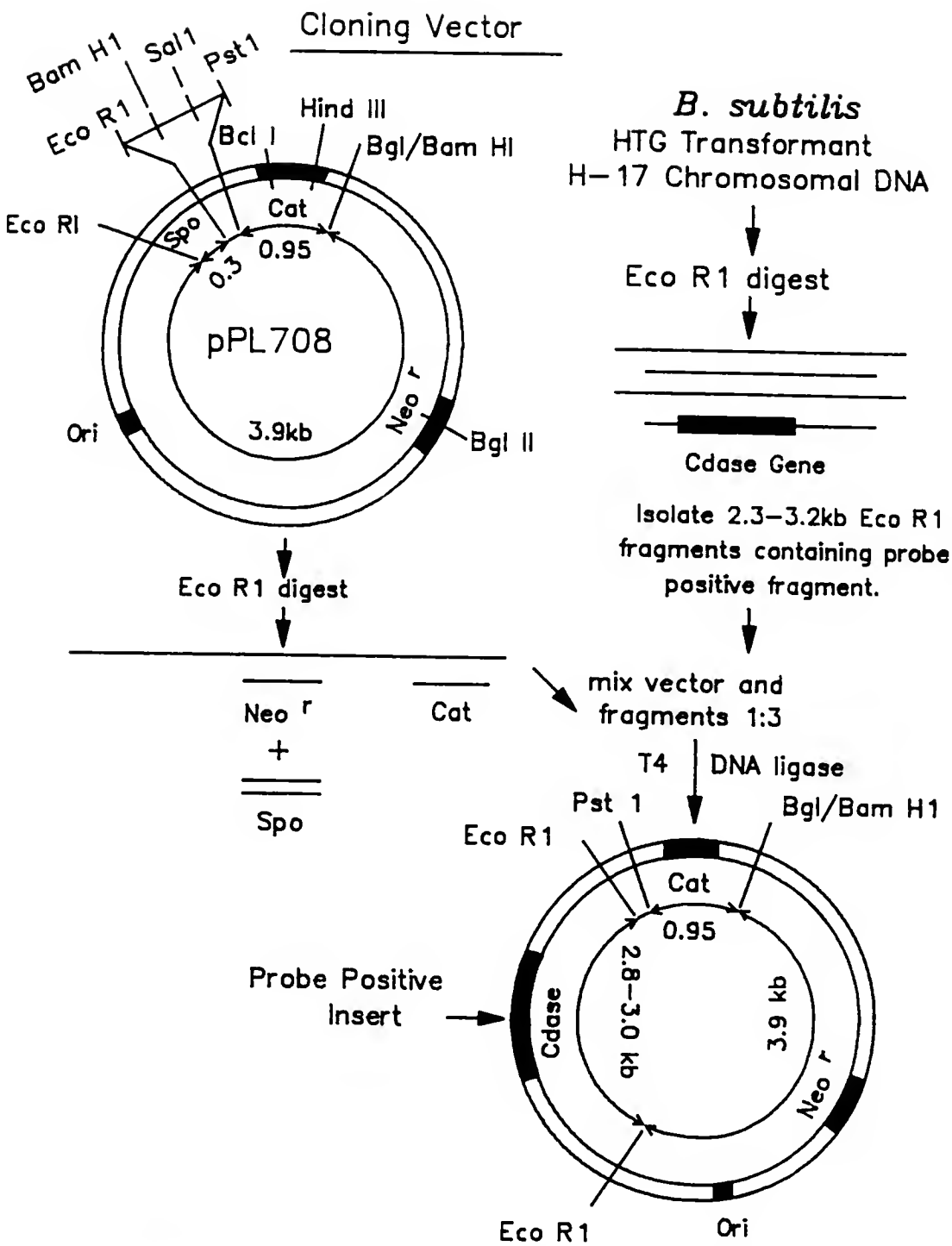
It appears that the high copy number of pUC18 plus the strong constitutive promoter activity by the *lac*Z promoter on pUC18 may have contributed to structural instability of the recombinant plasmids. The gene dosage effect from high level expression of the H-17 CDase gene product probably was detrimental to the host cell, which lead to selection for deletions which remove either the promoter, or all or part of the deleterious gene. In the absence of the toxic CDase gene product, the host cell has a selective advantage which allows it to grow faster than cells without deletions. This was evident in immunologically-screened colony lifts (Figure 19), in which only portions of most colonies, if examined closely, were actually producing the antigen. The same was also evident with the colony lifts in Figure 22. The majority of each colony was immuno-negative because those cells outgrew cells producing a toxic gene product. Transformants carrying Eco RI inserts may have remained immuno-positive because the Eco RI fragment is much smaller than either the Pst I or Bam HI fragment, and therefore more stably maintained.

The H-17 CDase, although not secreted extracellularly by *B. subtilis*, is considered to be an exoenzyme, in that it appears to be cell wall-bound or periplasmic in *B. subtilis*. All the exoenzymes from bacilli that have been cloned and sequenced have a signal peptide or prepeptide at the amino terminal, which must be cleaved to produce a mature enzyme [76]. The general pattern among *Bacillus* signal peptides involves an N-terminal hydrophilic/basic sequence of about

10 residues, followed by the hydrophobic core, a stretch of about 20 uncharged residues probably required for membrane translocation. In comparison to *E. coli*, *Bacillus* signal peptides are longer, usually between 30 and 40 residues, and are more positively-charged at the amino end. When the signal peptide emerges from the ribosome, it interacts with receptor proteins on the inner side of the cell membrane. As the polypeptide chain traverses the membrane, a specific protease cleaves the signal peptide, thereby allowing the enzyme to assume its final native configuration outside the membrane. The cleavage point of the signal peptide is usually within a sequence of hydrophobic residues, often containing alanine, valine, or glycine [66,76]. This is consistent with the first two residues at the N-terminus of the mature H-17 CDase, which are valine. If the post-translational processing system in *E. coli* does not recognize the signal sequence of the H-17 CDase, then membrane/cell wall translocation, and/or signal peptide cleavage to give a mature protein may not occur. This would result in high level intracellular accumulation of the CDase gene product, which could be toxic to the host and lead to rapid selection of plasmid deletion derivatives. Consequently, the CDase may have accumulated as a crystalline-like structure (inclusion bodies) in which the enzyme is in a non-native state of little or no biological activity. It is interesting to note that when the α-amylase (an exoenzyme) from *B. subtilis* was cloned in *E. coli*, it too was deleterious to the host [76].

The alternative host-vector system involved *B. subtilis* YB886 and the plasmid vector pPL708. Gene libraries were constructed from the Pst I and Bam HI DNA probe-positive fragments. However, the transformation frequency for each library was extremely low, probably because the recombinant plasmids were too large due to the long inserts. The transformation frequency of the gene library constructed from DNA probe-positive Eco RI fragments was sufficient to screen colonies for H-17 CDase production. Figure 23 illustrates the general strategy for cloning the Eco RI fragments in pPL708. Since *B. subtilis* YB886 produces its own CDase, substrate media were not employed to screen transformants. Colony lifts of *B. subtilis* YB886 transformants were screened immunologically. Unfortunately, the host colonies gave slightly more background since the antiserum was purified with a whole cell acetone powder from *E. coli* rather than from *B. subtilis.* However, four colonies in Figure 24 demonstrated a significantly stronger antigenic signal than other colonies. Plasmid DNA from each transformant was digested with Eco RI to liberate the insert from pPL708, and analyzed by agarose gel electrophoresis. Figure 25 shows the Southern blot of each digestion. Although each transformant carried an Eco RI fragment on pPL708, the DNA probe hybridized to only one insert. Plasmid DNA from 100 ml of an overnight culture of the transformant carrying this probe-positive insert was purified by cesium chloride density gradient centrifugation. Southern blot hybridization (Figure 26) of an Eco RI digest of this purified plasmid demonstrates that the plasmid is stably maintained in *B. subtilis*

Figure 23. General strategy for cloning DNA probe-positive Eco RI fragments into the Eco RI site of plasmid vector pPL708.




Competent *B. subtilis* YB886 cells transformed with recombinant vector and plated onto LB-kanamycin agar.

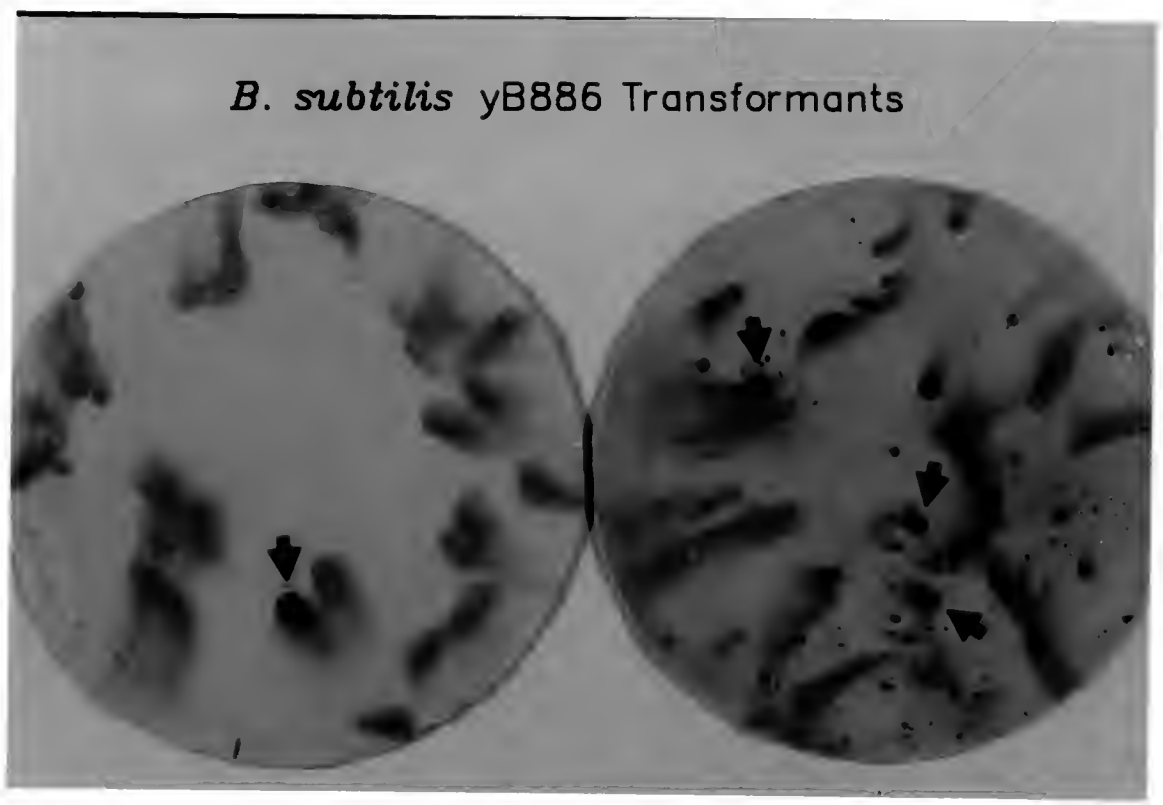



Figure 24. Immunologically-screened colony lifts of *B. subtilis* YB886 transformed with Eco RI library constructed in pPL708. Arrows indicate four colonies with strong antigenic signal.

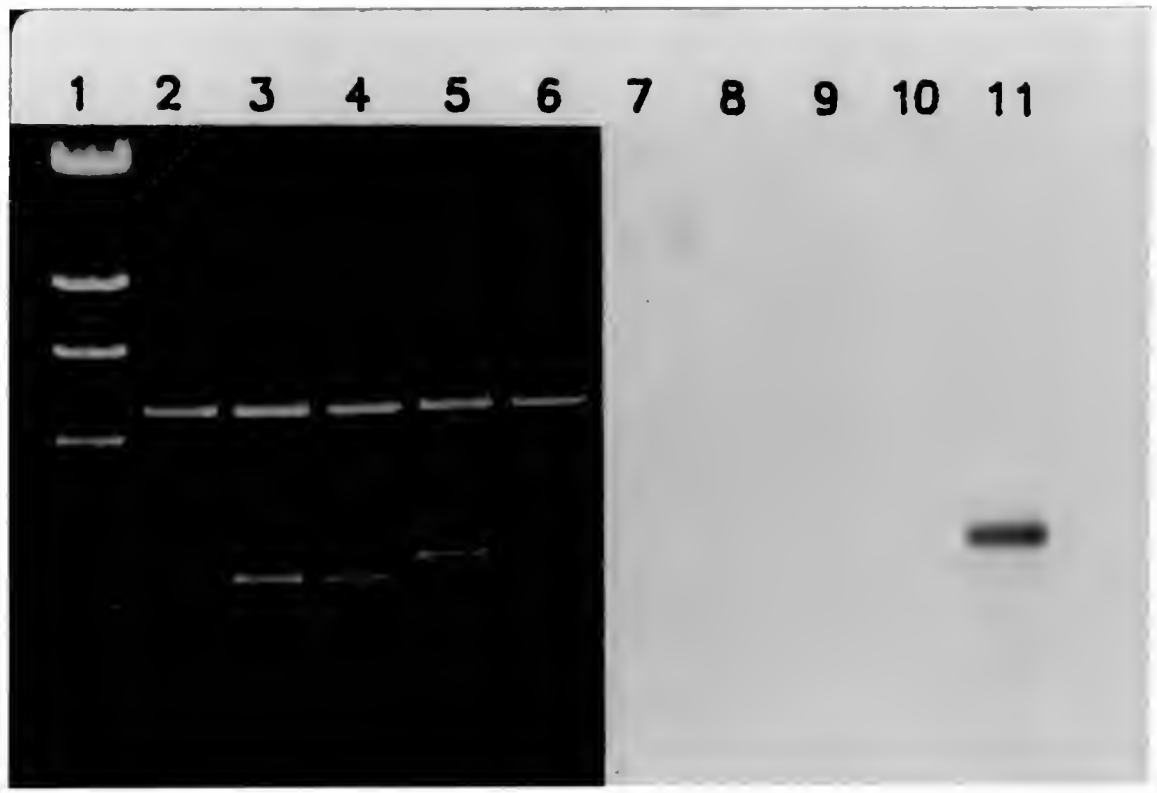



Figure 25. Southern blot hybridization of DNA probe to Eco RI digested plasmid DNA from four *B. subtilis* YB886 immuno-positive transformants. Lane 1: Lambda Hind III standards. Lane 2: pPL708 linearized by Eco RI digestion. Lanes 3-6: Eco RI digests of pPL708 carrying an Eco RI insert. Each lane represents plasmid DNA from one transformant. Lanes 7-11 represent DNA blotted from lanes 2-6, respectively.

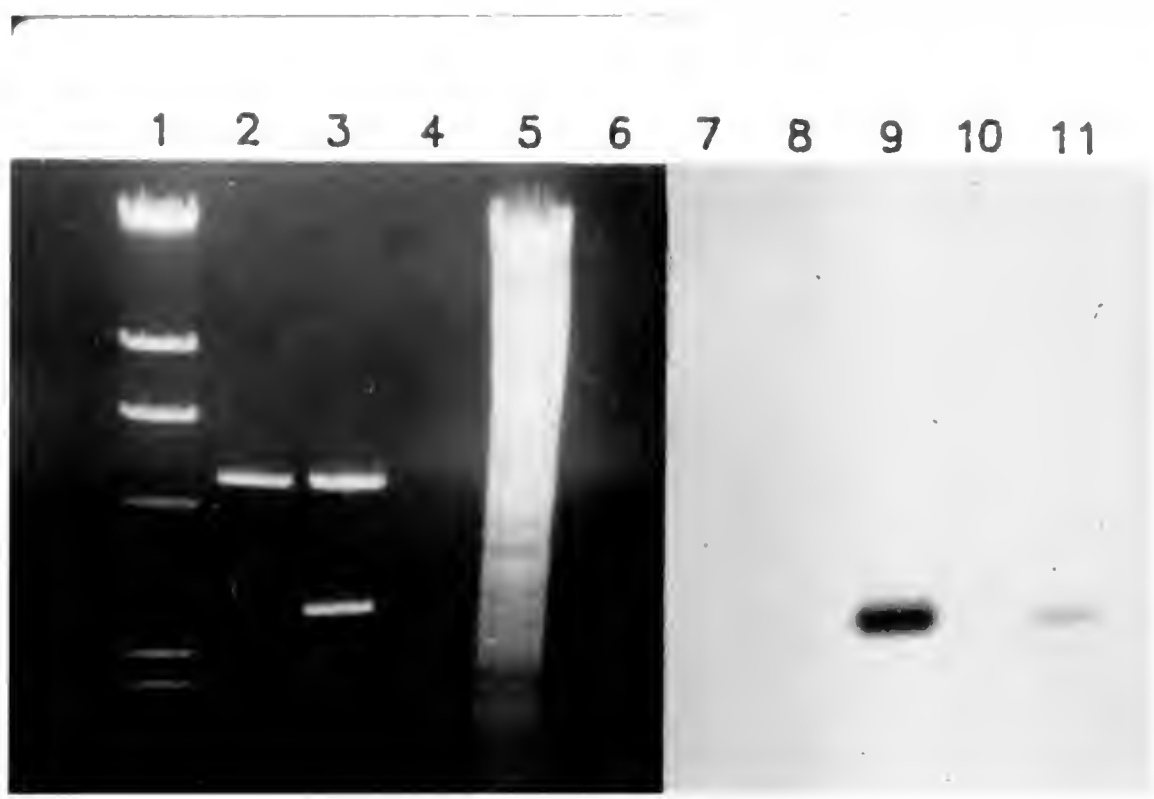



Figure 26. Southern blot hybridization of DNA probe to Eco RI digest of cesium chloride density gradient-purified pPL708 carrying the probe-positive Eco RI insert. Lane 1: Lambda Hind III standards. Lane 2: pPL708 linearized by Eco RI digest. Lane 3: Eco RI digest of density gradient-purified plasmid. Lane 5: Complete Eco RI digest of H-17 chromosomal DNA. Lanes 7-11 represent DNA blotted from lanes 1-5, respectively.

YB886 over many generations. Furthermore, the cloned Eco RI fragment is the same size (approximately 3 kb) as the DNA-probe positive fragment from an Eco RI chromosomal digest of H-17 DNA (Figure 26).

A drawback in cloning Eco RI fragments into pPL708 is that the plasmid has two Eco RI sites that flank the 0.3 kb sequence that contains the *spo*2 promoter (Figure 11). Consequently, there is a high probability of losing this promoter when ligating Eco RI fragments into pPL708 (Figure 23). The H-17 CDase gene appears to have been cloned with its own promoter on the 3 kb Eco RI fragment. Table 9 shows the specific activities of cell-free extracts from the *B. subtilis* host carrying pPL708 with no insert, and pPL708 with the probe-positive 3 kb Eco RI insert. Although the specific activities for the clone are slightly greater, preliminary results indicate total CDase activity to be thermolabile. This would seem logical if the H-17 gene is a mesophilic gene that originated in *B. subtilis* 25S. Expression of the H-17 CDase gene could be enhanced if the gene could be placed downstream from the *spo*2 promoter. This would require subcloning the H-17 CDase gene into the Pst I or Bam HI site downstream from the *spo*2 promoter, possibly by ligating Pst I or Bam HI adapters to the ends of the 3 kb Eco RI fragment.

The increase in CDase specific activity from the *B. subtilis* clone also suggests the entire structural H-17 CDase gene is on the probe-positive 3 kb Eco RI fragment. When this Eco RI fragment was subcloned into pUC18 and transformed

into *E. coli* XL1-Blue, expression of the gene was not repressed in the absence of IPTG. Figure 27 shows immunologically-screened colony lifts of *E. coli* XL1-Blue in which one membrane was incubated on LAT agar containing IPTG while the other was incubated on LAT agar without IPTG. If expression of the CDase gene was under control of the *lac*Z promoter, then little or no gene product should be detected in the absence of IPTG. The results indicate that the H-17 CDase gene is efficiently transcribed off its own promoter in *E. coli*, and that the high copy number of pUC18 may be responsible for the detrimental gene dosage effect.

Ochterlony gel diffusion, shown in Figure 28, verified that the 3 kb Eco RI fragment cloned in pPL708 produced the H-17 CDase gene product. As expected, the purified antiserum cross-reacted with the cell-free extracts from the *B. subtilis* YB886 clone and from *B. subtilis* H-17, but not with that from *B. subtilis* YB886 carrying pPL708 with no insert. Surprisingly, the antiserum also cross-reacted with the cell-free extract from *B. caldolyticus* C2, but not with that of *B. subtilis* 25S. One explanation may be that the 25S CDase acquired sufficient alterations in antigenic determinants in its transition to a thermostable form in *B. subtilis* H-17. Consequently, the enzyme resembled the C2 CDase in its antigenic determinants. Although Figure 28 indicates cross-reactivity of the polyclonal antiserum to the C2 and cloned enzyme, distinct lines of precipitation indicated non-identity between these two enzymes. This would indicate that the two enzymes do indeed share common antigenic determinants, but are non-identical. A second explanation may

be that the C2 CDase gene was actually transferred to *B. subtilis* 25S during the transformation to thermophily. In this case, the H-17 CDase is the product of the C2 CDase gene in *B. subtilis* H-17, and there could actually be two CDase genes in this HTG transformant. To further clarify this situation, the CDase gene on the cloned Eco RI fragment will be labelled, and then used to probe Southern blots of genomic DNA digests from *B. subtilis* 25S and *B. caldolyticus* C2. This will hopefully identify the origin of the H-17 CDase gene, and perhaps clarify why the H-17 CDase is thermostable.

Table 9. Comparison of cyclomatodextrinase activity between *B. subtilis* YB886 carrying pPL708 with no insert, and *B. subtilis* YB886 carrying pPL708 with probe-positive 3 kb Eco RI fragment.

| | Specific Activity (μmoles reducing groups/min/mg protein) | |
|---|---|---|
| Cell Free Extract | α-Cyclodextrin | ß-Cyclodextrin |
| *B. subtilis* YB886 | 0.61 | 0.64 |
| *B. subtilis* YB886 with cloned Eco RI fragment | 0.98 | 0.93 |

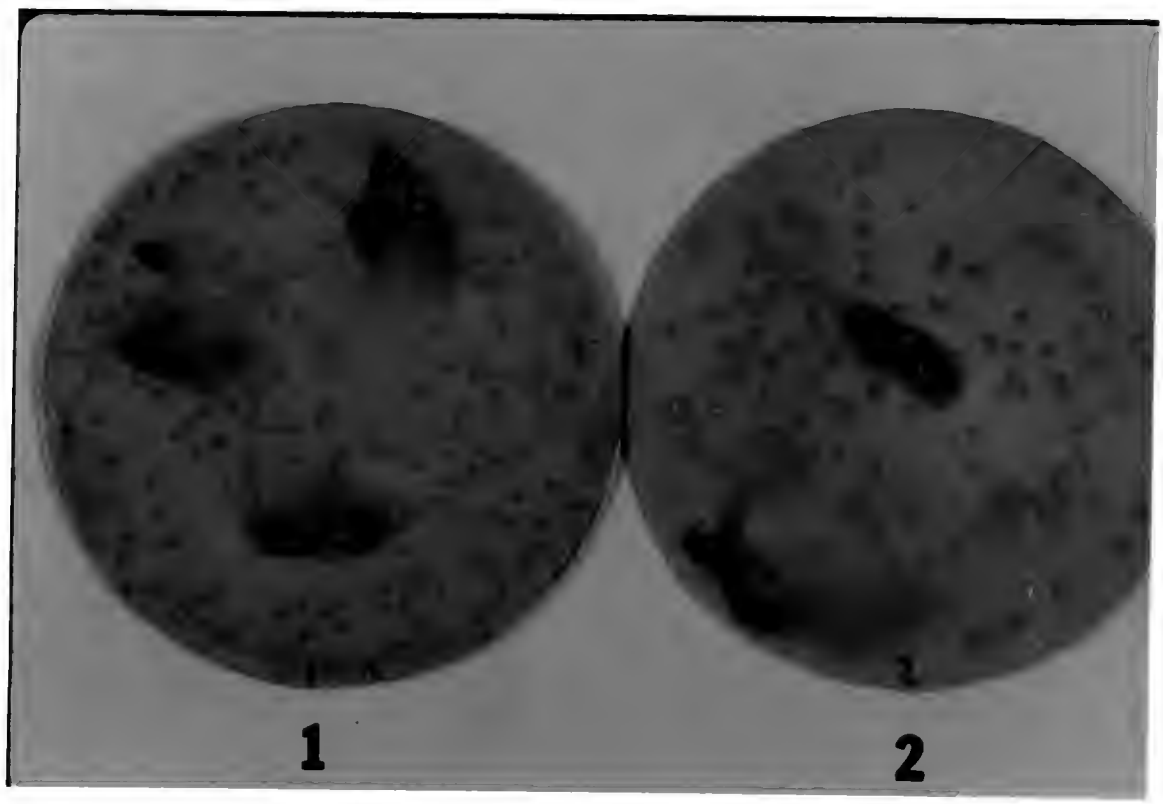



Figure 27. Immunologically-screened colony lifts of *E. coli* XL1-Blue transformed with pUC18 carrying the DNA probe-positive Eco RI fragment purified from pPL708. Left membrane (1) was incubated at 37°C for 7 h on LAT agar supplemented with 20 mM IPTG, while right membrane (2) was incubated under identical conditions without IPTG.

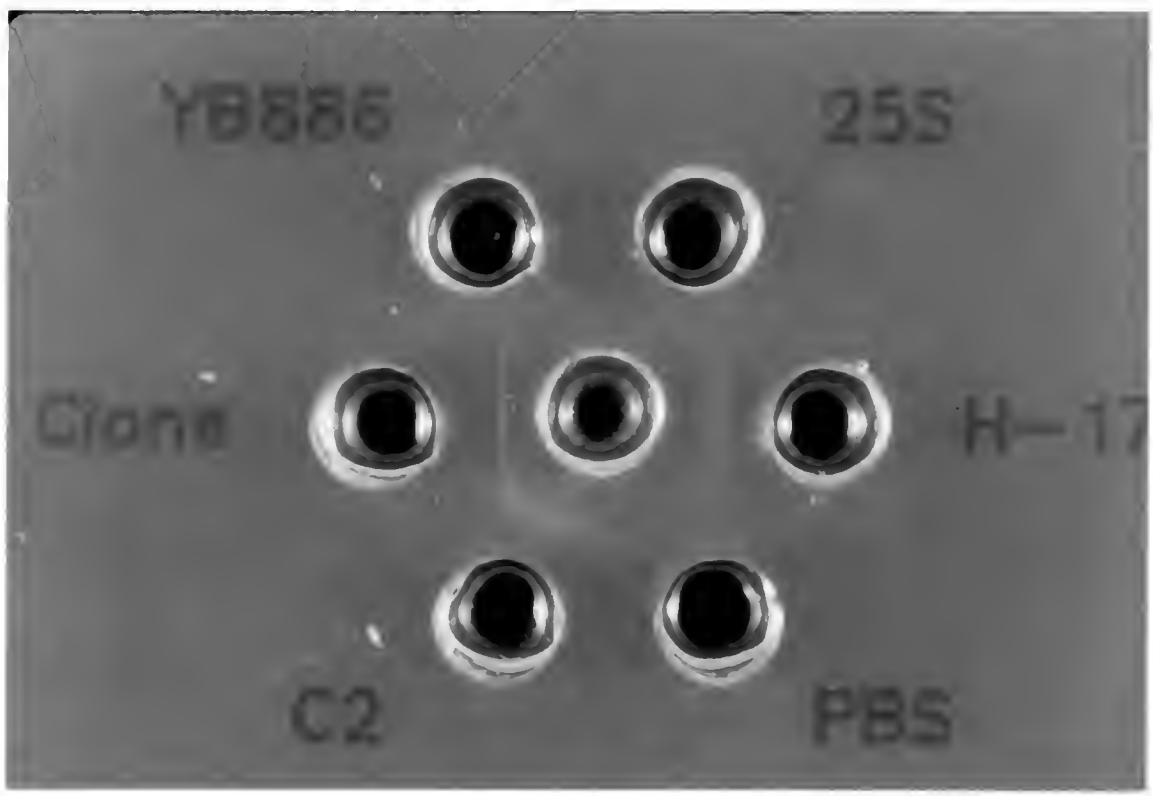



Figure 28. Ochterlony gel diffusion of purified rabbit polyclonal antisera against late log phase cell-free extracts. Center well: Purified antisera. Outer wells (clockwise from lower left): Cell-free extract from *B. caldolyticus* C2; *B. subtilis* YB886 clone carrying pPL708 with DNA probe-positive Eco RI insert; *B. subtilis* YB886 carrying pPL708 with no insert; *B. subtilis* 25S; *B. subtilis* H-17; Phosphate-buffered saline.

# CHAPTER 6
# SUMMARY AND CONCLUSIONS

The CDases produced by *B. subtilis* 25S, *B. caldolyticus* C2, and *B. subtilis* H-17 are all highly related to each other both biochemically and biophysically. They each share common or very similar properties such as $M_r$, subunit structure, pH optimum, isoelectric point, cation inhibition, and substrate specificity and affinity. All behave as low active, exo-acting, maltogenic enzymes against starch polymers, but show a highly-active endo- and exo-type attack on cyclodextrins and maltodextrins. These characteristics, which closely resemble properties of similar enzymes from other *Bacillus* species, support the theory that thermophilic, mesophilic, and psychrophilic bacteria from the genus *Bacillus* evolved from common ancestors [44,114,115]. Despite these similarities, there is however, a significant difference between the 25S, C2, and H-17 CDase thermostability. This difference is probably attributable to alterations in the hydrophobic amino acid content between each enzyme, and confirms previous results for the histidinol dehydrogenase enzymes from 25S, C2 and the HTG transformants [41].

The results from this study support the previously proposed hypothesis that thermostable proteins produced by the HTG transformants are the result of either specific mistranscription or mistranslation of the *B. subtilis* 25S genes [39]. There

are, however, several important and as yet unanswered questions. First, how does the presence of a reducing agent or chelating agent produce a difference in thermostability among the CDases? Second, are the requirements of these extrinsic factors for thermostability also a reflection of specific amino acid changes due to mistranscription or mistranslation of the 25S CDase gene? Third, are the requirements for 2-Me or EDTA not directly due to mistranscription or mistranslation, but are the consequence of an altered three-dimensional conformation due to an increase in hydrophobic residues? Fourth, why does 2-Me confer a greater thermostability for the H-17 CDase, while EDTA confers a greater thermostability for the C2 CDase?

There appears to be a complex combination of different molecular mechanisms which produces an interactive, cooperative network of intramolecular interactions that are responsible for the differences in thermostability among the three enzymes. Since the CDases are not extracellular enzymes, perhaps 2-Me and EDTA are not required for thermostability, *in vivo*! An intracellular environment may protect these enzymes from thermostability inhibitors that are encountered *in vitro* studies. Conversely, thermostability *per se* may be attributable to a more simple mechanism, for example, a subtle change in proline content. Although supporting the previous hypothesis, the biochemical results alone provide no definitive insights into how proteins were made thermostable by the HTG transformants. Future research would involve both amino acid sequencing of the

CDases and determination of the amino acid sequence derived from the nucleotide sequence of the CDase gene. This would determine which amino acid substitutions resulted due to either mistranscription or mistranslation.

While the H-17 CDase was cloned in pUC18, its gene product was expressed in *E. coli* in an inactive form and was toxic to the cell. The problem of structural instability of the recombinant plasmid in *E. coli* was resolved by cloning the gene in pPL708 and expressing it in *B. subtilis*. By subcloning the gene into a *Bacillus* expression vector, the levels of enzyme could be greatly increased, as long as toxicity to the host is avoided. Preliminary results indicated the cloned H-17 CDase to be thermolabile in *B. subtilis*. Perhaps the cloned gene may also need to be expressed in a thermophilic *Bacillus* host in order to obtain a thermostable enzyme that still has industrial potential. Thus far, all genes encoding for thermostable enzymes that have been cloned and expressed in a mesophilic host, have produced an enzyme that retained its thermostable properties. Theoretically, the H-17 CDase gene originated from a mesophile. This may explain the expected discrepancy in which the H-17 CDase becomes thermolabile when transferred back to a mesophilic host. Current research involves determining the origin of the H-17 CDase gene. Southern blot analysis of genomic DNA from the parents, *B. subtilis* 25S and *B. caldolyticus* C2 may clarify the events that occurred in the transformation to thermophily.

Although the H-17 CDase may have limited industrial applications, the results show that protein thermostability may be achieved via a unique, alternative approach, that is, by conversion of the entire cell of a mesophile into that of a thermophile. Therefore, the generation of HTG transformants from GRAS status mesophiles may be useful for the synthesis of thermostable enzymes with commercial value. One potential application of the H-17 CDase is the industrial production of high maltose syrups. The enzyme's neutral pH optimum would allow it to be employed immediately prior to starch liquefaction by highly thermostable *B. licheniformis* α-amylase, without any change in pH. However, a drop in temperature would be required. Alternatively, the enzyme could be used simultaneously with a neutrophilic, thermostable α-amylase with a lower temperature optimum. A possible drawback may be the necessary addition of a foodsafe reducing agent in order for the CDase to maintain thermostability. However, the H-17 CDase has been characterized for thermostability under relatively dilute substrate concentrations (less than 5% w/v). Commercial starch conversion begins with 35-37% starch dry solids, a very high concentration. High levels of substrates tend to increase thermostability by stabilizing enzyme conformation and protecting the catalytic site [9]. The H-17 CDase has not been thermally-characterized under conditions of extremely high substrate concentrations. Future research which focusses on this aspect, may show it unnecessary to add a foodsafe reducing agent in an industrial condition, in which substrate concentrations are extremely high.

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# BIOGRAPHICAL SKETCH

Bradley Martin Krohn was born May 16, 1959, in Springfield, VT. After graduating from Springfield High School in 1977, he attended Williams College, Williamstown, MA, and received his B.A. in biochemistry in 1981. He then moved to Sarasota, FL, where he pursued a career in restaurant management for four years. He realized a scientific career was in his better interests, so in 1985 he enrolled in the Food Science and Human Nutrition Department at the University of Florida. After one year postbaccalaureate, he was accepted into the graduate program. Upon graduation in December, 1991, he will work as a postdoctoral associate in the New Products group of the Monsanto Company, St. Louis, MO. He plans to pursue a career in applied molecular biology and microbiology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of a scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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