The cloning and characterization of an α-amylase and a branching enzyme from *Butyrivibrio fibrisolvens* H17c and their expression in *Escherichia coli*

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CERTIFICATION OF SUPERVISOR

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Dedicated to my parents, Louis and Betty Rumbak

and

my friend, Mark J. Gibbons.

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maggie and milly and molly and may
went down to the beach (to play one day)

and maggie discovered a shell that sang
so sweetly she couldn't remember her troubles, and

milly befriended a stranded star
whose rays five languid fingers were;

and molly was chased by a horrible thing
which raced sideways while blowing bubbles:and

may came home with a smooth round stone
as small as a world and as large as alone.

For whatever we lose (like a you or a me)
it's always ourselves we find in the sea

from e.e. cummings
selected poems 1923-1958

Contents

Abstract

Butyrivibrio fibrisolvens H17c is an important anaerobic bacterium found in the rumen of most ruminants. The aim of this thesis was to establish a genebank of *B. fibrisolvens* H17c DNA in *E. coli* and to isolate and characterize genes encoding enzymes involved in the degradation of the major plant polysaccharides.

A library of chromosomal DNA fragments from *B. fibrisolvens* was established in the *E. coli-Bacillus subtilis* shuttle vector pEB1. The library was screened for the expression of *B. fibrisolvens* genes in *E. coli*. *E. coli* clones expressing glutamine synthetase, carboxymethylcellulase, β-glucosidase and amylolytic-type activities were isolated.

A gene (*amyA*) expressing amylolytic activity and encoding an α-amylase was located on a 5.0 kb DNA fragment and expressed from its own promoter in *E. coli*. It was shown that more than 86% of the amylolytic actvity was located in the periplasm of the *E. coli* host and Tn*phoA* mutagenesis indicated the presence of a functional signal peptide. The nucleotide sequence of *amyA* was determined and encoded a protein of 976 amino acids with a calculated *M*r of 106,964. High sequence similarity was demonstrated between the *B. fibrisolvens* α-amylase and other α-amylases in the three highly conserved regions which constitute the active centre. Conserved regions were all located in the N-terminal half of the *B. fibrisolvens* amylase and no homology to other amylases was detected for the remainder of the protein. Approximately 40% of the C-terminal region of the protein could be deleted without loss of enzymatic activity. The *B. fibrisolvens* α-amylase degraded amylose, amylopectin and soluble starch with maltotriose as the major initial hydrolysis product.

A gene (*glgB*) encoding a glycogen branching enzyme, the activity of which produced clearing on starch azure plates, was isolated. The *glgB* gene was expressed from its own promoter in the host *E. coli* and encoded a protein of 639 amino acids with a calculated *M*r of 73,875. The deduced amino acid sequence of the *glgB* gene showed high sequence homology (46-50%) to other branching enzymes. The branching enzyme was purified to homogeneity and the properties of the purified enzyme were investigated. Optimal activity of the branching enzyme was at pH 7.2 and 37°C. The branching enzyme was shown to transfer chains of between 5 to 10 glucose units using α-1,4 glucans as substrates, and to stimulate the "de novo" synthesis of a polysaccharide similar to glycogen.

Abbreviations

A	adenosine
A_{420}	absorbance at 420 nm
aa(s)	amino acid(s)
Ap	ampicillin
ATCC	American Type culture collection
ATP	adenosine 5'-triphosphate
AMP	adenosine 5'-monophosphate
bp(s)	base pair(s)
BSA	bovine serum albumin
C	cytidine
C-	carboxy terminal (end of a protein)
Cm	chloramphenicol
CsCl	cesium chloride
CMC	carboxymethylcellulose
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic acid
DTT	1,4-dithio-L-threitol
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
g	standard gravitational acceleration
G	guanosine
G1	glucose
G2	maltose
G3	maltotriose
G4	maltotetraose
G5	maltopentaose
G6	maltohexaose
G7	maltoheptaose
G8	maltooctaose
GARP	goat anti rabbit IgG conjugated horseradish peroxidase
h	hour(s)
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase pairs
kDa	kilodalton(s)
Km	kanamycin
LB	Luria-Bertani broth
M10	*B. fibrisolvens* non-rumen fluid medium
min	minute(s)
mRNA	messenger RNA
M_r	relative molecular mass
MUC	4-methylumbelliferyl β-D-cellobioside
MUG	4-methylumbelliferyl β-D-glucoside

N-	amino terminal (end of a protein)
nt	nucleotides
OD_{600}	optical density at 600 nm
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
ORF	open reading frame
p	plasmid
PAGE	polyacrylamide gel electrophoresis
Pho	alkaline phosphatase
phoA	gene coding for alkaline phosphatase
PNPP	*p*-nitrophenyl phosphate
P_R	rightward promoter (λ)
r	(superscript) resistance
RBS	ribosome binding site
RNA	ribonucleic acid
s	second(s)
s	(superscript) sensitivity
SDS	sodium dodecyl sulfate
sp(p)	specie(s)
T	thymidine
TAE	tris-acetate EDTA buffer
TBE	tris-borate EDTA buffer
TEMED	N,N,N',N'-tetramethylethlenediamine
Tn	transposon
Tris	Tris(hydroxymethyl)aminomethane
Tween	polyoxyethylene soribitan monolaurate
U	units of enzyme activity
UV	ultraviolet (light)
UTP	uridine 5'-triphosphate
v/v	volume/volume
w/v	weight/volume
XP	5-bromo-4-chloro-3-indolyl phosphate
::	novel joint (fusion)
( )	plasmid carrier state

Chapter 1

General Introduction

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General Introduction

1.1 Introduction to *Butyrivibrio fibrisolvens*

1.1.1 General characteristics

The genus *Butyrivibrio* was proposed by Bryant and Small (1956) to describe isolates obtained from cows fed a wide variety of rations. These anaerobic, nonsporeforming, monotrichous, Gram-negative staining rods ferment a range of substrates which result in the production of large amounts of butyric acid. Members of this genus have been found to occur widely in nature and are among the most numerous bacteria isolated from the rumena of cattle, sheep, goats, reindeer, and bison, as well as faecal material obtained from rabbits, horses, and humans (Bryant and Small, 1956; Dehority, 1966; Dehority and Grubb, 1977; Orpin *et al.*, 1985; Varel and Dehority, 1989, and Brown and Moore, 1960). The cells of *Butyrivibrio* are typically short, curved rods 0.4-0.6 μm wide and 2-5 μm in length with tapered ends. These cells are found singly, in pairs, or short chains and are generally motile. Motility is by means of monotrichous flagellum attached in the polar or subpolar region. The cell wall structure of *Butyrivibrio* is interesting, as it consists of elements similar to both Gram-positive and Gram-negative bacteria. Initially this genus was classified as Gram-negative due to the reaction obtained when cells are stained by the standard technique, however it is now thought to be Gram-variable (Beveridge, 1990). During the lag and early exponential stage of growth the cell walls of *Butyrivibrio* cultures retain the gram-positive stain. At this stage the cell walls are composed of a plasma membrane covered by two layers; an inner peptidoglycan-containing matrix and an outer proteinaceous surface layer (S layer). As cells in the cultures age, the peptidoglycan layer becomes progressively thinner and more diffuse so that by stationary phase 95% of cells in the culture stain gram negatively (Beveridge, 1990). The extreme thinness of these cell walls (12-18 nm) is thought to account for their inability to retain the Gram stain (Cheng and Costerton, 1977).

Compounds characteristic of Gram-positive cell walls such as glycerol teichoic acid (Sharpe *et al.*, 1975) and lipoteichoic acid (Hewett *et al.*, 1976), have been isolated from strains of *Butyrivibrio.* Staining with ruthenium red has revealed the presence of extracellular knob-like structures surrounding cells of *Butyrivibrio* spp. (Cheng and Costerton, 1977; Cheng *et al.*, 1989). Similar stuctures have been observed in natural populations of rumen bacteria attached to forage cell walls (Akin, 1976), and it is suggested that these knobs mediate adhesion to cellulose fibres as well as cell-to-cell attachment (Cheng and Costerton, 1977).

Butyrivibrio strains show great variability in their phenotypic and physiological characteristics. All strains were originally classified as a single species, *B. fibrisolvens*, the name being used to describe the organisms' importance in the digestion of fibrous constituents of the food in ruminant rations (Bryant and Small, 1956). Most strains isolated subsequently were added to this species. The great heterogeneity that exists among the strains of *B. fibrisolvens* is illustrated by the number of studies that have attempted to reorganize the isolates into more uniform groups. Shane *et al.* (1969) proposed dividing cellulolytic strains into two groups based on their nutritional requirements and ability to utilize or produce lactate, acetate, and formate. Serological and immunochemical properties have been investigated in order to differentiate between isolates (Margherita and Hungate, 1963; Margherita *et al.*, 1964; Hazlewood *et al.*, 1976). Most strains of *B. fibrisolvens* produce extracellular polysaccharides (EPSs) when grown on a defined medium, and the differences in the EPSs compositional features have been used to sort isolates into different groups (Stack, 1988; Mannarelli *et al.*, 1990b). Several unusual sugars such as L-altrose, 4-*O*-(1-carboxyethyl)-D-galactose and 4-*O*-(1-carboxyethyl)-L-rhamnose, only found in the EPS of *Butyrivibrio* strains were identified (Stack *et al.*, 1988; Mannarelli *et al.* 1990b). *B. fibrisolvens* strains H17c and 49 also contain an acidic sugar of the lactyl ether type and are classified together as type IV B strains. Similar groups were also identified when the DNA relatedness of *B. fibrisolvens* strains were examined (Mannarelli, 1988; Mannarelli *et al.*, 1990b). Once again *B. fibrisolvens* strains H17c and 49 were grouped together and had a DNA relatedness of 96%.

As a result of the great variability found for G + C content as well as DNA hybridization values, the species *B. fibrisolvens* is suggested to comprise a number of distinct species and possibly several genera (Mannarelli, 1988). A new species *B. crossatus*, different from *B. fibrisolvens* in that it has lophotrichous flagella, produces no gas, and ferments only a few carbohydrates, was isolated from human rectal material (Moore *et al.*, 1976). The type strain has recently been shown to be closely related genetically to five strains of *B. fibrisolvens* isolated from the rumina of bison (Mannarelli *et al.*, 1990b). Cheng *et al.* (1989) characterized two strains, B-385-1 and 2-33, and suggest due to their biochemical characteristics and cell wall structure that these are strains of a different species of *Butyrivibrio*. These strains which are large and have subpolar tufts of flagella often replace *B. fibrisolvens* under acid conditions in the rumen (Bryant, 1984). They were originally not considered to belong to the *Butyrivibrio* genus (Hespell and Bryant, 1981).

1.1.2 Enzymes involved in the hydrolysis of complex substrates

One of the reasons for the success of *B. fibrisolvens* in terms of abundance and persistance during adverse conditions is its ability to ferment a wide range of substrates (Table 1.1). Strains of *B. fibrisolvens* such as H17c, are able to hydrolyze the major polysaccharides found in plant materials (Dehority, 1966). Total enzyme activity studies involving crude extracts of *B. fibrisolvens* have previously generated most of the information, however with the advent of recombinant DNA technology individual enzyme characteristics are now known.

Cellulases: *B. fibrisolvens* is one of the most important cellulolytic rumen bacteria in domestic animals fed poor quality forage, or in wild animals surviving austere nutritional conditions (Margherita and Hungate, 1963; Shane *et al.*, 1969; Orpin *et al.*, 1985). However in domestic animals under normal conditions other ruminal bacteria such as *Ruminococcus albus*, *R. flavefaciens*, and *Fibrobacter succinogenes*, are more important in cellulose degradation. The hydrolysis of cellulose to glucose usually requires the combined activity of four enzymes *viz.*, endoglucanases, cellobiohydrolases, cellodextrinases and β-

glucosidases. Two endo-β-1,4-glucanase genes from *B. fibrisolvens* have been cloned and expressed in *E. coli* (Berger *et al.*, 1989; Hazlewood *et al.*, 1990). These enzymes have high activity towards β-1,4-glucans, but low activity towards xylan and cellobiosides. The deduced amino acid sequences of these two genes share no homology although each show seperate similarity to other endoglucanases (Berger *et al.*, 1989; Hazlewood *et al.*, 1990). In addition, a cellodextrinase gene (*ced1*) and β-glucosidase gene (*bglA*) have been cloned and sequenced from *B. fibrisolvens* H17c (Berger *et al.*, 1990; Lin *et al.*, 1990). The enzyme encoded by *ced1* rapidly hydrolyzes short chain cellodextrins to produce either cellobiose or cellobiose and glucose as endproducts. The *bglA* encoded enzyme degrades short chain dextrins predominantly to glucose. The deduced amino acid sequences of these enzymes show homology to other cellodextrinases and β-glucosidases respectively.

Table 1.1 Substrates fermented by *B. fibrisolvens* (after Stewart and Bryant, 1988).

Acid from:	
starch	d
cellulose	d
xylan	+
pectin	+
maltose	d
cellobiose	d
sucrose	d
D-xylose	d
L-arabinose	+
glucose	+
fructose	+
galactose	+
mannose	+
lactose	d
Aesculin hydrolysis	d
H_2S production	d
Fermentation products:	
Major	F,B,A
Minor/some strains	L,S
Gas	H_2CO_2

Abbreviations: A = acetate, B = n-butyrate, F = formate, L = lactate, S = succinate, d = reaction varies between strains, + = positive reaction.

Xylanases (hemi-cellulases): *B. fibrisolvens* is one of the most important rumen bacteria involved in the degradation of xylan (Hespell *et al.*, 1987). A number of

cellulolytic rumen bacteria while being capable of hydrolyzing a considerable amount of xylan are unable to utilize the resulting endproducts (Coen and Dehority, 1970). In contrast, *B. fibrisolvens* can grow on media containing xylan as the only added carbohydrate source (Hobson and Purdom, 1961; Dehority, 1966). Hemicellulose (xylan) accounts for 20-40% of the total carbohydrate fraction of forages, and unlike cellulose does not have a homogeneous composition. The predominant polymer is xylan which is composed of xylose with arabinose and other sugar side chains (Dehority, 1973). Xylan degradation is a multistep process involving xylanases and xylosidases, as well as arabinosidases and acetyl esterases which remove side groups. *B. fibrisolvens* strains (eg. H17c and 49) that possess the key enzymes necessary to degrade xylan have been described (Hespell *et al.*, 1987; Hespell and O'Bryan-Shah, 1988). In these strains the xylanase and acetyl xylan esterase activities are extracellular whereas xylosidase activities are cell associated. Recently, a multienzyme aggregate with predominant xylanolytic activity has been characterized from the supernatant of *B. fibrisolvens* H17c cultures (Lin and Thomson, manuscript submitted). No xylosidase or glucosidase activity could be found associated with the complex although activity in the culture supernatant was detected. Both constitutive and regulated expression of xylanases in *B. fibrisolvens* strains have been reported (Sewell *et al.*, 1989; Mannarelli *et al.*, 1990a). Two xylanase genes *xynA* and *xynB* from *B. fibrisolvens* strains 49 and H17c respectively, have been cloned, sequenced, and expressed in *E. coli.* (Mannarelli *et al.*, 1990a; Lin and Thomson, 1991). On alignment the deduced amino acid sequences of these gene products were found to be 32% identical. An unusual gene (*xylB*) from *B. fibrisolvens* GS113 encoding both xylosidase and α-L-arabinofuranosidase activities has been sequenced (Utt *et al.*, 1991). The expression of xylanase and xylosidase activities in this strain is regulated, and the *xylB* gene is proposed to reside in an operon with two other ORFs which share deduced amino acid sequence similarity with other xylan degrading enzymes. Factors such as the bifunctional nature of the xylosidase and the multienzyme xylanase complex could optimize the digestion of hemicelluloses by *B. fibrisolvens*.

Pectinases: *B. fibrisolvens* is one of the important pectinolytic species in the rumen which can degrade pectin from grasses (Dehority, 1969). Pectin, an important constituent of the middle lamella joining plant cells, is composed of chains of α-1,4-linked galacturonic acid units in which some groups are esterified with methanol and others substituted with acetyl groups (see Chesson and Forsberg, 1988). The pectinolytic enzymes are mainly divided into pectin esterases which catalyze the removal of methanol, and depolymerizing enzymes which are either hydrolases or lyases. An extracellular pectinolytic enzyme, expectate lyase, produced by *B. fibrisolvens* 718 cleaves substrates from the reducing end resulting in the formation of unsaturated trigalacturonate (Wojciechowicz *et al.*, 1982). This enzyme is unusual as other major pectinolytic species in the rumen produce mainly endopectate lyases. A pectin esterase was also detected in the culture fluid. An intracellular pectinolytic enzyme that degrades de-esterfied substrates such as digalacturonate has also been isolated from the same *B. fibrisolvens* strain (Heinrichová *et al.*, 1985). This enzyme hydrolyzes monosaccharide units from the non-reducing end to produce D-galacturonic acid, and is classified as an exo-D-galacturonanase.

Amylases: *B. fibrisolvens* is an unusual cellulolytic bacterium in that it can utilize starch as well as cellulose and xylan as carbon sources. In the Svalbard reindeer *B. fibrisolvens* is the dominant starch-fermenting rumen bacterium during the harsh winter conditions (Orpin *et al.*, 1985). *B. fibrisolvens* is the predominant organism in domestic animals fed a high-roughage diet and one of the principle amylolytic bacteria in animals fed a high-concentrate diet (see Dehority and Orpin, 1988). Despite the importance of starch in producer diets for ruminants, the mechanism of starch degradation by rumen bacteria has been little studied. As this forms one of the main thrusts of this study, rumen bacterial amylases are reviewed in greater detail later.

Other enzymes: A number of *B. fibrisolvens* strains have been isolated which produce extracellular proteases (Fulgham and Moore, 1963). In general, the predominant proteolytic bacteria belong to the major genera of saccharolytic

ruminal bacteria such as *B. fibrisolvens, Bacteroides ruminicola* and *B. amylophilus*. The distribution of proteolytic activity of *B. fibrisolvens* strains as well as the general properties of proteolytic activity produced by strains 49 and H17c have been described (Cotta and Hespell, 1986; Strydom *et al.*, 1986). The characteristics of the proteases from *B. fibrisolvens* 49 and H17c are very similar. They are both mainly extracellular, serine-like, and activity is positively correlated with growth rate. In *B. fibrisolvens* H17c ten protease bands of varying *M*r were detected using SDS-PAGE analysis (Strydom *et al.*, 1986).

Lipases and esterases from *B. fibrisolvens* strains which degrade triglycerides and aliphatic esters to their component fatty acids and glycerol have been investigated (Lanz and Williams, 1973; Hespell and O'Bryan-Shah, 1988; see review Harfoot and Hazlewood, 1988). In addition, a natural occurring general fatty acid auxotroph, *Butyrivibrio* S2, which requires a range of saturated and unsaturated fatty acids for growth has been studied (Hazlewood and Dawson, 1979; Hazlewood *et al.*, 1983). Phospholipase and galactolipase activities are associated with this strains plasma membrane.

1.1.3 Genetics of *B. fibrisolvens*

Information available on the genetics of *B. fibrisolvens* and rumen bacteria in general is limited (see Hazlewood and Teather, 1988). However, a number of features can be deduced from the examination of the cloned *B. fibrisolvens* genes. All of the *B. fibrisolvens* genes cloned to date have been expressed in the host organism *E. coli*, with most of these genes having been expressed from endogenous promoters. This heterologous expression implies that the DNA sequences responsible for the regulation of transcription and translation, for example promoter sequences, ribosomal binding sites, stop and start codons, must be compatible with the *E. coli* biosynthetic machinery. Moreover, the secretion of the enzyme encoded by the *B. fibrisolvens end1* gene (Berger *et al.*, 1989) into the periplasmic space in *E. coli* implies that this gene product possesses a signal peptide recognized by the hosts protein export system. The gene

product of *ced1* (Berger *et al.*, 1990) has no recognizable signal sequence, but in some way this cellodextrinase is secreted into the *E. coli* periplasm. How this enzyme is secreted in *B. fibrisolvens* is unknown. The signal sequences of the *B. fibrisolvens* extracellular enzymes are not always recognized in *E. coli*, and some expressed proteins are accumulated in the cytoplasm, for example the xylanases (Mannarelli *et al.*, 1990a). Information on the regulation of gene expression and the secretion of proteins in *B. fibrisolvens* is not yet available and requires further investigation.

Other than the isolation and characterization of a few genes of interest, the ability to genetically manipulate rumen bacteria is limited at the moment. Suitable genetic transfer systems are required for the mobilization of cloned genes. Endogenous plasmids encoding advantageous functions (antibiotic resistance) may provide a basis for cloning vectors and a system for DNA transfer as in conjugation. Naturally occuring plasmids have been isolated from *B. fibrisolvens* (Teather, 1982; Mann *et al.* 1986), *B. ruminicola* (Flint and Stewart, 1987), *Ruminococcus* spp. (references in Hazlewood and Teather, 1988), and *Selenomonas ruminantium* (Dean *et al.*, 1989; Martin and Dean, 1989). Flint *et al.* (1988) demonstrated that a 19.5 kb plasmid from *B. ruminicola* was associated with tetracycline resistance. However the functions specified by most of these plasmids is unknown. A small cryptic plasmid from *B. fibrisolvens*, pOM1, was ligated to the *E. coli* vector, pBR325, in order to construct an *E. coli-B. fibrisolvens* shuttle vector (Mann *et al.*, 1985; 1986). These plasmids although stably maintained in *E. coli* have not yet been successfully transformed into *B. fibrisolvens*. Conjugation has been used to transfer broad host plasmids from *E. coli* to a *B. fibrisolvens* strain resistant to the antibiotic streptomycin (Teather, 1985). However, the method using *B. fibrisolvens* sphaeroplasts in the presence of PEG was inefficient, and in the few transformants obtained the plasmids were unstable. Russel and Wilson (1988) reported that an antibiotic resistance plasmid from *E. coli* has been transferred to *B. ruminicola* by conjugation.

Besides the problem of establishing genetic transfer systems for rumen bacteria, various constraints exist for the establishment of a genetically engineered bacterium in a natural ecosystem like the rumen. Energy sources are usually limiting and competition intense as microbial populations are relatively high and there is a short turnover time in the rumen (Patterson, 1989). Factors like substrate affinity, maintenance energy expenditures, resistance to toxic substances, attachment to solid surfaces, and the ability to tolerate periods of nutrient starvation can be critical to the survival of rumen bacteria (Russell and Wilson, 1988). As enumeration studies have shown that non-rumen bacteria do not compete well in the rumen (Russell and Wilson, 1988), native rumen bacteria would therfore be the choice for genetic manipulation of this microbial ecosystem. Because *B. fibrisolvens* has great biochemical diversity, low maintenance energy expenditures (Russell and Baldwin, 1979), can attach to substrates, and is able to withstand cycles of nutritional abundance and starvation, it was chosen for this study. Strain H17c was specifically selected because it can degrade the major plant polysaccharides (Dehority, 1966). The present study was aimed at gaining further insight into the role this organism plays in the rumen. The study of the genes at a molecular level may contribute to the understanding of gene regulation in this anaerobic rumen organism, and open the way to manipulation of gene expression using techniques such as site-directed mutagenesis. At the time of this study the cellulases of *B. fibrisolvens* H17c were already being investigated using recombinant DNA technology (E. Berger, PhD thesis, Dept. of Microbiology, UCT). It was decided to construct an additional genebank of *B. fibrisolvens* H17c DNA using an *E. coli-B. subtilis* shuttle vector, and to screen in *E. coli* for genes involved in starch and cellulose degradation, as well as nitrogen regulation.

1.2 Bacterial amylolytic enzymes

Amylolytic enzymes capable of hydrolyzing the α-glucosidic linkages of starch are produced by microorganisms, animals and plants. Different aspects of these starch degrading enzymes have been reviewed, namely the activity of these

enzymes (Fisher and Stein, 1960; Takagi *et al.*, 1971; Ingle and Erickson, 1978), their molecular aspects (Vihinen and Mäntsälä, 1989), and applications to starch related industries (Norman, 1978; Kennedy *et al.*, 1988).

1.2.1 Starch

Starch consists of a mixture of linear (amylose) and branched (amylopectin) homopolymers of α-D-glucose. Amylose is made up of chains of α-D-glucopyranose units predominantly linked by α-1,4 bonds, with very occasional α-1,6 branch points (Hizukuri *et al.*, 1981). It has an average degree of polymerization of between 10^2 and 4 X 10^5 and in solution assumes a helical structure. Amylopectin contains short linear chains of α(1,4) linked α-D-glucopyranose residues joined by α(1,6) linkages to form a highly branched structure. Amylopectin is similar to but less branched than glycogen, a storage polysaccharide found in animals and bacteria. Using amylolytic enzymes the fine structure of amylopectin has been elucidated (Whelan, 1966; Gunja-Smith *et al.*, 1970). It was shown that amylopectin has an asymmetrical bimodal distribution of unit chain lengths, a major peak with an average length of 20, and a lesser peak in excess of 50 units. Models have been proposed for the structure of starch, all of which include the presence of three types of 1-4 linked glucan chains:

(a) Those linked to the molecule by the reducing group only (A chains)

(b) Those similarly linked, but also carrying side-chains linked to the 6 position of one or more residues (B chains)

(c) Those as for B, but carrying a free reducing end (C chains).

Models differ in the arrangement of the three chain types; currently it is thought that a cluster model with the ratio of A:B chains of 1:1 and a single C chain per molecule explains the structure (see Chesson and Forsberg, 1988). Some properties of starch change with source and age, for example the ratio of amylose:amylopectin, the degree of branching, the length of branches and the absorption spectrum of the iodine-starch complex. Amylopectin normally comprises about 80% of starch, although this value ranges between 48% in

Amylomaize and 100% in Waxy maize (Williams, 1968). Starches from various sources usually have a characteristic granule structure both in the external morphology and in the internal semi-crystalline structure. The amylase substrate is therefore complex and amylolytic enzymes have different specificities presumably to catalyze the hydrolysis of the various substrate forms.

1.2.2 Enzymes involved in the degradation of starch

A number of different amylolytic enzymes can hydrolyze starch and they are classified according to their mode of action (Fig. 1.1). Amylases are broadly divided into endoamylases, exoamylases and debranching enzymes.

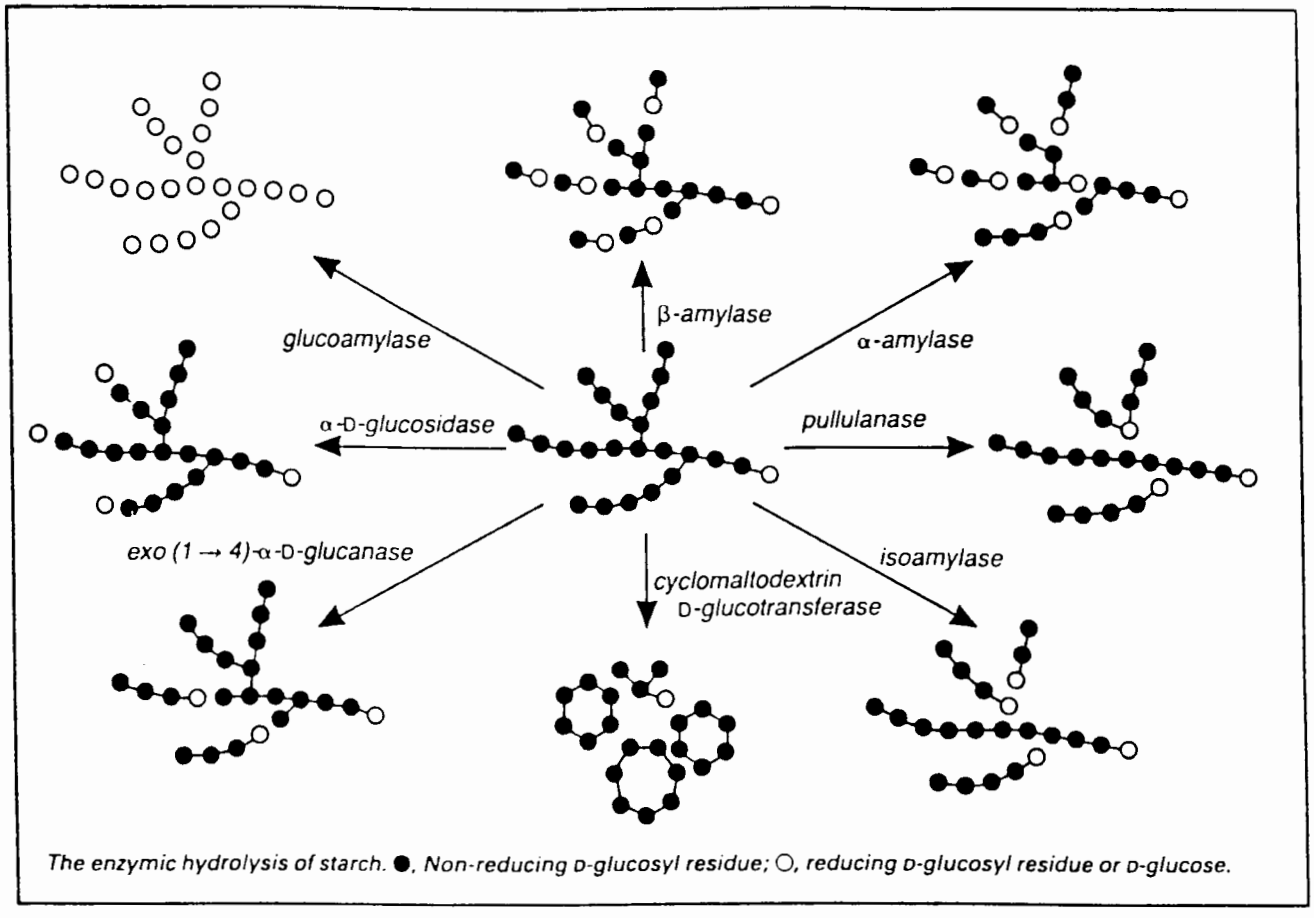


Fig. 1.1 Schematic representation of the enzymes involved in the degradation of starch and their enzymatic mode of action (after Kennedy *et al.*, 1988).

The **endoamylases** cleave α-1,4 glucosidic linkages in amylose, amylopectin and related polysaccharides. They hydrolyze linkages randomly located in the inner regions of the substrate. This results in a rapid decrease in both the molecular weight and the viscosity of starch solutions as well as a decrease in the iodine staining power of the substrate. Endoamylases are able to bypass but not cleave the α-1,6 branch points in amylopectin (Robyt and Whelan, 1968a). The main products of hydrolysis are initially oligosaccharides of varying chain length which are later broken down to yield low molecular weight oligosaccharides and branched chain products. Most of the endoamylases are α-amylases (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase), so called as the hydrolysis products have an α-configuration on the reducing glucose unit (Robyt and Whelan, 1968a). These enzymes are most relevant to this study and their biochemical and molecular characteristics will be detailed further on.

Exoamylases also cleave α-1,4 glucosidic linkages in starch and related polysaccharides but their manner of attack differs from endoamylases. Hydrolysis of the α-glucosidic bonds occurs from the non-reducing chain end in a stepwise manner resulting in the successive release of low molecular weight products such as glucose and maltose. Due to this action a slow decrease in the molecular weight, viscosity and iodine staining power of starch solutions occurs. Hydrolysis products generally have a β-anomeric configuration of the reducing glucose unit (Robyt and Whelan, 1968b). Exoamylases have been shown to fall into different classes, namely β-amylases, glucoamylases and α-glucosidases.

β-Amylases (EC 3.2.1.2, 1,4-α-D-glucan maltohydrolase) release β-maltose units from the non-reducing end of starch. These enzymes are unable to bypass or cleave the α-1,6 glucosidic branch points and produce high molecular weight β-limit dextrins. β-Amylases are mainly of plant origin (Robyt and Whelan, 1968b) although several β-amylases from bacteria have been characterized, cloned and sequenced (Vihinen and Mäntsälä, 1989). Comparisons of the deduced amino acid sequences show an approximately 32% sequence identity and the presence of homologous regions in β–amylases from diverse genera (Kitamoto *et al.*, 1988).

β-Amylases are mainly extracellular enzymes which are stabilized by starch and used industrially to produce maltose containing syrups.

Glucoamylases (EC 3.2.1.3, α-1,4-D-glucan glucohydrolase) release β-glucose from starch and related substrates and have a low degree of specificity. In addition to α-1,4 linkages they are able to cleave α-1,3 and α-1,6 linkages in maltooligosaccharides although at a reduced rate. These extracellular enzymes occur in multiple forms and can also catalyze a reverse-type reaction producing maltose and isomaltose from molecules of glucose. Glucoamylases are commercially important enzymes used in the production of high glucose and fructose syrups, and ethanol. Digestion of, and adsorption to, raw starch granules are characteristics of glucoamylases which have potential industrial application (Hayashida, 1988). Although produced mainly by fungi, a number of bacterial glucoamylases have been characterized, cloned and sequenced (Vihinen and Mäntsälä, 1989).

α-Glucosidases (EC 3.2.1.20, α-D-glucosidase glucohydrolase) are intra- or extracellular enzymes that hydrolyze α-1,4 and α-1,6 linkages in short chain dextrins produced by the action of other amylolytic enzymes on starch. These enzymes, which release α-glucose, occur widely in nature, generally in association with amylases. α-Glucosidases also catalyze transglycosylation, whereby a glucose residue is transferred to a sugar acceptor eg. fructose, mannose (Chiba, 1988). Microbial α-glucosidases have been reviewed (Kelly and Fogarty, 1983; Vihinen and Mäntsälä, 1989) and differ from glucoamylases in both the anomeric configuration of the glucose released and the rapid hydrolysis of low molecular weight oligosaccharides. Large polysaccharides are digested slowly if at all. These enzymes have industrial applications, for example if used in conjunction with glucoamylase the α-glucosidases could hydrolyze the glucoamylase reversal products in the manufacture of high glucose syrups (Norman, 1979).

The classification of the **exo-α-amylases** is not clearly defined. These enzymes generally hydrolyze α-glucans in an exoglucolytic manner from the non-reducing ends to produce specific maltooligosaccharides. Maltose, maltotriose, and maltotetraose are examples of the specific hydrolytic products, but all with an α-configuration at the reducing end (Nakakuki *et al.*, 1984). Both exo- and endo-type activities have been reported for the maltotetraose forming amylase from *Pseudomonas stutzeri* (Schmidt and John, 1979). This enzyme has an exo-activity but can hydrolyze a water insoluble, cross-linked blue starch normally only considered to be hydrolyzed by α-amylases. Deduced amino acid sequences of the maltohexaose-producing amylases from *Bacillus* spp. (Tsukamoto *et al.*, 1988; Shirokizawa *et al.*, 1990), the maltopentaose-producing amylase from a Gram-positive bacterium (Candussio *et al.*, 1990), and the *P. stutzeri* G4-amylase (Fujita *et al.*, 1989) all show homology with regions conserved in α-amylases (Nakajima *et al.*, 1986) but not in β-amylases or glucoamylases. Thus these amylases seem to be intermediates between endo- and exoamylases. Other bacteria producing exo-α-amylases are reviewed by Vihinen and Mäntsälä (1989). Although not currently used in industry these enzymes could produce reagents for the determination of serum amylase activity, as well as nutrient feeds for infants, the aged and patients with renal failure or patients in a state of calorific deprivation (Kennedy *et al.*, 1988).

Debranching enzymes cleave the α-1,6 glucosidic linkages that form the branch points in starch and related polysaccharides either through a direct or indirect action (Lee and Whelan, 1971). Enzymes of the former kind occur in higher plants and microorganisms whereas those of the latter kind occur in yeasts and mammals. There are two main types of direct debranching enzymes, namely isoamylases and pullulanases:

Isoamylases (EC 3.2.1.68, glycogen 6-glucanohydrolase) have been isolated from a few bacterial strains (Vihinen and Mäntsälä, 1989). These enzymes can hydrolyze the α-1,6 linkages in amylopectin, glycogen, branched dextrins and oligosaccharides but not in pullulan. Pullulan is a related α-glucan consisting of

maltotriose units linked by α-1,6 glucosidic bonds. The extracellular *P. amyloderamosa* isoamylase has been characterized, cloned and sequenced (Yokobayashi *et al.*, 1970; Amemura *et al.*, 1988). The deduced amino acid sequence has three regions homologous with those conserved in α-amylases as well as a region of significant homology to pullulanases in the C-terminal domain. Transcription of the *P. amyloderamosa* isoamylase gene is induced by maltose and not repressed by glucose (Fujita *et al.*, 1989). The action of isoamylases is thought to act endo-lytically towards amylopectin, but exo-lytically towards glycogen removing most branched linkages (Yokobayashi, 1988). Isoamylase has been successfully used to investigate the structure of these substrates (Gunja-Smith *et al.*, 1970).

Pullulanase (EC 3.2.1.41, pullulan 6-glucanohydrolase) hydrolyzes the α-1,6 branch points in pullulan and other branched polysaccharides but not in native glycogen. The pullulanases have been classified into four types according to their action on pullulan: pullulanase, which hydrolyzes the α-1,6 glucosidic linkages to produce maltotriose; isopullulanase (EC 3.2.1.57), which hydrolyzes the α-1,4 glucosidic linkages to produce isopanose; neopullulanase, which hydrolyzes the α-1,4 linkages to produce panose; glucoamylase, which produces glucose from the non-reducing ends (Saha and Zeikus, 1989). A few different bacterial pullulanases have been cloned and in some cases sequenced (Vihinen and Mäntsälä, 1989; Kuriki and Imanaka, 1989). As was shown for isoamylases, the deduced amino acid sequences of pullulanases have regions with high homology to the conserved regions of α-amylases. The conventional pullulanase cleaves α-1,6 linkages in pullulan at random, although some pullulanase-amylase enzymes have been described that cleave both the α-1,6 linkages in pullulan and the α-1,4 linkages in starch (Saha and Zeikus, 1989). The reason why pullulanases cleave α-1,4 linkages in starch is not clear and requires further investigation. Pullulanases are used in the food and the brewing industries in conjunction with other amylases.

Cyclodextrin glycosyltransferases (EC 2.4.1.19, 1,4-α-D-glucan 4-α-D-(1,4-α-D-glucano)-transferase) are unusual enzymes in that they cleave α-1,4 bonds within starch and produce cyclodextrins through the reformation of the α-1,4 bond (Fig. 1.1). Cyclodextrins (CD), also known as Schardinger dextrins, are a homologous series of cyclic oligosaccharides containing six (α), seven (β), or eight (γ) glucosyl residues linked by α-1,4 bonds (French, 1957). Cyclodextrin glycosyltransferase (CTGase) is mainly an extracellular enzyme that has only been isolated from bacteria (Vihinen and Mäntsälä, 1989). These enzymes have been extensively investigated due to their importance in the food and pharmaceutical industries. CGTases from different sources have different specificities, some producing predominantly α-cyclodextrins with others producing mainly γ-cyclodextrins (Vihinen and Mäntsälä, 1989). A number of CGTases mainly from *Bacillus* spp. and *Klebsiella pneumoniae* have been cloned and sequenced (Binder *et al.*, 1986; Takano *et al.*, 1986; Kimura *et al.*, 1987). The *B. circulans* CGTase has been crystallized and the three-dimensional structure determined (Hofmann *et al.*, 1989). Comparisons of the primary structure of CGTases with α-amylases, show them to have similar chain folding properties as well as homologous conserved regions thought to be the active centres. The function of the C-terminal region in CGTases is unclear. It was previously thought to be an additional active site (Kimura *et al.*, 1987) but is now thought to be important in maintaining stuctural integrity and substrate binding (Hellman *et al.*, 1990). Enzymes involved in the degradation of CDs are mostly endo-acting α-amylases and some glucoamylases. A cyclodextrinase from *B. macerans* that can degrade CDs but not synthesise them has been described (DePinto and Campbell, 1968).

1.2.3 The biochemical and molecular characterisitics of α-amylases

α-Amylases are amongst the earliest known enzymes and were first described in 1811 (Fisher and Stein, 1960). The occurence of these amylases is widespread in nature and they have been isolated from microorganisms, animals and plants. α-Amylases constitute a well characterized group of proteins and a large amount of information on their properties, purification, crystallization, cloning, nucleotide

and amino acid sequences is available (see reviews, Fisher and Stein, 1960; Takagi *et al.*, 1971; Ingle and Erickson, 1978; Vihinen and Mäntsälä, 1989). The uses of α-amylases in industry are many and varied (Norman, 1979) and this enzyme is an example where new information has developed in response to commercial needs and scientific cooperation. In a search for enzymes with unusual properties, microorganisms which grow under extreme conditios have been investigated. α-Amylases with thermostable (up to 140°C)(Koch *et al.*, 1990), acid-stable (pH 2.0), alkaline-stable (pH >10.0), and halophilic (2 M salt concentration) properties have been characterized from a number of bacterial species (Vihinen and Mäntsälä, 1989). Raw-starch-digesting α-amylases from *B. subtilis* have recently been isolated and their characteristics investigated (Hayashida *et al.*, 1988; 1990). These enzymes have potential industrial importance as the use of untreated starch would mean saving energy and efficient biomass utilization. The terms saccharifying and liquefying have been used to describe the properties of α-amylases, despite this classification being vague. These terms describe the extent to which starch is hydrolyzed and the end products of hydrolysis in addition to referring to the stage at which the amylases are used in industrial starch processing. In general, saccharifying amylases produce a greater amount of reducing sugars from starch than liquefying amylases (Takagi *et al.*, 1971).

Properties of bacterial α-amylases

Molecular mass, pH and temperature. Most bacterial α-amylases range in size from 40,000 to 60,000 Daltons in size (see reviews). Although α-amylases with very small (10,000) and very large (139,000) *M*r have been reported, this data still requires confirmation from sequence analysis. Exo-α-amylases of 180,000 (Candussio *et al.*, 1990) and 102,598 (Shirokizawa, 1990) Daltons have been deduced from the DNA sequence. The former protein is thought to have arisen by gene fusion as the sequence of the N-terminal domain is similar to α-amylases and that of the C-terminal domain has elements similar to β-amylases. Bacterial α-amylases generally exist as monomeric proteins although dimers and trimers

of *B. subtilis* enzymes have been reported (Robyt and Ackerman, 1973). These latter enzymes form aggregates on interaction with zinc ions. pH optima of α-amylases vary between 2.0 and 10.5 depending on the source (Vihinen and Mäntsälä, 1989) and are influenced by calcium ions. The enzyme is more stable over a wider range of pH in the presence of Ca^{2+}. Like pH, the optimum temperature of α-amylases (from 25°C to 100°C) varies with the source (Vihinen and Mäntsälä, 1989). The temperature optima of some halophilic α-amylases are Ca^{2+} or NaCl dependant. Other factors such as the purity of the enzyme, the presence of calcium ions and the substrate itself influence the thermostability. Starch stabilizes the enzyme because it usually contains a small amount of calcium and the enzyme-starch complex provides greater rigidity of structure and protection. Thermostable α-amylases are usually more stable against denaturing agents than thermolabile enzymes. The stability of the thermophilic *B. licheniformis* amylase is increased by hydrophobic solvents such as octane (Asther and Meunier, 1990). Similarly the thermostable *B. stearothermophilus* α-amylase retains activity after treatment with acetone, ethanol, or 1% SDS (Vihinen and Mäntsälä, 1990). BSA also stabilizes some α-amylases, thought to be due to partial proteolytic protection. Thermostable enzymes, due to their increased structural rigidity, appear more resistant to proteolysis (Vihinen and Mäntsälä, 1989).

Effect of inhibitors and calcium on α-amylases. Metal cations, sulfhydryl group reagents and chelating compounds such as EDTA inhibit most α-amylases. Calcium, which stabilizes amylases, also inhibits some amylases at high concentrations (see Vihinen and Mäntsälä, 1989). This is unusual as α-amylases are in fact classified as metalloenzymes containing calcium as a cofactor (Fisher and Stein, 1960). The extracellular α-amylase from *Clostridium acetobutylicum* is not stimulated by calcium ions or inhibited by EDTA, although the enzyme contains 7 calcium atoms per molecule (Paquet *et al.*, 1991). Removal of calcium from the *B. subtilis* α-amylase by chelation with EDTA at pH 8.0, or by electrodialysis (Imanishi, 1966; Toda and Narita, 1968) resulted in a loss of enzymatic activity with no irreversible denaturation as restoration of the calcium

fully reactivated the amylase. The *B. subtilis* enzyme needs 3 or 4 gram atoms of calcium per mole, for full activity. It is thought that a tight metal-chelate structure is formed producing intramolecular cross-links similar in function to disulfide linkages (Imanishi, 1966). Native enzyme molecules usually bind more than three calcium atoms per mole, and up to ten can be present. The additional atoms are bound with weaker affinity and have a stabilizing effect rather than being essential for enzymatic activity. The conformation of the calcium-free enzyme usually becomes more labile resulting in a greater sensitivity to changes in pH, temperature, proteolytic degradation and denaturing agents. The *Aspergillis oryzea* α-amylase (Taka-amylase A) has one essential calcium ion that is tightly bound, possibly to the sulfhydryl groups of the cysteine residues (Toda *et al.*, 1968). The sulfhydryl group does not seem to be part of the active site of the enzyme but is thought to maintain the active configuration by calcium chelation. Similarly, the *B. subtilis* var. *amylosaccharitícus* α-amylase has a single masked sulfhydryl moiety which together with calcium is essential for enzymatic activity (Toda and Narita, 1968). In some α-amylases, other ions such as strontium and magnesium can replace the calcium with no great change in activity (Vihinen and Mäntsälä, 1989), but calcium could not be replaced by other divalent cations in the refolding process of reduced Taka-amylase A. Conserved regions in α-amylases are suggested to be involved in calcium binding (Buisson *et al.*, 1987).

Catalytic properties and mode of action of α-amylases. The action of α-amylases has been described as random, unlike exo-amylases which hydrolyze a prescribed number of glucosidic linkages from the non-reducing end of the α-glucan chain. The random mode of attack initially gives rise to products of varying molecular mass which are later hydrolyzed to maltooligosaccharides. As discussed previously, α-amylases are classified into saccharifying and liquefying amylases depending on their mode of action. These two types also immunologically distinct in the *Bacillis* spp., with anti-serum raised against the one type only reacting with amylases of the same type (Welker and Campbell, 1967). The *B. subtilis* saccharifying and liquefying amylases both preferentially

hydrolyze maltooligosaccharides with the saccharifying amylase removing a maltotriosyl unit from the non-reducing end, and the liquefying amylase removing a maltohexaosyl or maltopentaosyl unit also from the non-reducing end (Okado, 1968). The former enzyme shows greater affinity than the latter for glucose chains of 2 - 23 units.

The catalytic activity of α-amylase has been investigated using chemical modification, kinetic analysis and difference spectrophotometry. As starch is not a homogeneous substrate and varies with both source and the method of

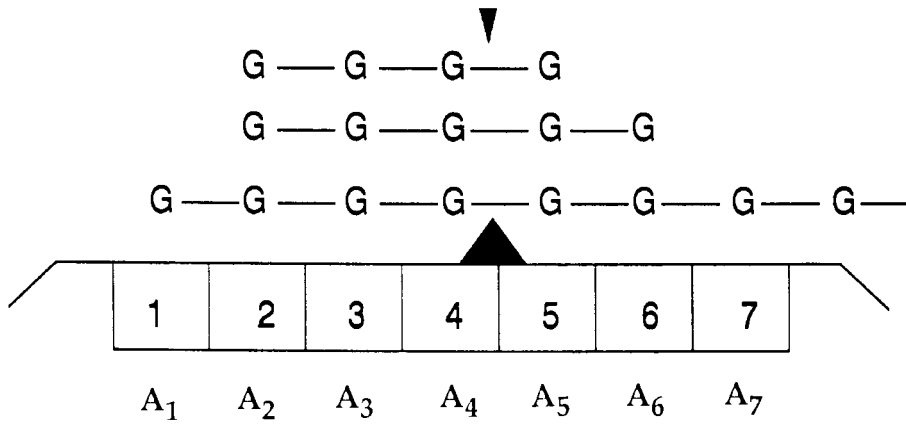


Fig. 1.2 Schematic representation of the active site and subsites with the productive binding mode of substrates of an α-amylase. Subsites per glucose residue (G) of the substrates are numbered 1-7 from the non-reducing end. The catalytic residue (▲) is located between subsites 4 and 5, and each subsite has its own subsite affinity (A_i) towards a glucose residue. The downward arrowhead denotes the position of oligosaccharide cleavage (after Hiromi, 1988).

purification, small oligosaccharides such as maltotriose, maltotetraose, α-, β-, γ-cyclodextrins, and α-*p*-nitrophenyl maltooligosaccharides have been used as investigative substrates (Yoshida *et al.*, 1967; 1969; Onishi *et al.*, 1973; 1975). Amylolytic enzymes have subsites, each of which interacts with a successive glucose residue with an affinity that varies according to the subsite (Hiromi, 1988). The catalytic site is located between these subsites (Fig. 1.2). The number of subsites comprising the active site and the arrangement of subsite affinities are

characteristic of specific amylases. The three-dimensional structure of the liquefying *Bacillus* α-amylases have been modelled and seven glucose residues are thought to be bound by the enzymes (Vihinen *et al.*, cited in Vihinen and Mäntsälä, 1989). Glucose, maltose, and β-cyclodextrin competitively inhibit the hydrolysis of amylose by these liquefying enzymes (Onishi, 1971), possibly due to the formation of a specific inhibitor-enzyme complex in which the inhibitor binds to the active site. The porcine pancreatic α-amylase (PPA) has five subsites and a shift in the optimum pH or mode of attack (random or multiple) can occur depending upon which subsites are occupied (Ishikawa *et al.*, 1990 and Kondo *et al.*, 1990 respectively). Through chemical modification, kinetic and spectral techniques, tryptophan and tyrosine have been found to be present in the subsites of *Bacillus* spp. α-amylases (Onishi *et al.*, 1975; Kochhar and Dua, 1985). Catalytic roles for aspartic acid, glutamic acid, and histidine as well as a binding role for tryptophan residues have been reported for the *B. amyloliquefaciens* α-amylase (Kochhar and Dua, 1984; Dua and Kochhar, 1985). The Taka-amylase (Matsuura *et al.*, 1984) and the PPA (Buisson *et al.*, 1987) are the only α-amylases that have been analyzed by X-ray crystallography. The folding of the PPA molecule is very similar to that of Taka-amylase A and differs only in the orientation of the C-terminal domain. For both enzymes aspartic acid residues have been proposed to be catalytic and residues located on the walls of the active site cleft to be involved in substrate binding. Calcium-binding residues have been proposed for the PPA with the calcium ion stabilizing the active cleft. Correlation between the three-dimensional structure and conserved amino acid regions of α-amylases will be discussed later.

Molecular characterization of bacterial α-amylases

The advent of recombinant DNA technology has led to the investigation of α-amylases at a molecular level. The genetic structure and regulation of α-amylases have become more accessible to research and the possibility of genetically engineering amylases has become feasible. An understanding of the

molecular characteristics is needed before these enzymes can be optimally manipulated for industrial or agricultural use.

Amylase synthesis and secretion in the native host. Most of the work on α-amylase synthesis and expression has been carried out usingthe genus *Bacillus* (see review Ingle and Erickson, 1978). In *Bacillus* the rate of α-amylase biosynthesis is thought to be controlled by both substrate induction and catabolite repression. Starch and related α-glucans are reported to cause an increase in the rate of amylase synthesis in *B. stearothermophilus, B. licheniformis,* and *Clostridium* spp. (Walker and Campbell, 1963; Saito and Yamamoto, 1975; Madi *et al.*, 1987). It has not been fully established however if induction occurs; some strains of *Bacillus* produce α-amylases constitutively. Catabolite repression of amylase synthesis appears to be more uniform and occurs in most species. In strains of *Bacillus*, carbohydrates that increase the growth rate, for example glucose, repress amylase synthesis (see Ingle and Erickson, 1978). The mechanism by which this occurs is obscure. Although regulation of amylase synthesis appears to occur at the transcriptional level, cAMP does not affect transcription in the same way as it does in *E. coli* catabolite-regulatory systems (Preist, 1977). The genetic regulation of amylase synthesis in *Bacillus* will be discussed in a seperate section. α-Amylases are generally produced as extracellular enzymes. Factors controlling both their synthesis and secretion are important especially in their industrial production. The mechanism of exoenzyme secretion in bacteria has not been fully elucidated. Both extracellular and membrane bound α-amylases are produced by *Bacillus* and *Clostridium* strains (Nagata *et al.*, 1974; Annous and Blaschek, 1990). The production of both the membrane-bound and extracellular enzymes of *B. subtilis* are under the direction of the same gene (Nagata *et al.*, 1974). The temporal appearance of the membrane-bound α-amylase suggests that this enzyme is intermediate between the synthesis and release of the exo α-amylase (Mäntsälä and Zalkin, 1979). These findings, together with what is known about the secretion of penicillinase in *B. licheniformis*, support the model proposed by Ingle and Erickson (1978) for the secretion of α-amylases in *Bacillus*. These authors postulated that the

simultaneous transcription and translation of amylolytic-specific mRNA probably does not occur. The untranslated cytoplasmic mRNA becomes associated with membrane-bound ribosomal subunits where the translational process is initiated. A polar phospholipoprotein segment which lodges in the membrane, is synthesized. As the remainder of the molecule is synthesized the enzyme transverses the membrane and enters the external environment. The signal peptide is cleaved by a specific protease and the enzyme released. A similar mechanism of translation is proposed for amylase secretion in *C. acetobutylicum* (Annous and Blaschek, 1990). More research is required however to provide substantive evidence for this type of model.

α-Amylases from cloned genes. Due to its industrial importance, the availability of simple screening techniques as well as *amy* minus host strains, a large number of bacterial amylases have been cloned and sequenced. The structural *amy* genes have originated from a wide spectrum of bacteria (Table 1.2) with an emphasis on *Bacillus* strains (Vihinen and Mäntsälä, 1989). The cloned genes have predominantly been of chromosomal origin although both plasmid (Mielenz, 1983) and chromosomal (Jørgenson *et al.*, 1991) *amyA* genes have been reported in *B. stearothermophilus*. Most of the cloning has been carried out using *E. coli* and *B. subtilis* as the host strains. In *E. coli* the expressed protein is usually transported into the periplasm whereas in *B. subtilis* extracellular recombinant α-amylases are produced. Once a gene has been cloned using an appropriate vector, it can be transferred to the host of choice. The initial cloning of a *B. licheniformis amyA* gene in *E. coli* and subsequent transfer to *Zymomonas mobilis* (Brestic-Goachet, 1990), where the enzyme is secreted into the medium rather than the periplasm, illustrates this point. Plasmid stability can be a problem in host strains but this can be overcome via integration of the cloned gene into the chromosome of the host. *B. amyloliquefaciens amyA* genes have been integrated and amplified into the *B. subtilis* genome from which stable production of the enzyme occurred (Kallio *et al.*, 1987). The incorporation of eight *amyA* genes produced a corresponding increase in the amount of α-amylolytic activity. High expression of a cloned gene can be obtained by in-

frame fusion of the gene to an efficient promoter. The *B. licheniformis* α-amylase gene has been cloned into a *Bacillus* secretion vector which resulted in an increase of expression (Sibakov, 1986). Overexpression of the *B. stearothermophilus* α-amylase in *E. coli* resulted from induced transcription controlled by the *E. coli lac* promoter (Suominen *et al.*, 1987). Hybrid proteins with specific characteristics can be selected for by fusion or homologous recombination of related cloned genes (Matsuzaki *et al.*, 1974). Hybrids between the *B. stearothermophilus* and B. licheniformis *amyA* genes were constructed through selection in *E. coli*, the expressed proteins had intermediate properties (Gray *et al.*, 1986).

Table 1.2 A selection of cloned α-amylases from bacteria showing the host strain in parentheses. The length in amino acids of the deduced or enzymatically determined signal sequence is indicated.

Bacterial strain (host)	Signal sequence	Reference
Aeromonas hydrophila (*E. coli*)	21	Gobius and Pemberton, (1988)
B. amyloliquefaciens (*B. subtilis*)	31	Takkinen *et al.* (1983)
B. licheniformis (*E. coli*)	29	Gray *et al.* (1986)
B. stearothermophilus (*B. subtilis*)	34	Nakajima *et al.* (1985)
Bacillus sp. B1018 (*E. coli*)	27	Itkor *et al.* (1990)
B. subtilis (*E. coli*)	32	Yang *et al.* (1983)
C. thermohydrosulficurium (*E. coli*)	31	Melasniemi *et al.* (1990)
Dictyoglomus thermophilum (*E. coli*)	NP	Fukusumi *et al.* (1988)
Micrococcus sp. (*E. coli*)	ND	Kimura and Horikoshi (1990)
Streptomyces griseus (*S. lividans*)	28	Vigal *et al.* (1991)

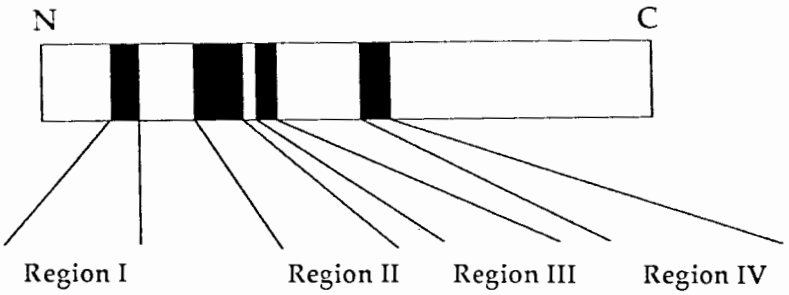
NP = not present, ND = not determined.

α-Amylase gene architecture. The nucleotide sequence analysis of cloned amylase genes has revealed details about the primary structure of these genes

and deduced amino acid sequences of the enzymes. Most cloned amylases have been expressed from their own promoters in the host strain. These promoters usually have regions at -35 and -10 which are recognized by sigma factors in both *E. coli* and *B. subtilis*. The amylase genes of *Dictyoglomus thermophilum* (Fukusumi *et al.*, 1988; Horinouchi *et al.*, 1988) and *Pseudomonas stutzeri* (Fujita *et al.*, 1989) are exceptions to this however, and are expressed from *E. coli* promoters present on the cloning vectors. Inverse repeat sequences which may act as regulatory signals such as transcription termination signals are usually present (Takkinen *et al.*, 1983; Yuuki *et al.*, 1985). The amino acid sequences of the N-terminal amino region have indicated the presence of signal peptides in most cloned α-amylases (Table 1.2). The length of the signal peptide varies between 29 and 41 amino acids (Vihinen and Mäntsälä, 1989). These signal sequences, if recognized by the host strain, usually direct the export of the translated α-amylases to the periplasm in *E. coli* and extracellularly in *B. subtilis*. The *D. thermophilum* amylases are exceptions; their signal sequences, if present, are not recognized in *E. coli* and these enzymes are localized in the cytoplasm rather than the periplasm (Horinouchi *et al.*, 1988). The cleavage site of the signal peptides is generally the same in host and donor strains, for example the *B. stearothermophilus* α-amylase expressed in *E. coli* (Ihara *et al.*, 1985), and *B. amyloliquefaciens* α-amylase expressed in *B. subtilis* (Takkinen *et al.*, 1983). Itoh *et al.* (1990) investigated the effect of peptides inserted between the signal peptide and the mature protein on the secretion and processing of exported α-amylases in *E. coli* and *B. subtilis*. One peptide insertion of 21 amino acids enhanced the amylase production although most insertions had the opposite effect. In addition, the signal peptide cleavage site of the precursor protein differed between *E. coli* and *B. subtilis*.

Amino acid sequence alignments of α-amylases from plants, mammals, and bacteria have indicated the presence of three (Rogers, 1985) or four (Nakajima *et al.*, 1986) conserved regions. Other than these regions, which are spaced at similar intervals along the protein (Fig. 1.3), the overall homology of the diverse α-amylases is generally low (10%). Mammalian amylases however show

sequence similarity of between 80 and 90% and *Bacillus* liquefying amylases of between 64 and 67%.



Amy consensus	DAVINH	GFRLDAAKH	EVID	FVDNHD
Amy TAA	117 DVVAN*H*	202 GLRIDTV*KH*	230 e*VLD*	292 FVEN*Hd*
Amy PPA	96 DAVI<u>N</u>*H*	193 GFRLdASK<u>H</u>	233 *EVI*D	295 FVDN*Hd*
Amy B.s	97 DAVINH	172 GFRFDAAKH	208 EILQ	264 WVESHD

Fig. 1.3 Schematic diagram of the relative location of conserved regions I to IV (Nakajima *et al.*, 1986), and the amino acid consensus sequences for those regions. N and C indicate the NH_2 and COOH termini of a generalized amylolytic protein. The consensus (Amy consensus), Taka-Amylase A (Amy TAA; Toda *et al.*, 1982), pig pancreatic amylase (Amy PPA; Pasero *et al.*, 1986), and *B. subtilis* amylase (Amy B.s.; Yang *et al.*, 1983) sequences of the conserved regions are shown. Substrate binding residues (bold italics), catalytic residues (bold lower case), and calcium binding sites (underlined) of the TAA (Matsuura *et al.*, 1984) and PPA (Buisson *et al.*, 1987) are indicated.

The conserved regions, which are present not only in α-amylases but also in debranching enzymes, CGTases, and exo-α-amylases, are proposed to play an important role in enzymatic activity. This is supported by studies on the three dimensional structure of Taka-amylase A and PPA (Matsuura *et al.*, 1984 and Buisson et al, 1987, respectively). Residues identified as important in catalytic activity, substrate binding, and calcium binding are located in these conserved regions (Fig. 1.3). One conserved region comprises only four amino acids of which only one residue (Glu) is constant in a comparison of 11 sequences (Nakajima *et al.*, 1986). This residue is implicated in substrate binding (Buisson *et al.*, 1987) or catalytic activity (Matsuura *et al.*, 1984). Conserved regions tend to be located in the N-terminal domain of α-amylases. Some of these enzymes have

extended C-terminal domains, the function of which is unclear but which have been implicated in separate amylolytic activity and substrate binding. The *B. polymyxa* amylase gene contains in-phase α- and β-amylase-coding sequences in the 3' and 5' regions respectively (Uozumi *et al.*, 1989). Moreover, the conserved regions of α-amylases are partially duplicated in the *C. thermohydrosulfuricum* α-amylase-pullulanase gene product (Melasniemi *et al.*, 1990). It has been suggested that in raw-starch digesting enzymes the C-terminus is involved in substrate binding (Itkor *et al.*, 1990).

Genetics of α-amylases. Studies on the genetic regulation of α-amylases have dealt mainly with the genus *Bacillus*, in particular *B. subtilis* (reviews Ingle and Erickson, 1978; Yamane, 1988; Vihinen and Mäntsälä, 1989). α-Amylase synthesis in *B. subtilis* is subject to both temporal regulation and catabolic repression. This is illustrated by the expression of α-amylase at the onset of stationary phase in *B. subtilis* and the strong repression of synthesis in the presence of glucose (Yamaguchi *et al.*, 1974). The structural gene coding for α-amylase, *amyE*, and the closely linked regulatory element, *amyR*, have been mapped to the position 25° on the *B. subtilis* chromosome where the gene order is *lin-2-tmrA-amyR-amyE-tmrB-aroI-narB* (Nomura *et al.*, 1978). Allelic *amyE* and *amyR* loci are present in different *B. subtilis* strains. *amyE*n and *amyE*m, coding for different sized polypeptides, are two alleles of *amyE* from *B. subtilis* 168 and *B. subtilis* var. *natto* respectively (Yamane *et al.*, 1984). Two further alleles of *amyE*n, amy2633 and amy1212, differ in their ability to hydrolyze maltotriose (Emori *et al.*, 1990). This has been ascribed to one of the five amino acid residue changes within the two enzymes.

Regulation of *amyE* synthesis occurs at the level of transcription. Thus *amyE* mRNA has been found both to accumulate at the onset of stationary phase and to be repressed in the presence of glucose (Nicholson *et al.*, 1987). Transcription of *amyE* occurs from a promoter located in the 5' linked *amyR* region. Genetic analysis has revealed at least three alleles of the *amyR* locus: *amyR*1 (*B. subtilis*

168), *amyR2* (*B. subtilis* var. *natto*) and *amyR3* (*B. subtilis* var. *amylosaccharitícus*) (Yoneda *et al.*, 1974). These latter two alleles conferred an amylase hyperproducing phenotype on *B. subtilis* 168 transformants. In addition, the *cis*-acting *amyR2* locus conferred resistance to glucose-mediated repression of α-amylase synthesis whereas temporal activation of expression was unaffected (Nicholson and Chambliss, 1985). The nucleotide sequence of *amyR2* has two base differences in the promoter region when compared with *amyR1* as well as an additional A-T rich region within an inverse repeat sequence 5' to the promoter region (Yamazaki *et al.*, 1983). In an analysis of the *B. subtilis amyE* promoter region, Weickert and Chambliss (1989) found that deletion of the 5' A-T rich inverted repeat of *amyR1* and *amyR2* had no effect on expression or transcription of α-amylase. Previously it had been suggested that the *amyR2* inverted repeat was responsible both for the hyperproduction phenotype (Yamazaki *et al.*, 1983) and for repression in the presence of glucose (Takano *et al.*, 1987). The role of transcription terminators for upstream operons was therefore suggested for these inverted repeat structures (Weickert and Chambliss, 1989). This postulate was supported by the finding that a similar inverted repeat sequence in the *B. amyloliquefaciens* α-amylase gene functioned as a transcription terminator for an upstream 2.2 kb operon (Kallio *et al.*, 1986). The hyperproduction of *amyR2* is now thought to be related to weakened catabolic repression. A 34 bp promoter-operator region containing the transcription start site has been postulated to be sufficient to regulate activation of transcription, catabolic repression and thus hyperproduction in *amyR2* mutants (Weickert and Chambliss, 1989). Site-directed mutagenesis of this region identified nucleotide positions important for glucose regulation and an *amyR1* phenotype could be transformed to an *amyR2* phenotype by mutations at some of these positions. Two independently isolated *cis*-acting mutants which conferred the Gra$^-$ (glucose-resistant-amylase) phenotype have identical single base-pair mutations close to the *amyE* transcriptional start-point (Nicholson and Chambliss, 1985; Nicholson *et al.*, 1987). The sequence near the mutation shows structural similarity to the operator regions of the *E. coli gal* and *lac* operons. A *ccpA* gene (catabolite control protein) which codes for a protein with homology to *gal* and

lac repressors may be a trans-acting negative regulator controlling *amyE* expression in response to glucose (Henkin *et al.*, 1991). Disruption of this unlinked gene results in the same Gra- phenotype obtained from the single bp mutations.

Expression of *amyE* in *B. subtilis* is subject to multiple levels of control. Stationary growth phase activation is independent of glucose repression as neither the *cis*-acting *gra*-mutations nor the *trans*-acting *ccpA* mutation result in synthesis of α-amylase during exponential growth. Other genes and their products are also involved in α-amylase synthesis (Vihinen and Mäntsälä, 1989). Mutations such as *sacU, amyB, pap* and *sacQ* have pleiotropic effects which include an increase in α-amylase production. Similarly mutants conferring antibiotic resistance such as *tmrA* (tunicamycin), C-108 (D-cycloserine) and A-2 (ampicillin) cause an increase in α-amylase production. These factors act synergistically, for example *B. subtilis* PP13 which presumably contains the genetic elements *amyR3, amyS, tmr, papS1* and *pap*M118 produces 250 times more enzyme than the parental strain (Yoneda, 1980). Mechanisms controlling these effects are not yet understood.

1.2.4 Amylases of ruminal bacteria

The major species of ruminal bacteria that utilize starch as a growth substrate include *Bacteroides ruminicola, Butyrivibrio fibrisolvens, Ruminobacter amylophilus* (formerly *Bacteroides amylophilus*), *Selenomonas ruminantium, Streptococcus bovis* and *Succinomonas amylolytica* (Russel, 1984). Amylases characterized from ruminal bacteria usually have properties similar to α-amylases isolated from mammalian and other bacterial sources (Hobson and Macpherson, 1952; Walker, 1965; Walker, 1966; McWethy and Hartman, 1977; Cotta, 1988). The final products of amylolytic starch hydrolysis tend to be small maltooligosaccharides, dominated by maltotriose. Experiments with radio-labelled maltodextrins to investigate the action pattern of the *S. bovis* α-amylase, revealed that maltotriose arose from the hydrolysis of the third α(1,4) glucosidic linkage from the non-

reducing end of each dextrin (Walker, 1965). Moreover, only the *S. bovis* extracellular α-amylase has been shown to cleave maltotriose to maltose and glucose (Walker and Hope, 1964). The amylases from *S. bovis*, *C. butyricum* and *R. amylophilus* can hydrolyze and adsorb to raw starch granules producing the same end products as obtained from soluble starch (Walker and Hope, 1964; McWethy and Hartman, 1977). These amylases are more active against maize than potato starch granules. In a recent study, pure cultures of amylolytic bacteria were shown to exhibit different abilities to digest starch in the endosperm of barley, maize and wheat kernels (McAllister *et al.*, 1990). *S. bovis* had the greatest activity against wheat whereas *B. fibrisolvens* was virtually unable to digest the starch in wheat, but was far more active against barley and maize. It was suggested that this latter bacterium digests the protein rather than starch in wheat, and was shown to initially colonize the cell wall material rather than the endosperm (*S. bovis*) or starch granules (*R. amylophilus*) of cereal grains. Variations in cereal grains with respect to starch and protein structure affect the extent to which amylolytic species can utilize cereal starch (McAllister *et al.*, 1990). Effective fermentation of cereal starch requires not only amylases but also the combined action of additional enzymes such as proteases.

α-Amylase synthesis and expression in some ruminal bacteria appears to be regulated. The amylolytic activity of starch- or maltose-grown cultures of *S. bovis*, *B. ruminicola* and *B. fibrisolvens* was greatly reduced in glucose-grown cultures (Cotta, 1988). In contrast *R. amylophilus*, which cannot grow on glucose, seems to produce amylases constitutively. The distribution of amylolytic activity in the former ruminal bacteria also changed in response to carbohydrates present in the growth medium. Growth of these bacteria on starch resulted in amylolytic activity associated with the cell pellet whereas growth on maltose resulted in activity associated with the extra-cellular fluid (Cotta, 1988). In *S. bovis* the cell-bound and extracellular α-amylases have similar properties (Walker, 1965) and may be of the same origin as found for *B. subtilis* (Nagata *et al.*, 1974). The mechanism and significance of the change in enzyme location is unknown. It is possibly related to the formation of an enzyme-substrate complex in starch-

grown cultures (Cotta, 1988) because amylolytic ruminal bacteria attached to starch have a higher specific activity than unattached bacteria (Minato and Suto, 1976). No information on the molecular characterization of ruminal amylases could be found, and it appears that this field of study has been unexplored.

1.2.5 Agricultural applications of amylases

Starch is an important component of ruminant diets and provides an effective source of readily fermentable energy unlike cellulose, which is more refractive to digestion. Amylolytic bacteria increase in domestic ruminants fed high-concentrate diets with *Butyrivibrio, Bacteroides, Selenomonas* and *Streptococcus* the predominant species. In cattle fed both high-forage and high-concentrate diets, amylolytic bacteria comprised 64 to 81% of the total bacterial population (Dehority and Orpin, 1988). High-starch diets are, however, associated with a number of digestive disorders. For example, lactate acidosis is associated with a proliferation of the amylolytic species, *S. bovis*, and is thought to occur as a result of the rapid fermentation of starch leading to an accumulation of lactic acid (Slyter, 1976). Forms of starch such as those from maize, sorghum, and others rich in amylose are poorly degraded in the rumen compared with cereal starches or starches containing low/normal levels of amylose (Szylit *et al.*, 1978). This can lead to a significant proportion of dietery starch escaping ruminal fermentation which reduces its utilization. In addition, amylolytic inhibitory compounds such as tannins which are present in some starches reduce their feed value (Glennie *et al.*, 1982). An understanding of the biochemical, physiological and genetic mechanisms of starch degradation by ruminal bacteria could theoretically be used in devising strategies (genetic manipulation) to enhance or alter the starch-utilizing capacity of some species. Increased ruminant production as well as relief from some digestive disorders would thus be facilitated.

1.3 Glycogen Biosynthesis and Importance in Rumen Bacteria

The biosynthesis and accumulation of glycogen and glycogen-like polymers is wide spread amongst bacteria and has been extensively reviewed (Preiss and Walsh, 1981; Preiss, 1984; Preiss and Romeo, 1989). However, for rumen bacteria little is known about glycogen biosynthetic pathways or the relevance of reserve polysaccharides. The following section deals with the bacterial glycogen biosynthetic enzymes, their regulation and the possible importance of storage polysaccharides in rumen bacteria.

1.3.1 Structure of bacterial glycogens

Bacterial glycogens are similar in structure to those found in mammals. They usually possess 90% α-1,4-glucosidic linkages with the remaining 10% being made up of α-1,6-linkages. The degree of branching as well as the related average chain length and iodiphilic properties of glycogen vary depending on the source (Archibald *et al.*, 1961). The glycogen from *Streptococcus mitis* (Walker and Builder, 1970) and *Enterobacter aerogenes* (Zevenhuizen, 1966) is highly branched. In *Clostridium butyricum* (Hobson and Nasr, 1951) only a limited amount of α-1,6 branches has been reported. In the rumen bacteria, *Bacteriodes succinogenes* (Stewart *et al.*, 1981) and *Ruminococcus albus* (Cheng *et al.*, 1977), the maximum wavelength of the iodine-polysaccharide complex ranges from 425 nm to 550 nm respectively.

1.3.2 Metabolism of glycogen

Bacteria accumulate α-1,4 glucans mainly via the ADP-glucose pathway. Other pathways utilizing sucrose, maltose, or glucose-1-phophate via a phosphorylase reaction are possible. These are however, limited by a number of constraints including bacterial strain, carbon source, glucose repression, and insufficient enzyme activity to account for the amount of glycogen stored (see review Preiss and Romeo, 1989).

In 1964 the reactions of the ADP-glucose pathway for glycogen synthesis were elucidated (Greenberg and Preiss, 1964; Sigal *et al.*, 1964; Shen and Preiss, 1964). ADP-glucose is made via a reaction (1) of ATP and glucose-1-phosphate catalyzed by ADP-glucose pyrophosphorylase (ATP:α-glucose-1-phosphate adenylyltransferase, E.C. 2.7.7.27).

$$\text{ATP} + \alpha\text{-glucose-1-phosphate} \rightarrow \text{ADP-glucose} + PP_i \quad \text{.....(1)}$$

The glucosyl unit from ADP-glucose is transferred to an α-glucan primer to form an additional α-1,4-glucosyl linkage. The reaction (2) is catalyzed by glycogen synthase (ADP-glucose:1,4-α-D-glucan 4-α-glucosyl transferase, E.C. 2.4.1.21).

$$\text{ADP-glucose} + \alpha\text{-1,4-glucan} \rightarrow \alpha\text{-1,4-glucosyl-glucan} + \text{ADP} \quad \text{.. (2)}$$

Later, it was shown that the α-1,6-glucosyl linkages are formed in a reaction catalyzed by the branching enzyme (α-1,4-glucan: α-1,4-glucan -6-glycosyltransferase, E.C. 2.4.1.18) (Sigal *et al.*, 1965).

Complete glycogen or starch degradation requires at least two enzymes: a phosphorylase specific for α-1,4-glucosidic linkages and a "debranching" enzyme capable of hydrolyzing the α-1,6-glucosidic linkage. In plants amylolysis is the dominant activity of starch breakdown (see section 1.2) with phosphorylase and transglycosylase enzymes playing a minor role (Lee and Whelan, 1971). Yeasts and mammals have an indirect debranching enzyme, amylo-1,6-glucosidase/4-α-glucanotransferase, which removes α-1,6 linkages from a phosphorylase limit dextrin of glycogen (Lee and Whelan, 1971). In *E. coli* glycogen degradation is thought to occur by the following system (Preiss and Walsh, 1981). Phosphorylases convert the glycogen into a glucose-1-phosphate phosphorylase limit dextrin. The direct debranching enzyme (Jeanningros *et al.*, 1976), then converts the limit dextrin to maltotetraose and linear maltodextrins. These maltodextrins are then degraded to glucose-1-phosphate. A glycogen

phosphorylase encoded by *glgY(P)* has been identified and found to be part of the glycogen operon in *E. coli* (Romeo and Preiss, 1988; Yu *et al.*, 1988). The enzymes involved in the action on phosphorylase limit dextrins in *E. coli* have not yet been identified. A possible candidate is an α-amylase which has been isolated from *E. coli* and found to be active towards maltodextrins (Freundlieb and Boos, 1986). Amylases produced by other bacteria are mostly extracellular, and the catabolism of the intercellular reserve polysaccharide remains to be determined. A debranching enzyme, isoamylase, which is active towards glycogen has been isolated from several bacteria and may be involved in glycogen catabolism. In *Streptococcus mitis* a transglucosylase has been thought to supplement phosphorylase activity in the catabolism of storage polysaccharides (Walker, 1966). The transglucosylase is considered to transfer glucosyl residues that are inaccessible to phosphorylase action.

1.3.3 Characterization and Regulation of Bacterial Glycogen Biosynthetic Enzymes

The physical and chemical properties as well as the regulation of bacterial ADP-glucose pyrophosphorylases and glycogen synthases have been extensively reviewed (Preiss and Walsh, 1981; Preiss, 1984; Preiss and Romeo, 1989), and will not be discussed in detail here. Rather, a brief overview of these enzymes will be presented. As the branching enzyme is relevant to this thesis it will be discussed in greater detail.

ADP-glucose pyrophosphorylase

Allosteric control of bacterial glycogen synthesis occurs at the first step of the reaction, the synthesis of ADPglucose. Glycolytic intermediates are activators of ADPglucose synthesis, whereas energy metabolites, AMP, ADP, and P_i are inhibitors (Preiss and Walsh, 1981). The ADPglucose pyrophosphorylases can be divided into 7 groups based on their differences in activator specificity (Preiss, 1984). The most prevalent activators are fructose-6-P, fructose 1,6-P2, 3-phosphoglycerate and pyruvate. The variation in activator specificity is related

to the type of carbon metabolic pathway that occurs in the bacterium. The energy charge of the cell is also central to the regulation of glycogen synthesis as ATP is one of the substrates of ADPglucose pyrophosphorylase.

Structural genes *(glgC)* of the *E. coli* and *Salmonella typhimurium* ADPglucose pyrophosphorylases have been cloned and sequenced (Okita *et al.*, 1981; Baecker *et al.*, 1983; Leung and Preiss, 1987a, 1987b). The enzymes have a subunit *M*r of 48 478 and 48 490 respectively, and are highly conserved. The native enzymes occur as homotetramers. Analyses involving chemical modification of the activator sites of *E. coli* ADPglucose pyrophosphorylases indicate that activators and inhibitors must bind at different sites (Preiss and Romeo, 1989). Allosteric activator sites occur in the N-terminal portion of the enzyme and involve charged amino acid residues such as arginine and lysine. The substrate and inhibitor binding sites of *E. coli* ADPglucose pyrophosphorylases have been investigated using photo-affinity labelling agents 8-azido -ATP, -ADP and -AMP (reviewed in Preiss and Romeo, 1989). The substrate binding sites seem to be situated in close proximity in the tertiary structure of the enzyme. A partial overlap of inhibitor sites with both the substrate- and activator-binding sites has also been indicated. Site-directed mutagenesis studies on the substrate-binding site of the *E. coli* ADPglucose pyrophosphorylases were consistent with the idea that adenine nucleotide substrates share a common site with the inhibitor AMP and this site is linked to the activator-binding sites. Therefore the speculation that inhibitor- activator- and substrate-binding sites are proximal to each other in the enzymes tertiary structure is supported and suggests an important interaction of the three separate sites in modulating activity. However, further work on the three-dimensional topography and crystalline structure of the enzyme as well as the structural relationships of the ligand-binding sites is needed (Preiss and Romeo, 1989). Studies involving mutant strains of *E. coli* and *S. typhimurium* show a positive correlation between the affinity of the ADPglucose pyrophosphorylase for activators with glycogen accumulation. The allosteric activator specificity was also shown to involve two amino acid substitutions in mutational studies (Preiss and Romeo, 1989).

The bacterial ADPglucose pyrophosphorylases differ from those of mammals in two major respects: ADP-glucose rather than UDP-glucose is the effective glycosyl donor in bacteria, and the regulation of synthesis in bacteria occurs at the ADPglucose pyrophosphorylase step rather than glycogen synthase step (Preiss and Walsh, 1981).

Glycogen Synthase

Bacterial glycogen synthases unlike the mammalian glycogen synthases exhibit no specific regulatory properties. The enzyme appears to be specific for ADPglucose, and regulated solely by the energy charge and the availability of ADPglucose and primer (Greenberg and Preiss, 1965; Shen and Preiss, 1964; Fox *et al.*,1976). Activity shown with other sugar nucleotides is generally less than 5% of the ADPglucose activity (Cattaneo *et al.*, 1979; Holmes and Preiss, 1979). Two distinct sulphhydral residues in the enzyme are present. One is protected by either ADP or ADPglucose and the other is protected by glycogen (see Preiss and Romeo, 1989). These sulphhydral groups are probably associated with the substrate-binding sites. The bacterial glycogen synthases are usually found in the particulate fraction of the cell, bound to the glycogen. Glycogen synthase can be released from the glycogen by pretreatment with amylase (Fox *et al.*, 1976). Requirements for glycogen synthase activity are usually an α-1,4-glucan primer and ADPglucose. Addition of Mg^{2+} ions stimulates the activity, probably due to the release of inhibition by ADP via the formation of a MgADP complex (Preiss and Greenberg, 1965). Various α-glucans such as glycogen, amylose and amylopectin can serve as primers. Maltodextrins such as maltotriose or maltose but not glucose can also act as primers (Preiss and Greenberg, 1965; Greenberg and Preiss, 1965; Fox *et al.*, 1976). The combined action of glycogen synthase and branching enzyme on the endogenous primer associated with the synthase account for the *de novo* unprimed synthesis of *E. coli* glycogen (Kawaguchi *et al.*, 1978; Cattaneo *et al.*, 1978). This unprimed reaction is stimulated by salts, and while it was previously thought to involve an additional glucoprotein synthase (Barengo *et al.*, 1975), only glycogen synthase is now implicated (Holmes and Preiss, 1979; Cattaneo *et al.*, 1979).

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Immunological studies have shown glycogen synthases of the enteric bacteria *E. coli*, *E. aurescens*, and *S. typhimurium* to be closely related, whereas there is a limited or no relationship between glycogen synthases from *Enterobacter aerogenes*, *Klebsiella pneumonia*, *Serratia marcescens*, *Aeromonas hydrophila*, *Rhodobacter spp.* and *Rhodospirillum molishianum* (Holmes *et al.*, 1982).

The structural gene for glycogen synthase, *glgA*, from both *E. coli* and *S. typhimurium* has been cloned, and the gene from *E. coli* sequenced (Okita *et al.*, 1981; Leung and Preiss, 1987a; Kumar *et al.*, 1986). The subunit *M*r is 52 412, and the native enzyme appears to exist as dimers, trimers and tetramers (Fox *et al.*, 1976). A conserved amino acid Lys-X-Gly-Gly motif was observed in the comparison of amino terminal sequences of the *E. coli* and mammalian glycogen synthases (Furukawa *et al.*, 1990). This motif may be involved in sugar nucleotide binding.

Branching Enzymes.

The synthesis of α-1,6-glucosidic linkages in α-1,4-glucosidic linked glucans is thought to be catalyzed by α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase (branching enzyme, E.C. 2.4.1.18). The branching enzyme is a transglycosylase which catalyses the transfer of a single oligosaccharide chain from a 1,4- to a 1,6-linkage (Larner 1953, Verhue and Hers 1966). Branching enzymes can be divided in two broad groups:

(1) The Q-enzyme of plants which transforms amylose into amylopectin by the introduction of approximately 5% α-1,6-glucosidic linkages.
(2) The branching enzymes found in organisms that are concerned with glycogen biosynthesis. These enzymes normally introduce 10% α-1,6-glucosidic linkages to form the high degree of branching found in glycogen.

A number of reviews are available on starch and glycogen biosynthesis and regulation which include branching enzymes (Preiss and Walsh, 1981; Preiss, 1984; Preiss and Romeo, 1989). The occurrence of branching enzymes has been reported in bacteria, fungi (Matsumoto, 1983), yeasts (Gunja *et al.*, 1960), animals and plants (see above reviews for references). Two methods have mainly been utilized to investigate branching enzyme activity. The one method is qualitative and measures the effect of the branching enzyme on the absorbance of the iodine-complex of various polysaccharides (Gunja *et al.*, 1960). The other method is more sensitive and quantitative. It measures the stimulatory effect of branching enzyme on chain elongating enzymes. The stimulation is due to the provision of additional non-reducing ends in the α-glucan by the branching enzyme which can be used as activity sites for the chain elongating enzymes (Brown and Brown, 1966a; see review Preiss and Walsh, 1981). This latter method is more representative of the physiological process especially when glycogen synthase instead of phosphorylase is used as the elongating enzyme. Chain length units transferred by the branching enzyme have been studied using methods formulated for investigations on amylopectin and glycogen structure (Gunja-Smith *et al.*, 1970; Carter and Lee, 1971).

Bacterial, animal and plant branching enzymes have been the most intensively investigated and will be discussed here.

Bacterial Branching Enzyme

The activity of the branching enzyme has been determined both on its own and in conjunction with other glycogen biosynthetic enzymes (Zevenhuizen, 1964; Walker and Builder, 1971; Boyer and Preiss 1977; Cattaneo *et al.*, 1978; Kawaguchi *et al.*, 1978). Bacterial branching enzymes are active against both amylose and amylopectin resulting in changes in the absorption and wavelength maximum of the iodine-glucan complex spectra. Reactivity of bacterial branching enzymes against glycogen is variable, the *E. coli* enzyme having only limited or no activity (Boyer and Preiss 1977), whereas the *S. mitis* enzyme is more active (Walker and Builder 1971). In the "de novo" unprimed synthesis of

enzyme is more active (Walker and Builder 1971). In the "de novo" unprimed synthesis of glycogen the branching enzyme plays an important role. The branching enzyme is the synthesis activator while the endogenous glucan associated with the glycogen synthase is used as a primer (Cattaneo *et al.*, 1978; Kawaguchi *et al.*, 1978). The stimulation of glycogen synthase catalysis by branching enzymes has been reported for *E. coli*, *S. typhimurium* and *S. mitis* branching enzymes (Boyer and Preiss, 1977, Steiner and Preiss, 1977, and Walker and Builder, 1971 respectively). During *in vivo* glycogen synthesis the branching enzyme is thought to act in a co-ordinate rather than a successive manner with glycogen synthase (Boyer and Preiss, 1977). The presence of salts, which stimulates the unprimed synthesis, may induce a conformational association between the branching enzyme and glycogen synthase necessary for unprimed activity (Cattaneo *et al.*, 1978; Kawaguchi *et al.*, 1978). In *E. coli* the relative ratios of branching enzyme to glycogen synthase affects the product formed (Boyer and Preiss, 1977). Ratios of 1:1 produce a predominantly linear α-glucan whereas the product formed using ratios of 20:1 resemble native glycogens. Branching enzymes due to their glycosyltransferase nature change the average chain lengths of α-glucan substrates. The *E. coli* enzyme transfers a minimum of 5-7 glucose units (Boyer and Preiss, 1977) while the *Arthrobacter globiformis* branching enzyme introduces chain lengths of 14 units (Zevenhuizen, 1964). Action of branching enzymes against β-limit dextrins indicates that these enzymes can also transfer branched chains. The minimum length of maltodextrin against which the *S. mitis* branching enzyme can react is 20 (Walker and Builder, 1977). This value has not been reported for other bacterial enzymes. Branching enzyme activity is stimulated by citrate and inhibited by mercuric chloride (Zevenhuizen, 1964; Walker and Builder, 1971).

Mutants of *E. coli* deficient in branching enzyme have been isolated and reported to accumulate a linear polysaccharide similar to amylose (Lares *et al.*, 1974). The branching enzymes of some enteric bacteria are closely related as determined in an immunological investigation (Holmes *et al.*, 1982). In this report an antibody prepared against the *E. coli* branching enzyme cross reacted with enzymes from

Serratia marcescens. No relationship to the phytobacteria tested could be determined. Glycogen synthesis in *S. mitis* is reported to be an unstable characteristic (Van Houte and Jansen, 1970). Some strains that do not store glycogen are however, reported to have branching enzyme activity (Walker and Builder, 1971).

The *E. coli* branching enzyme has been purified to apparent homogeneity, and occurs primarily as a monomer subunit with an apparent *M*r of 84 kDa (Boyer and Preiss 1977). This enzyme was cloned as part of a glycogen operon (Okita *et al.*, 1981) and the nt sequence determined (Baecker *et al.*, 1986). The *M*r of the deduced amino acid sequence was 84,231 and agrees closely with the electrophoretically determined *M*r of the protein.

Mammalian Branching Enzymes

Branching enzymes from rabbit skeletal muscle (Brown and Brown, 1966a; Brown and Brown, 1966b; Gibson *et al.*, 1971) and rat liver (Krisman, 1962; Verhue and Hers, 1966; Larner, 1953) have been extensively studied. The rabbit muscle branching enzyme has been purified to near homogeneity, and seems to be a monomer protein with a *M*r of 77,000 (estimated from SDS-PAGE) (Caudwell and Cohen, 1980). These properties are similar to bacterial branching enzymes. The liver and muscle branching enzymes are active against amylose and amylopectin molecules. The length of the chain units transferred by the mammalian branching enzymes have been investigated (Larner, 1953; Verhue and Hers, 1966; Brown and Brown, 1966b). Both the liver and muscle branching enzymes have a specificity to transfer chains of 7 glucose units in length, although a minimum of 6 glucose units can be transferred. These chains are moved from the outer main chains and reattached in an α-1,6-glucosidic linkage to form a new side chain. Polysaccharides with an outer chain length of 11-21 glucose units could be branched by the liver branching enzyme, however, a maltodextrin containing 16 glucose units was unaffected. The mammalian branching enzymes are able to stimulate the rate of polysaccharide synthesis catalyzed by phosphorylase or UDP-glycogen synthase. The glycogen

synthesized with UDPglucose as the doner is different from that prepared with glucose-1-phosphate (Parodi *et al.*, 1969). Unprimed synthesis is greatly activated when the branching enzyme and phosphorylase are both present (Brown *et al.*, 1961). When UDP-glycogen synthase is used as the chain elongation enzyme, the branching enzyme can stimulate the synthesis with limited amounts of primer (Brown and Brown, 1966a). Mammalian branching enzymes generally have a broad pH optimum 6.8-7.8, and like bacterial branching enzymes are activated by citrate.

A severe hereditary disorder of glycogen metabolism in humans is associated with the lack of branching enzymes, namely type IV glycogen storage disease (Brown and Brown, 1966c). Purified rabbit muscle branching enzyme is able to introduce further branches into the amylopectin-like polysaccharide isolated from patients with this disease (Gibson *et al.*, 1971). Thus, an increased knowledge of the regulation of branching enzyme synthesis and activity could have medical implications.

Plant branching enzymes (Q-enzyme)

Plant branching enzymes have both a number of similarities and dissimilarities to those of bacteria and mammals. Some of these will be reviewed here. Reported molecular sizes of spinach (Hawker *et al.*, 1974), potato (Borovsky *et al.*, 1975a), and maize endosperm (Boyer and Preiss, 1978) Q-enzymes range between 72 and 89 kDa. Additionally, the purified potato Q-enzyme is composed of only one sub-unit. Both these aspects are in common with bacterial and mammalian branching enzymes. Hawker *et al.* (1974) reported a comprehensive study on spinach leaf starch synthase and Q-enzymes. The Q-enzymes stimulate the unprimed activity catalyzed by both phosphorylase and α-glucan synthase. Therefore, like mammalian and bacterial branching enzymes, the Q-enzymes provide an increased number of non-reducing chain ends for the chain elongating enzymes to use as additional sites. It has been speculated that the *in vivo* formation of amylopectin may also occur by the combined, and

simultaneous action of α-glucan synthase and plant Q-enzymes (Hawker *et al.*, 1974).

One of the main differences of action of Q-enzymes when compared to mammalian and bacterial branching enzymes, is in the end product. Plant Q-enzymes form amylopectin, which is less branched than glycogen, the end product formed in bacteria and mammals. The main substrate of plant branching enzymes is amylose although extra branches have been reported to be introduced into amylopectin (Drummond *et al.*, 1972; Hawker *et al.*, 1974; Borovsky *et al.*, 1975a). In a series of investigations by Borovsky *et al.* (1975a, 1975b, 1976, 1979) the mode of interaction between potato Q-enzymes and substrates was reported. The action of the Q-enzyme was found to be by random endo-type transglycosylation of the substrate chains. This transglycosylation is thought to occur by inter-chain transfer of a chain fragment from a donor chain to an acceptor chain, where an α-1,4-bond is broken in the donor chain and an α-1,6-bond formed in the acceptor chain. Intra-chain transfer has however, not been experimentally excluded. During the transglycosylation reaction the minimum length of amylose chain that can act as an acceptor is greater than 40 glucose units. It is proposed by Borovsky *et al.* (1976) that this is the minimum length required for a stable secondary and tertiary structure of the substrate, which must be established for Q-enzyme interaction. This substrate is thought to have a complex conformation, perhaps a double helix, formed by two substrate chains. The Q-enzyme associates with the complex and introduces inter-chain linkages which both stabilizes the chain interactions and accelerates the branching action. A novel arrangement of unit chains in amylopectin is predicted due to the random action of the Q-enzyme.

Multiple forms of branching enzymes occur in spinach leaf (Hawker *et al.*, 1974), maize endosperm (Boyer and Preiss, 1978a) and algae (Fredrick 1980). Numerous reports of multiple starch synthases and branching enzymes are available (see review Preiss and Walsh, 1981). The function of these multiple forms is not clear. In maize kernels the different branching enzyme fractions of

mutant strains were found to produce different degrees of branched α-glucans (Boyer and Preiss, 1978b). For algae, the branching isozymes have been separated into two formally recognized types: the Q-enzyme which can branch amylose into amylopectin; and the branching enzyme type that can further branch amylopectin into substrates such as phytoglycogen (Fredrick, 1980). Thus it seems that branching isozymes are associated with different degrees of branching although the mechanism of action has not yet been determined. Cyanobacteria like *Anacystis nidulans* are reported to have branching enzyme isozymes capable of both types of activities (Fredrick, 1968), and are reported to store photoglycogen (Weber and Wöber, 1975). Bacteria and mammals in general do not have multiple forms of branching enzymes.

1.3.4 Genetic Regulation of Glycogen Synthesis

The synthesis of glycogen biosynthetic enzymes is under complex regulation influenced not only by nutrient depletion but also by the rate of bacterial growth (reviewed in Preiss, 1984). Allosteric regulation of ADPglucose pyrophosphorylase at the first step of glycogen synthesis (see section 1.3.2) as well as regulation at the level of gene expression seems to be involved (Preiss and Romeo, 1989).

Co-ordinate derepression of glycogen biosynthetic enzymes occurs in both *E. coli* and *S. typhimurium* when cultures grown in rich media go from logarithmic to stationary phase (Preiss and Walsh, 1981; Steiner and Preiss, 1977). This implies a linked regulation under certain conditions. Mutant strains of *E. coli* which accumulate high levels of glycogen and are derepressed in the levels of the glycogen enzymes support this idea (Preiss, 1984). Linked (*glgR*) and trans-acting (*glgQ*) mutations have been identified (Preiss *et al.*, 1983; Preiss, 1984; Preiss and Romeo, 1989). The *glgQ* mutation affects the levels of all three enzymes, whereas the *glgR* mutation affects only the levels of ADPglucose pyrophosphorylase and glycogen synthase.

The *E. coli* and *S. typhimurium glg* genes are closely linked on the bacterial chromosome (Latil-Damotte and Lares, 1977; Steiner and Preiss, 1977). The *E. coli* genes have been cloned on a single genomic fragment (Okita *et al.*, 1981) and the nucleotide sequences of *glgA*, *glgC* and *glgB* determined (Kumar *et al.*, 1986, Baecker *et al.*, 1983, and Baecker *et al.*, 1986 respectively). This gene cluster also encodes the degradative enzyme glycogen phosphorylase, GlgY(P), and contains an additional open reading frame, *glgX*, which encodes a protein which has amino acid sequence homology to the *E. coli* branching enzyme as well as to glucan hydrolases and transferases (Yu *et al.*, 1988; Romeo *et al.*, 1988). Neither of these two proteins are required for glycogen synthesis. The *glg* genes are located next to the *asd* (aspartate semialdehyde dehydrogenase) gene at 75 min on the genomic map of *E. coli* K12. The gene order is illustrated in Fig.1.4.

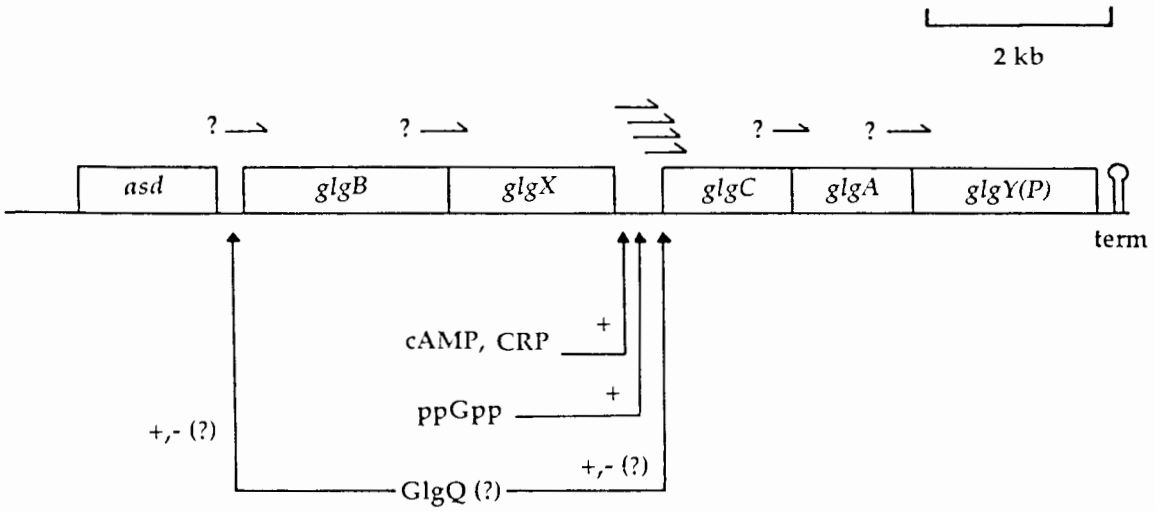

Fig. 1.4. Schematic representation of the 10.5 kb fragment containing the *glg* and *asd* genes of *E. coli* K12. Direction of transcription is indicated by arrows above the map. A question mark represents an unknown origin of transcription. Possible regions of transcriptional regulation are indicated by arrows below the map together with the (possible) transcriptional regulators. A putative transcriptional terminator is represented by the word term (Yu *et al.*, 1988). (after Preiss and Romeo, 1989).

The mechanism by which the transcription of these genes is regulated has been investigated at the molecular level (Romeo and Preiss, 1989; Romeo *et al.*, 1990). No promoter sequences similar to any consensus sequences have been detected for the *E. coli glg* genes. *In vitro* and *in vivo* expression of the *glgC* and *glgA* genes

is under positive control of the global regulatory compounds cAMP and ppGpp. Transcription of the gene coding for the branching enzyme, *glgB*, is unaffected by either compound. Thus it was proposed (Romeo *et al.*, 1990) that although the *glgB* gene is located in a glycogen regulon, it resides in an operon separate from the other two genes. The initiation codon of *glgA* overlaps with the stop codon of *glgC* suggesting co-ordinate transcription. A 0.5 kb region of DNA upstream of the *glgC* translational start site has been identified to be important for transcriptional regulation (see Fig. 1.4). Four discreet transcriptional starts of *glgC* as well as *cis*-acting sites required for cAMP, ppGpp, and *glgQ* gene product effects are known to reside in this region (Romeo and Preiss, 1989; Romeo *et al.*, 1990). The *glgQ* mediated regulatory effects on *glgB* must be in a site separate from these as the *glgB* promoter region is further upstream. The *glgQ* encoded factor does not seem to be involved in either the catabolic repression or the stringent response regulatory systems, which suggests a third independent regulatory system. In a recent investigation on the regulation of stationary phase and starvation gene expression, a sigma factor (σ^s), was recognized as a central regulator (Lange and Hengge-Aronis, 1991). Glycogen synthesis in *E. coli* is greatly reduced by the disruption of this regulatory gene. This implies that this novel sigma factor is one of the factors involved in the regulation of glycogen biosynthesis.

1.3.5 Accumulation and importance of bacterial glycogen reserves with reference to rumen bacteria

Accumulation of storage products in bacteria

In bacteria reserve polymers generally provide both carbon and energy required during times when no external source of nutrients is available. Reserve materials within the cell may be utilized to provide the energy necessary for survival. Once the cell has ceased to grow energy is required for processes such as osmoregulation, pH maintenance, motility, protein and nucleic acid turnover, sporulation and luminescence (Dawes and Ribbons, 1962). Endogenous metabolism is the process by which this maintenance energy is provided and the

compounds metabolized are generally referred to as endogenous substrates. Three criteria have been proposed (Wilkinson, 1959) to distinguish energy storage compounds from other compounds. A compound must be:

(i) accumulated intracellularly when an excess energy supply for growth is available,
(ii) utilized when an exogenous supply of carbon or energy is no longer sufficient for maintenance of growth and viability,
(iii) an energy source which can be utilized by the cell enabling it to survive in its natural environment.

The endogenous substrates recognized in bacteria include carbohydrates (glycogen and polyglucose compounds), lipids (for example poly-β-hydroxybutyrate), protein peptides, amino acids, RNA, and inorganic polyphosphate (volutin). Glycogen is a storage product which is widespread amongst bacteria, it is not restricted to any class and is reported in archaebacteria, Gram-positive and Gram-negative species, cyanobacteria as well as aerobic and anaerobic bacteria. Table 1.3 lists some of the rumen and anaerobic bacteria in which glycogen or glycogen-like compounds are stored.

Table 1.3 The occurrence of glycogen-type reserve polysaccharides in a selection of rumen and anaerobic bacteria.

BACTERIA	REFERENCE
Bacteroides amylogenes	Doetsch *et al.*, 1957
Bacteroides fragilis	Lindner *et al.*, 1979
Bacteroides succinogenes	Stewart *et al.*, 1981
Clostridiun pasteurianum	Mackey and Morris, 1971
Megasphaera elsdenii	Brown *et al.*, 1975
Ruminococcus albus	Cheng *et al.*, 1977
Selenomonas ruminantium	Wallace, 1980

Glycogen is usually accumulated in either stationary phase or under limited growth conditions in the presence of excess carbon and energy (Preiss, 1984). There are however, some bacteria (*S. mitis*, Gibbons and Kapsimalis, 1963) that accumulate glycogen during exponential growth. In batch cultures the rumen bacterium *Ruminococcus albus* accumulates polysaccharide in the late log and early stationary phases but by late stationary phase almost no polysaccharide inclusions are present (Cheng *et al.*, 1977). Under conditions of ammonia limitation the rumen bacteria *Selenomonas ruminantium* accumulates carbohydrate reserves such as glycogen (Mink and Hespell, 1981). In general, glycogen accumulation in bacteria occurs when nitrogen (Strange *et al.*, 1961; Dawes and Senior, 1973), phosphate (Zevenhuizen, 1966), or sulphate (Zevenhuizen, 1966) concentrations become limiting. Light and unfavourable pH values have also been reported to induce glycogen synthesis (Zevenhuizen, 1966; Sarma and Kanta, 1979). In sporulating bacteria the onset of sporogenesis causes an increase of glycogen accumulation (Mackey and Morris, 1971).

Glycogen is however, not essential to growth and many mutants unable to synthesize glycogen grow as well as wild type strains (Steiner and Preiss, 1977)

The importance of glycogen reserves in bacteria

Glycogen is thought to play a role in the survival of bacteria during unfavourable conditions like starvation. There are reports where bacteria that accumulate glycogen have prolonged survival rates over those that do not (Strange, 1968; Van Houte and Jansen, 1970). Nevertheless, *S. lutea* cells containing glycogen are less viable than cells without glycogen during starvation in phosphate buffer (Burleigh and Dawes, 1967). The presence of glycogen during nutrient limitation is also thought to delay the degradation of cellular constituents such as protein and RNA. In *E. coli*, cells containing glycogen do not degrade their RNA and protein components whereas, cells without glycogen immediately release ammonia from their cell constituents (Dawes and Ribbons, 1963). However, in the presence of $MgCl_2$ *E. coli* cells with and without glycogen

have the same survival rate (Strange, 1968). This is probably due to the stabilizing effect of $MgCl_2$ on nitrogen-containing constituents of the cell. Glycogen is not always the primary endogenous substrate utilized during starvation. In *Zymomonas spp.* RNA is preferentially broken down in starvation and not endocellular carbohydrates (Dawes and Large, 1970). *Bdellovibrio bacteriovorus* which does not store glycogen uses ribosomes as the target constituent during starvation (Hespell *et al.*, 1974). In many sporulating organisms glycogen or granulose reserves serve as the carbon and energy source for spore formation and maturation (Robson *et al.*, 1974).

All the information indicates that the exact role glycogen plays in bacteria is not yet clear. Glycogen does seem to satisfy all conditions as an energy storage reserve however, its importance in the survival of bacteria during starvation is still ambiguous. This is true especially in rumen bacteria.

Starvation and glycogen in rumen bacteria

The rumen is a naturally occurring continous-culture where energy is abundant for short periods of time but followed by longer periods of starvation. The nature and the quality of organic matter is also constantly changing due to ingestion of additional feed by the ruminant and continued microbial fermentation. There are times when the free-sugar concentrations in the rumen fluid drop to very low levels, usually within a few hours after feeding (Takahashi and Nakamura 1969). Large variations can occur in the rumen contents, depending on time after feeding, diet, and composition of microbial population present. These factors affect the growth of various ruminal bacterial species and their ability to survive. Domesticated ruminants are usually fed on a regular basis, once or more a day, thus it may not be necessary for their rumen bacteria to survive long periods of starvation (Wachenheim and Hespell, 1985). However in wild ruminants this is not always the case and animals may endure long periods of starvation depending on environmental conditions. The strategies of rumen microorganisms for coping with periods of starvation are important not

only for maintaining viability but to ensure the organism can respond rapidly and efficiently to the subsequent influx of fermentable energy sources.

Rumen bacteria can prepare for starvation by laying down large amounts of cytoplasmic polysaccharide storage granules, up to 75% of the dry weight of the organism has been reported for *Bacteroides succinogenes* (Stewart et al 1981). Cell carbohydrate, nucleic acids, protein and other cell polymers may be used as endogenous substrates during starvation. A number of rumen bacteria have been reported to store a glycogen-like polysaccharide when excess soluble carbon is present (see Table 1.3). Starvation studies on rumen bacteria (Mink and Hespell, 1981a, 1981b; Wachenheim and Hespell, 1985) have shown that these bacteria have fairly poor survival capacity during starvation. The reason for this is not clear and may involve unknown factors. Most survival studies have dealt with cold shock, dehydration, osmotic stress, heat and thermal treatments. Few starvation studies have been done on anaerobic bacteria and enhanced survival due to glycogen accumulation has been shown mainly for aerobic bacteria (Van Houte and Jansen, 1970). Some rumen bacteria do not utilize intracellular polysaccharides as the primary endogenous substrate during starvation. *Megashaera elsdenii* (Mink and Hespell, 1981b) and *Ruminococcus flavefaciens* (Wachenheim and Hespell, 1985) show no specific preference for cell constituent degraded. *Selenomonas ruminantium* in glucose-limited conditions shows a preferential degradation of intracellular carbohydrate in the first 12 h, but in nitrogen-limited conditions RNA initially shows the greatest decrease (Mink and Hespell, 1981a; Mink *et al.*, 1982).

Chapter 2

Construction and screening of a *B. fibrisolvens* H17c genomic library in *E. coli* using a shuttle vector

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2.0 Summary

A library of *B. fibrisolvens* chromosomal DNA fragments was established using the plasmid pEB1, an *E. coli-B. subtilis* positive selection shuttle vector. The presence of inserts was confirmed by analysis of a random selection of clones. The library was screened using a variety of methods and clones expressing glutamine synthetase, carboxymethylcellulase, exoglucosidase, and amylolytic activities were isolated. Restriction maps of the plasmids encoding glutamine synthetase and amylolytic activities were constructed. Southern hybridization was used to confirm that the insert DNA from the clones expressing amylolytic activity originated from *B. fibrisolvens* H17c.

2.1 Introduction

The *in vitro* cloning of microbial genes using recombinant DNA techniques to characterize enzyme functions and investigate gene expression in a host strain such as *E. coli*, has been successful for a number of rumen bacteria. Cellulase genes from *B. fibrisolvens*, *Ruminococcus* spp. and *B. succinogenes* have been cloned and their expression in *E. coli* characterized (see reviews, Hazlewood and Teather, 1988; Béguin, 1990). An important step in the investigation of *B. fibrisolvens* genes is the construction of genomic libraries that are representative of the source genome, in a suitable vector-host system. The choice of this system depends on a number of factors, such as the size of the DNA fragment to be cloned, and considerations of gene expression (reviewed by H. Zappe, Ph.D Thesis, Dept. of Microbiology, UCT). The plasmid pEB1 used in this study is an *E. coli-B. subtilis* shuttle vector, a gift from H. Zappe, Dept. of Microbiology, UCT. It was constructed (Lin *et al.*, 1990) by replacing the pBR322 fragment of the *E. coli-B. subtilis* shuttle vector pLP1202 (Robson and Chambliss, 1986) with pEcoR252, a derivative of pEcoR251. The *E. coli* pEcoR251 vector (a gift from M. Zabeau, Plant Genetic Systems, Ghent, Belguim) utilizes the insertional inactivation of the lethal *Eco*RI gene as a positive selective marker. Vector pEcoR252 is identical to pEcoR251 except that the *Pst*I site in the β-lactamase gene has been removed.

The establishment of a gene bank in a shuttle vector allows for a greater choice of recipient strains in which to screen for gene expression. *B. fibrisolvens* although previously described as a Gram-negative bacterium is now thought to be Gram-positive, but the precise classification is still unclear (see General Introduction). Having a choice of Gram-negative and Gram-positive host strains is an advantage especially if the gene product has a lethal effect or the mRNA is not transcribed or translated in a particular bacterium. Aspects such as post-translational processing or secretion may be possible in one strain but not in another.

The work described in this chapter involved the construction of a *B. fibrisolvens* H17c DNA fragment library in *E. coli*, and the initial screening for glutamine synthetase, cellulase, and amylase activities in *E. coli*. The aim of this project was to investigate genes encoding amylolytic activity. Other research groups in the Department of Microbiology, UCT. are studying glutamine synthetase and cellulase genes of anaerobic bacteria.

2.2 Materials and Methods

2.2.1 Bacteria and plasmids. The rumen bacterium *B. fibrisolvens* H17c strain obtained from Dr. R. B. Hespell (Department of Animal Science, University of Illinois, Urbana) was originally isolated by Dehority (1966). This strain was maintained at -70°C on slopes of M10 medium (Caldwell and Bryant, 1966) (Appendix A). *E. coli* strains K514 and LK111 (Appendix C) were used as recipient strains for recombinant plasmids. A restriction map of the plasmid vector pEB1 is given in Appendix E.

2.2.2 Media and growth conditions. All media, buffers and solution not described in the text are listed in Appendix A. *B. fibrisolvens* H17c was grown in M10 medium as described by Strydom *et al.* (1986). Samples (1 ml) of overnight cultures were centrifuged, washed with one quarter strength Ringer solution, resuspended in 10 ml M10 medium, and incubated at 37°C under stringent anaerobic conditions. *E. coli* strains were grown in Luria-Bertani (LB) medium and appropriate antibiotics were added for the selection of transformants.

2.2.3 Isolation of chromosomal DNA from *B. fibrisolvens*. The DNA was isolated using a method based on that of Marmur (1961) with modifications described by Berger *et al.* (1989). *B. fibrisolvens* chromosomal DNA was prepared from 500 ml overnight cultures which were harvested, resuspended in 4 ml Solution A (10 mM Tris-HCl, pH 8.0, 25% w/v sucrose containing 5 mg/ml

lysozyme) and mixed gently at 37°C for 30 min. Two ml of ice-cold Solution B (0.5 M EDTA, pH 8.0) were added and the mixture kept on ice for 5 min. Four ml of Solution C (10 mM Tris-HCl, pH 7.5, 1 mM Na_2EDTA, 2% w/v SDS) containing 5 mg/ml proteinase K were added, and the mixture was kept at 20°C for 10 min. CsCl (1 g/ml) and EtBr (250 µg/ml) were added, the refractive index adjusted to 1.395, and the DNA purified by isopycnic CsCl/EtBr ultracentrifugation (Appendix A).

2.2.4 Preparation of DNA

2.2.4.1 Partial digestion and sucrose gradient fractionation of *B. fibrisolvens* DNA. The chromosomal DNA was partially digested with *Sau*3A endonuclease (Maniatis *et al.*, 1982) in a two-fold dilution series to estimate the optimum DNA-enzyme ratio to produce fragments 4-10 kb in size. Approximately 400 ug of DNA was digested, phenol extracted, precipitated and resuspended in TE buffer (Appendix A). This DNA was heated to 60°C for 10 min prior to loading on a sucrose gradient (10-40% w/v in 20 mM Tris-HCl (ph 8.0), 5 mM EDTA, and 1 mM NaCl). The sample was centrifuged at 25 000 rpm for 24 h at 20°C in a Beckman SW28 rotor. The gradient was fractionated (0.5 ml), and 10 µl of every third fraction analysed by agarose gel electrophoresis (Appendix A). The gradient fractions containing fragments in the 6-11 kb range were pooled, the DNA precipitated, resuspended in TE, and the concentration of the DNA determined.

2.2.4.2 Preparation of vector DNA. The vector pEB1 was prepared by digesting 10 µg of the plasmid with 40 units of *Bgl*II endonuclease for 1 h at 37°C. A sample of the DNA was analysed by agarose gel electrophoresis to confirm complete digestion.

2.2.5 Construction of the *B. fibrisolvens* genomic library

2.2.5.1 Ligation of vector and insert DNA. pEB1 is a positive selection vector in *E. coli*, and the *Eco*RI gene is lethal unless insertionally inactivated. DNA concentrations of 5 pmole and 2.5 pmole in 1 ml of buffer and an insert:vector ratio of 1:1 were used to optimize recombinant formation (H. Zappe, Ph.D Thesis, Microbiology Dept., University of Cape Town). The ligation reaction contained DNA, 5 µl ligation buffer (10X), T4 DNA ligase (2 units), and water to 50 µl. The reaction was placed at room temperature for 16 h, phenol extracted, the DNA precipitated, and resuspended in 50 µl TE buffer.

2.2.5.2 Transformation of *E. coli*. *E. coli* K514 competent cells were prepared and transformed with 2 µl of the ligated DNA as described in Appendix A. Controls included unrestricted pEB1 and pBR325 DNA transformed as above to monitor the correct functioning of the cloning vector and to calculate competence. After 1 h expression of the transformation reaction in LB containing Ap (100 ug/ml) to allow for expression of the *Eco*RI αvδ β-lactamase genes, cells were plated on to LB agar plates containing Ap (100 µg/ml). The entire 50 µl of the ligation reaction was transformed into *E. coli* competent cells, and groups of approximately 500 Ap^r colonies were pooled, and recombinant pEB1 plasmid DNA was isolated using the maxi-prep method (Appendix A), but without CsCl gradient ultracentrifugation. DNA pellets were resuspended in 1 ml TE buffer and aliquots of 0.5 ml were stored at -70°C.

2.2.6 Transformation for specific clone isolation;. Suitable strains of competent *E. coli* cells were transformed with recombinant pEB1 plasmid pools, and transformants were analysed for plasmid linked activities. A clone complementing the *E. coli* YMC11 glutamine synthetase *(glnA)* deletion strain was isolated by selection on minimal agar (Appendix A) with $(NH_4)_2SO_4$ (1 g/l) as the sole nitrogen source. Clones expressing endoglucanase activity were isolated by transforming *E. coli* LK111 cells and plating on LB agar containing Ap and medium viscosity CMC (1g/l). Colonies were lifted with Whatman No.1

filter paper discs. The plates were rinsed and stained with Congo red (0.1% w/v; 15 min), followed by destaining with 1 M NaCl until zones appeared. Clones expressing CMCase activity were identified by a clear zone around and beneath the colony. Clones expressing exocellobiosidase and exo-β-glucosidase activities were identified by saturating the filter paper discs with 1 ml of either 4-methylumbelliferyl β-D-cellobioside (MUC) or 4-methylumbelliferyl β-D-glucoside (MUG) at a concentration of 0.02% (w/v) in 0.1 M sodium acetate buffer, pH 5.0. The discs were incubated for 10 min at 37°C and examined for fluorescent halos under UV light (254 nm).

Amylolytic activity expressed by clones was detected by transforming *E. coli* LK111 cells with the gene bank and plating transformants on LB agar plates containing (0.5%) starch azure and Ap (100 μg/ml). Colonies producing clear halos were selected for further study.

2.2.7 Restriction endonuclease mapping and Southern blot analysis. Recombinant plasmid DNA was prepared by the small-scale (miniprep) and large-scale (maxiprep) methods described in Appendix A. Restriction endonuclease mapping was carried out using standard procedures (Maniatis *et al.*, 1982). Southern hybridization using the cloned DNA as a probe was used to confirm that the insert DNA originated from *B. fibrisolvens*. Chromosomal and recombinant plasmid DNA were digested with restriction nucleases and the DNA fragments seperated by electrophoresis in TBE buffered agarose gels (0.8%). The DNA was transferred to Hybond N+ membranes (Amersham) by the DNA/RNA alkali blotting procedure (Appendix A). The non-radioactive digoxigenin (DIG) DNA labelling and detection kit (Boehringer Mannheim) was used for probe DNA labelling, hybridization and detection. The manufacturer's procedures were followed exactly.

2.3 Results

2.3.1 Construction of the *B. fibrisolvens* H17c genomic library. Approximately 7 500 *E. coli* K514 colonies containing recombinant DNA were obtained. To determine whether the library was representative of the source genome, the 99.9% probability (P) that a certain gene was contained amongst the hybrids was calculated according to equation 1 (Clarke and Carbon, 1976).

$$N = \frac{\ln(1-P)}{\ln(1-f)} \qquad \text{.....(1)}$$

where N is the number of recombinants and f is the fraction of the total genome that each insert represents. As the size of the *B. fibrisolvens* chromosome is unknown the average prokaryotic genome size of 6 x 10^6 bp (Starr *et al.*, 1981) and an average insert size of 6 kb were assumed. The number of colonies required for a 99.9% probability was at least 4 600 assuming that each transformant colony arises from an independent transformation event. The integrity of the gene bank was assessed by isolation of plasmids from a random selection of clones. Ten out of 12 clones had inserts with an average size of 5.4 kb.

2.3.2 Isolation of the *B. fibrisolvens glnA* gene. Eight transformants complemented the GlnA$^-$ phenotype of *E. coli* YMC11. Initial restriction enzyme mapping indicated that the plasmids from five of the clones had inserts of the same size, while the other three appeared to have overlapping fragments.

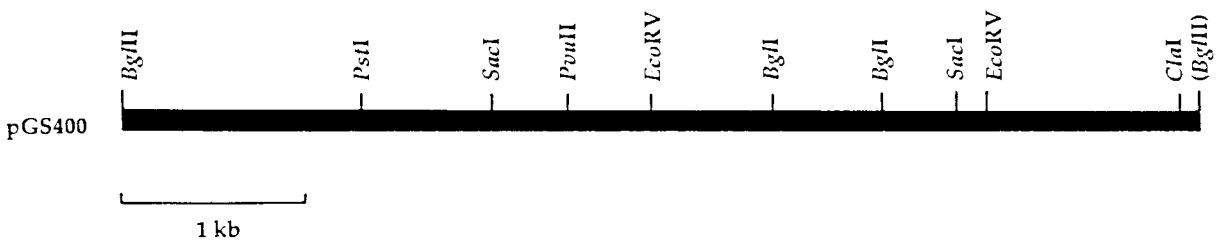


Fig. 2.1 Restriction endonuclease map of the DNA insert from the clone expressing glutamine synthetase activity.

A restriction endonuclease map of one of these clones, pGS400, was determined (Fig. 2.1). This clone is in the process of being sequenced and characterized by Dr. H. Goodman (Microbiology Dept., University of Cape Town).

2.3.3 Isolation of recombinant clones expressing CMCase, cellobiosidase, or β-glucosidase activities. A large number of clones expressing CMCase activity were isolated. Restriction endonuclease mapping of the inserts from a random selection indicated that the gene cloned was the same as a previously isolated *B. fibrisolvens* cellodextrinase (Berger *et al.*, 1990). Three transformant *E. coli* LK111 colonies had both cellobiosidase and β-glucosidase enzyme activities but no CMCase activity. Restriction endonuclease analysis of the recombinant plasmid DNA isolated from these clones demonstrated that the inserts had overlapping DNA fragments. The plasmid with the smallest insert, pBEX8, has been characterized and sequenced (Lin *et al.*, 1990). The *B. fibrisolvens* insert DNA was found to code for a β-glucosidase.

2.3.4 Isolation of *B. fibrisolvens* genes expressing amylolytic activity. *E. coli* LK111 was transformed with recombinant pEB1 plasmid pools and from approximately 7500 colonies screened, 7 transformants produced a distinct halo on starch azure plates. Halos were also produced by these colonies when plated onto starch plates stained with I_2/KI, and Phadebas substrate plates. Restriction enzyme analysis showed that the plasmids from two of the clones had identical 5 kb insert fragments, whereas the other five clones had inserts different to the former but overlapping fragments in common with each other. A plasmid of the former type, pBAMY100, and that plasmid of the latter type with the smallest DNA insert, pBGB100, were chosen for further study. The zones indicating amylolytic activity around *E. coli* LK111(pBAMY100) colonies were slightly bigger than the zones around *E. coli* LK111(pBGB100) colonies (Fig. 2.2). Amylolytic activity was always associated with transformation to Ap^r. *E. coli* LK111 transformed with the vector pBluescript as a control did not produce zones on starch azure plates.

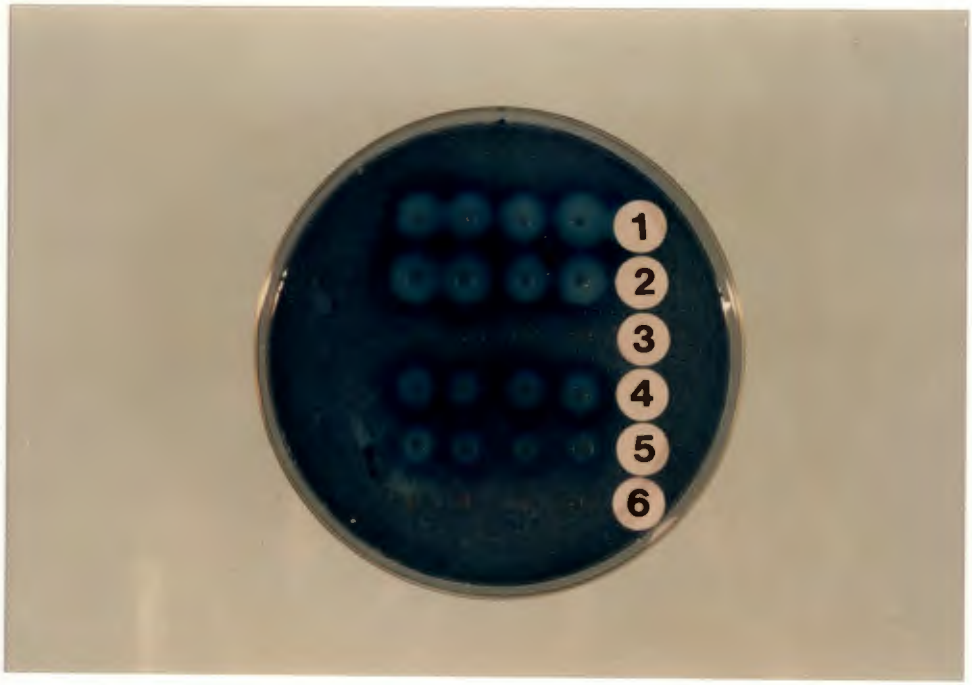


Fig. 2.2 A starch azure plate showing a comparison of the zones produced by the *E. coli* LK111 Ap^r transformants displaying amylolytic activity. Lanes 1 and 2, *E. coli*(pBAMY100); lane 3, *E. coli*(pBluescript); lanes 4 and 5, *E. coli*(pBGB100); lane 6, *E. coli*(pBluescript).

2.3.5 Restriction endonuclease mapping and Southern hybridization. Partial restriction maps of the insert fragments on pBAMY100 and pBGB100 were constructed (Fig. 2.3). The origin of the 5.0 kb DNA insert on pBAMY100 was determined by Southern blotting and DNA hybridization, using dioxigenin d-UTP labelled pBAMY100 as a probe. Two *Eco*RI fragments (1.2-kbp and 250-bp) internal to the pBAMY100 insert hybridized to two *B.fibrisolvens* H17c chromosomal fragments of the same size, confirming the origin of the cloned DNA fragment (lane 1, Fig. 2.4 A). The other two hybridization signals at 5 kb and 11 kb in lane 1 represent the flanking *Eco*RI fragments of the *B. fibrisolvens* chromosomal DNA. A similar Southern hybridization confirmed the origin of the insert DNA on pBGB100, using dioxigenin d-UTP labelled pBGB100 as a probe. Three *Acc*I fragments (1 kb, 800 bp, and 500 bp) internal to the pBGB100 insert hybridized to three *Acc*I digested *B. fibrisolvens* H17c chromosomal fragments of a similar size (lane 1, Fig. 2.4B). Internal *Hinc*II (500-, 300-, 200 bp) and *Sac*I (850 bp) insert fragments of pBGB100 also hybridized to fragments of equivalent size after *Hinc*II and *Sac*I endonuclease digestion of *B. fibrisolvens* chromosomal DNA (lanes 3 and 5 respectively Fig. 2.4B). Additional

hybridization signals in the chromosmal digests would be due to the flanking regions of *B. fibrisolvens* DNA. Labelled pEB1 DNA did not hybridize to *B. fibrisolvens* H17c DNA (result not shown). The hybridization signals also provided supporting evidence for the restriction endonuclease plasmid maps illustrated in Fig. 2.3.

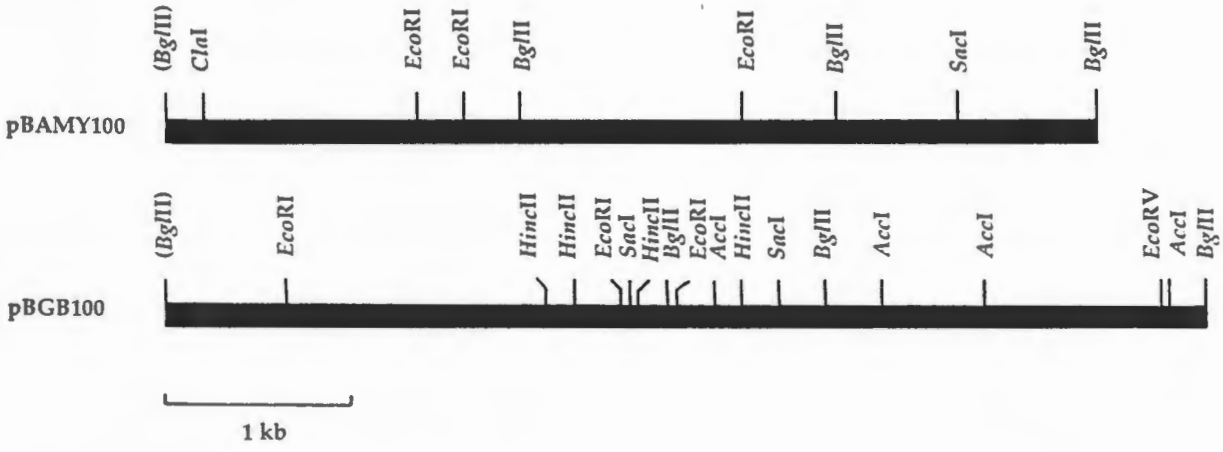


Fig. 2.3 Restriction endonuclease maps of the DNA inserts in the two clones, pBAMY100 and pBGB100, expressing amylolytic activity.

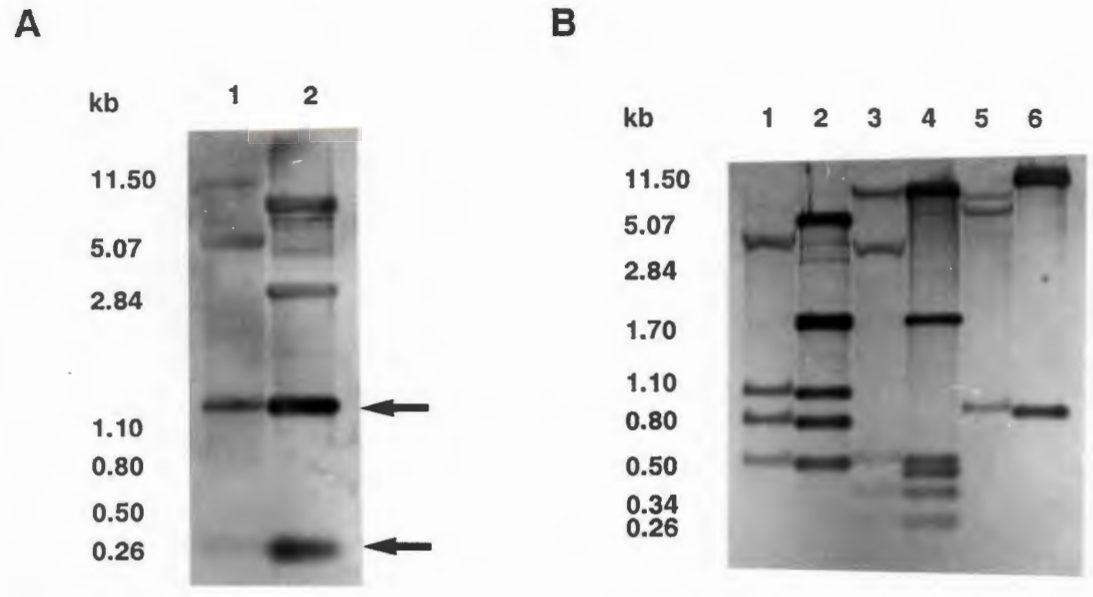


Fig. 2.4 Southern blot hybridization using nonradioactively labelled pBAMY100 (A) and pBGB100 (B) as probes. (A) *Eco*RI restriction endonuclease digestions of *B. fibrisolvens* chromosomal DNA (lane 1) and pBAMY100 (lane 2). Arrowheads indicate internal bands. (B) *B. fibrisolvens* chromosomal DNA digested with *Acc*I (lane 1), *Hinc*II (lane 3), and *Sac*I (lane 5) restriction endonucleases. Similarly pBGB100 DNA is digested with *Acc*I (lane 2), *Hinc*II (lane 4), and *Sac*I (lane 6) restriction endonucleases.

Molecular analysis and characterization of the cloned gene products of the recombinant plasmids pBAMY100 and pBGB100 are described in Chapters 3 and 4 respectively.

2.4 Discussion

A genomic library that appears to be representative of *B. fibrisolvens* H17c DNA was constructed using an *E. coli-B. subtilis* shuttle vector. A total of approximately 7 500 colonies were isolated, 83% of which contained inserts with an average size of 5.4 kb. Assuming a *B. fibrisolvens* genome size of 6 000 kb there is a 99.94 % probability that a given gene is represented in the bank. The presence of 17% parental plasmids in the gene bank despite the use of a lethal *Eco*RI gene as the positive selection measure is not uncommon. In two other gene libraries established in the Microbiology Department, University of Cape Town, using this selection technique, a similar result was obtained (H. Zappe, Ph.D. Thesis and E. Berger, Ph.D. Thesis). It is suggested that a low percentage of the vector DNA is damaged during manipulations resulting in a disruption of the *Eco*RI gene.

Screening of the *B. fibrisolvens* gene bank in *E. coli* resulted in the isolation of two different genes expressing amylolytic activity on starch azure plates. The presence of clones with different genomic overlapping fragments of the same chromosomal region as pBGB100 illustrated the random nature of the *Sau*3A partial digestion. These clones make the isolation of a linked region of DNA for further regulation studies or gene linkage analyses relatively simple. As no amylases had previously been cloned from *B. fibrisolvens* or indeed from any ruminal bacterium, it was decided to concentrate on these industrially and agriculturally important enzymes.

Even though the gene bank was established in a shuttle vector, the initial screening in *E. coli* yielded a sufficient number of *B. fibrisolvens* H17c genes for the scope of this study. Whilst not included in this chapter this *B. fibrisolvens* genomic library was also screened for protease genes in *E. coli*. No protease producing clones were isolated. As reported previously by E. Berger (Ph.D. Thesis, Dept. of Microbiology, UCT) protease gene expression may be lethal. It is possible that these genes would not be lethal when expressed in a suitable Gram-positive host. However the gene library ligation would have to be transformed directly into a suitable Gram-positive host. The expression of *B. fibrisolvens* and *R. albus* glucanase genes cloned on a shuttle vector were found to be lethal in *E. coli* but not in the Gram-positive host *Enterococcus faecalis* (Mann, 1988). The lethal action was suggested to be due to a combined interaction of the glucanases and the plasmid encoded tetracyline resistance proteins on the *E. coli* membranes. It would prove interesting to screen this genomic library in a *B. subtilis* host strain.

Chapter 3

An α-amylase from *B. fibrisolvens*: the DNA sequence analysis, enzymatic characterization in *E. coli*, and comparison of the deduced amino acid sequence with other amylases

Chapter 3

An α-amylase from *B. fibrisolvens*: the DNA sequence analysis, enzymatic characterization in *E. coli*, and comparison of the deduced amino acid sequence with other amylases

3.0 Summary

A *Butyrivibrio fibrisolvens* amylase gene was cloned and expressed using its own promoter on the recombinant plasmid pBAMY100 in *Escherichia coli*. The amylase gene consisted of an open reading frame of 2,931-bp which encoded a protein of 976 amino acids with a calculated M_r of 106,964. In *E. coli* (pBAMY100) more than 86% of the active amylase was located in the periplasm, and Tn*phoA* fusion experiments showed that the enzyme had a functional signal peptide. The *B. fibrisolvens* amylase is a calcium metalloenzyme, and three conserved putative calcium-binding sites were identified. The amylase showed high sequence homology with other α-amylases in the three highly conserved regions which constitute the active centers. These and other conserved regions were located in the N-terminal half, and no similarity with any other amylase was detected in the remainder of the protein. Deletion of approximately 40% of the C-terminal portion of the amylase did not result in loss of amylolytic activity. The *B. fibrisolvens* amylase was identified as an endo-α-amylase by hydrolysis of the Phadebas amylase substrate, hydrolysis of γ-cyclodextrin to maltotriose, maltose and glucose, and the characteristic shape of the blue value and reducing sugar curves. Maltotriose was the major initial hydrolysis product from starch, although extended incubation resulted in its hydrolysis to maltose and glucose.

3.1 Introduction

Starch is an important component of the ruminal diet and its digestion is essential for maximum conversion. Knowledge of the biochemical action and regulation of the enzymes involved in ruminal starch degradation could facilitate the manipulation of digestion in the rumen to the benefit of agricultural productivity. Relatively few amylases of ruminal bacteria have been characterized. Those that have been studied include amylases from *S. bovis, Clostridium butyricum* (Hobson and Macphearson 1952, Walker 1965, Walker and Hope 1964) and *Ruminobacter amylophilis* (formerly *Bacteroides amylophilis*)(McWethy and Hartman, 1977). In addition, Cotta (1988) carried out a general investigation of amylases fron the major starch-utilising bacteria found in the rumen including *B. fibrisolvens.* The expression of amylase in *B. fibrisolvens* was influenced by the carbohydrate source present in the growth medium and as such seems to be regulated. Increases in amylolytic activity of 32- and 26-fold were observed when starch rather than glucose was included in the growth medium. Moreover, growth in a starch-containing medium resulted in amylolytic activity associated with the cell pellets whereas growth in maltose resulted in the activity in the extracellular fluid. An α-amylase of the anaerobic ruminal fungus *Neocallimastix frontalis* has been investigated and found to be regulated by glucose (Mountfort and Asher, 1988).

α-Amylases are widely distributed amongst both prokaryotes and eukaryotes, and substantial nucleotide and amino acid sequence information is available. No molecular studies, however, of the amylases from ruminal bacteria have been reported. The nucleotide sequence of a gene coding for an amylase, (1,4-α–D-glucan-4-glucanhydrolase [E.C. 3.2.1.1]) from *B. fibrisolvens* H17c is reported in this chapter. The hydrolytic properties of this enzyme have been characterized and the secretion and location of the cloned amylase in *E. coli* were examined. Since the majority of cloned α-amylases are extracellular enzymes with a signal sequence responsible for the transport of the enzymes to the periplasm when

expressed in *E. coli* (Vihinen and Mäntsälä, 1989), the presence of a signal peptide in the *B. fibrisolvens* amylase was investigated utilizing the Tn*phoA* system (Manoil and Beckwith, 1985). This system has been used successfully to investigate both the topology of membrane bound proteins and functional signal peptides in *E. coli*. The fusion proteins formed in this system were also used to confirm translated regions of the ORF. The derived amino acid sequence for the encoded polypeptide was compared with amylases of prokaryotic and eukaryotic origin.

3.2 Materials and Methods

3.2.1 Bacterial strains and plasmids. The plasmid pBAMY100 (Chapter 2.3.5) was used as the primary source of DNA for further plasmid mapping, subcloning and templates for DNA shortening. The M13-derived Bluescript SK and KS plasmids (Stratagene, San Diego) were used for the preparation of nt sequencing templates. *E. coli* strains are described in Appendix B. Cloning and genetic manipulations were carried out in the *E.coli* strains C600 and LK111. Transformants of *E. coli* K12 G6MD3 (a gift from Dr. J. Preiss, Dept. of Biochemistry, Michigan State University, MI) were used for amylase assays. The *phoA E. coli* strain CC118 was used for the Tn*phoA* fusion experiment. Phage λb221*rex*::Tn*phoA*cI857 Pam3 (Gutierrez *et al.*, 1987) was a gift from Dr. C. Manoil (Appendix D).

3.2.2 Media, buffers and enzymes. Litner soluble starch, starch azure, α-, β- and γ-cyclodextrins (Schardinger dextrins), and linear malto-oligosaccharides (glucose(G1), maltose(G2), maltotriose(G3), maltotetraose(G4), maltopentaose(G5), maltohexaose(G6)) were purchased from Sigma Chemical Company (St Louis, Mo). Raw corn starch was obtained from B.D.H. (Poole, England), and Phadebas reagent from Pharmacia (Sweden). All media and buffers not described in the text are given in Appendix A. Restriction endonucleases, T4 DNA ligase and S1 nuclease were purchased from Boehringer

Mannheim Biochemicals. Exonuclease III was obtained from Bethesda Research Laboratories.

3.2.3 Growth conditions. *E. coli* strains were grown in Luria broth with 5 mM $CaCl_2$ and 100 μg/ml ampicillin for the selection of transformants. When *E. coli* G6MD3 was used, media were supplemented with 50 μg/ml diaminopimelic acid due to the *asd* deletion present in this strain. All cultures were grown at 30°C.

3.2.4 Restriction endonuclease mapping and sequencing strategy. The preparation of plasmid DNA and restriction endonuclease mapping of the clones was done using standard techniques (Maniatis *et al.*, 1982; see Appendix A). The amylase gene was located on a 3.52 kb *Cla*I-*Sac*I restriction fragment of pBAMY100 (Fig. 3.1). This fragment was subcloned into *Cla*I-*Sac*I restriction endonuclease digested pBluescript.SK and pBluescript.KS to give pBAMY200 and pBAMY300 respectively (Fig. 3.1). pBAMY200 was used to generate overlapping deletions for one orientation of the gene using the exonuclease III shortening technique (Henikoff, 1984). The *Cla*I-*Sac*I restriction endonuclease fragment of pBAMY200 was subcloned into *Cla*I-*Eco*RV digested pBluescript KS. This was done by blunt ending the *Sac*I site of the insert fragment and ligating it to the *Eco*RV site of the plasmid vector. The restriction endonuclease fragment to be blunt ended was initially cut with *Sac*I, then purified by phenol and ether extractions (Appendix A), and ethanol precipitated in the presence of 0.3 M sodium acetate. The sticky ends of the *Sac*I site were filled in using 1 unit/ug DNA of T4 polymerase enzyme (Boehringer Mannheim) and 250 uM of each dNTP in T4 polymerase buffer (Appendix A). The plasmid was then purified, digested with *Cla*I, gel purified and ligated to the plasmid vector. The resultant plasmid called pBAMY600 was used to generate overlapping deletions in the opposite orientation to those generated from pBAMY200.

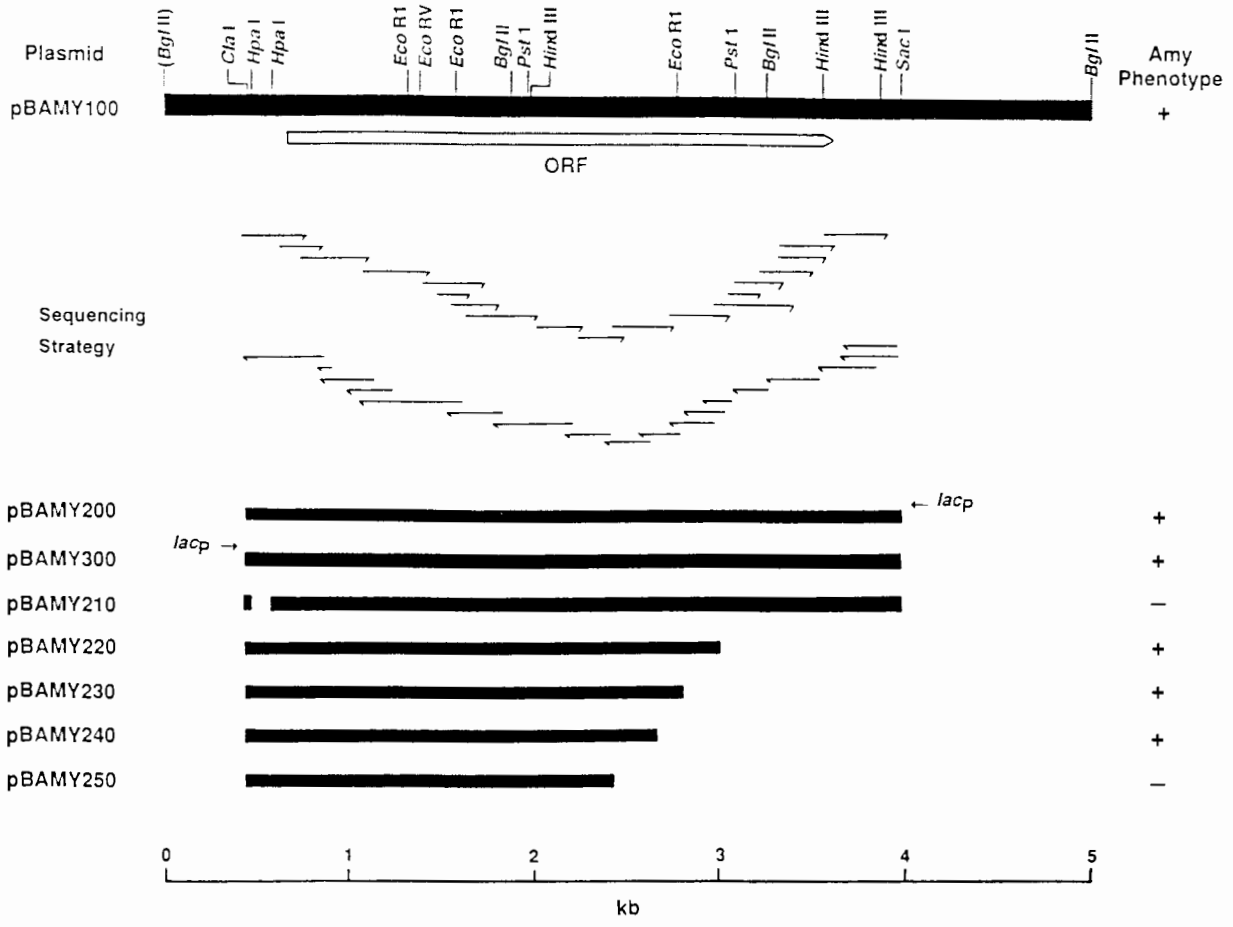


Fig. 3.1 Restriction endonuclease map of insert DNA of pBAMY100 and subclones encoding the *B. fibrisolvens* H17c amylase. The ORF and direction of transcription are indicated by an open arrow, lac_p with an open arrow indicates the direction of transcription of the vector *lac* promoter. The thin arrows represent the extent and direction of sequencing templates generated by exonuclease III digestion.

3.2.5 Exonuclease III digestion. Exonuclease III deletions of pBAMY200 and pBAMY600 were generated by an adaptation of the method of Henikoff (1984). Progressive deletions from the 5' end of the insert were obtained by unidirectionally digesting *Apa*I-*Cla*I restriction endonuclease digested pBAMY200. *Sac*I-*Xba*I restriction endonuclease digested pBAMY600 was used to generate progressive deletions from the 3' end of the insert. After digestion, the linearised plasmid DNA (16μg) was ethanol precipitated and resuspended in 100 μl Exo-buffer. Exonuclease III digestion was then carried out as described in Appendix A. The re-ligated deletion plasmids were transformed into *E. coli* LK111 and transformants were selected on LB agar containing Ap (100 μg/ml).

Shortened recombinant plasmids were selected by restriction endonuclease mapping before being purified by isopycnic CsCl density gradient ultracentrifugation (Appendix A).

3.2.6 Nucleotide sequence determination and protein sequence comparison. Sequencing was carried out by the dideoxynucleotide triphosphate chain termination method using the overlapping DNA fragments generated by exonuclease III digestion (Appendix A). The sequence of the entire insert *Cla*I-*Sac*I restriction endonuclease fragment was determined from both strands. Sequence data was analysed using the Genetics Computer Group Inc.(GCG) software package (version 6.2). The TFASTA subroutine was used to screen the GENEMBL (release 65.0), Swiss Protein (release 15.0), NBRF-N (release 36.0) and NBRF-P (release 25.0) databases for sequences having similarity to the amino acid sequence of the *B. fibrisolvens* amylase.

3.2.7 Preparation of cell-free extracts and cell fractionation. Cell-free extracts were prepared from 24 h, 100 ml *E.coli* cultures. Cells were harvested, rinsed with saline and resuspended in 2 ml of 50 mM sodium phosphate buffer containing 5 mM $CaCl_2$ pH 6.8. The cell suspension was disrupted by sonication on ice (30 s bursts for 3 min) using a MSE (soniprep 150) sonicator. The extract was clarified by centrifugation for 15 min at 27000 g at 4 ^{o}C. This fraction constituted the cell-free extract. Periplasmic and cytoplasmic extracts were prepared from mid-stationary phase *E. coli* C600 (pBAMY100) cultures (200 ml) by the osmotic shock procedure of Willis *et al.* (1974). Cells were harvested by centrifugation, and the supernatant stored at 4°C. The pellet was resuspended in a final concentration of 33 mM Tris-HCL (pH7.3) 33 mM NaCl, and incubated for 10 min. The suspension was then centrifuged (6 000 g; 5 min), and the pellet resuspended in 33 mM Tris-HCl (pH 7.3; 10 ml/g wet wt. cells), with an equal volume of TSE (33mM Tris-HCl, pH7.3; 40% w/v sucrose; 2 mM EDTA). After 5 min at room temperature the cells were harvested and resuspended in ice-cold deionized water. $MgCl_2$ was added to 1 mM within 1 min. The cells were fractionated by centrifugation and the supernatant constituted the periplasmic

fraction. The cells were treated as described for the cell-free extract to obtain the cytoplasmic fraction. β-galactosidase and β-lactamase activities were assayed (Pardee *et al.*, 1959 as modified by Miller, 1972, and Sykes and Nordstrom, 1972 respectively).

3.2.8 Tn*phoA* mutagenesis. Transposon insertions into *amyA* (pBAMY100) were made using λ::Tn*phoA* (Manoil and Beckwith, 1985; 1986) according an adaptation of the protocol of Gutierrez *et al.* (1987). An overnight culture of *E. coli* CC118(pBAMY100) grown in LB (2 ml) containing 10 mM $MgSO_4$, 0.2% maltose, and Ap (100μg/ml) was mixed with phage λ::Tn*phoA*, at a multiplicity of infection of approximately one, and incubated at 30°C for 15 min. The culture was diluted 1:10 with LB and incubated at 30°C for 4 h to allow outgrowth of the phage. The cells were concentrated and aliquots (100 μl) plated onto LB plates containing Km (250 μg/ml), Ap (100 μg/ml), 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine (XP; 40 μg/ml). After incubation for 2 to 3 days at 30°C, blue colonies (alkaline phosphatase positive) were selected and plasmid DNA prepared by the mini-prep method (Appendix A). This DNA was used to retransform *E. coli* CC118 and blue, alkaline phosphatase-positive colonies were chosen to prepare maxi-prep plasmid DNA (Appendix A) for mapping and sequencing. A 15 bp synthetic oligonucleotide primer, 5'-AAACGGCGAGCACCG-3', complementary to the nt positions 126-140 of the *phoA* gene, was used to sequence across the junction of the *phoA* and *amyA* genes. The nucleotide sequencing was to determine the position and orientation of the Tn*phoA* insertions.

3.2.9 Western blotting and activity gels. Cell free extracts of amylase-phosphatase fusion proteins prepared from *E. coli* CC118 were fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The transfer of proteins onto nitrocellulose membranes was done by a horizontal blotting method (E. P. Rybicki, pers. comm., Microbiology Dept., University of Cape Town) using New Transfer Buffer (Appendix A). Western blots were processed as described by Rybicki and von Wechmar (1982).

Rabbit antiserum raised against a commercial preparation of alkaline phosphatase was used as a probe. Blots were developed using goat anti-rabbit alkaline phosphatase antibody-enzyme conjugate (Miles Laboratories, Cape Town) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate solution.

Amylase activity gels were performed using crude extracts of *E. coli* G6MD3 cells containing the various clones fractionated on 10% SDS-PAGE gels. Amylase activity bands were detected *in situ* after electrophoresis and renaturation of proteins (Lacks and Springhorn, 1980).

3.2.10 Enzyme assays

Amylase assays. Amylolytic activity was determined at 45°C using 0.5% soluble starch as a substrate in 50 mM sodium phosphate buffer pH 6.8 containing 5 mM $CaCl_2$. Release of reducing sugar from soluble starch, after incubation for various time intervals with appropriate enzyme dilutions, was measured with 3,5 dinitrosalicylic acid (Bernfeld, 1955) (Appendix A). The activity of 1 U of amylase was defined as that amount of enzyme which liberated 1 µmol of reducing sugar per min under the specified conditions, using glucose as the standard. A water-insoluble cross-linked blue starch polymer (Ceska *et al.*, 1969) commercially available as the Phadebas^R Amylase Test (Pharmacia Diagnostics) was used as a qualitative assay. The manufacturer's instructions were followed exactly.

Analysis of blue value, reducing sugar and hydrolysis products. Iodine blue values were determined by a modification of the method of Mountford and Asher (1988). A sample of crude enzyme extract was added to a 50 mM sodium phosphate, 5 mM $CaCl_2$ pH 6.8 buffer containing 10 mg/ml soluble starch, the volume adjusted to 3 ml and the mixture incubated at 45^{o}C. Samples (0.1 ml) were withdrawn, mixed with 0.4 ml HCl and a 0.1 ml sample was added to 1 ml of iodine stain (0.05 g I_2/0.5 g KI per litre). The absorbance was measured at

620nm (A_{620}) against a water-iodine blank. The A_{620} of a substrate blank treated in an identical manner, except with no enzyme extract added, was also determined and the blue value calculated according to the formula:

A_{620}(sample) / A_{620}(substrate blank) X 100

Two additional 0.1 ml samples were used to determine the amount of reducing sugar liberated (see above section) and products of hydrolysis. High pressure liquid chromatography (HPLC) was used to identify the products of hydrolysis of soluble starch, amylose, amylopectin, pullulan, α-, β-, γ-cyclodextrins and malto-oligosaccharides. Reaction mixtures containing a dilution of crude enzyme were incubated with substrate (10 mg/ml) for various time intervals. Samples were boiled for 1 min to stop the reaction, centrifuged for 5 min and the supernatant loaded on to a Beckman HPLC system equipped with a model 156 refractive index detector and a Waters C18 separation column.

Alkaline phosphatase. Alkaline phosphatase activity was assayed by a modification of the method of Brickman and Beckwith (1975). Overnight cultures of *E. coli* CC118(pBAMY100::Tn*phoA*) were grown at 30°C in LB with Ap (100 μg/ml) and Km (250 μg/ml). A sample (1 ml) was pelleted in an Eppendorf microfuge, washed twice with saline, and the cell pellet resuspended and diluted in 1 M Tris-HCl buffer (pH 8.0). The absorbance at 600 nm (A_{600}) was recorded, the sample (1 ml) mixed with chloroform (50 μl) and 0.1% SDS (50 μl) and equilibrated for 5 min at 37°C. The alkaline phosphatase substrate (4 mg/ml p-nitrophenyl phosphate (PNPP) in 1 M Tris-HCl, pH 8.0) (100 μl) was added and the time taken for the sample to turn yellow was recorded. The reaction was terminated by the addition of 1 M KH_2PO_4 (100 μl) and the samples spun in a microfuge for 1 min at 4°C. The supernatant was removed and the absorbance at 420 nm (A_{420}) was measured. Alkaline phosphatase specific activity was expressed as Units/A_{600} and calculated using the formula:

Units / A_{600} = [A_{420} / A_{600} x min] X 1000 X dilution factor.

Sample fractions collected from *E. coli* CC118(pBAMY100) were used as a negative control.

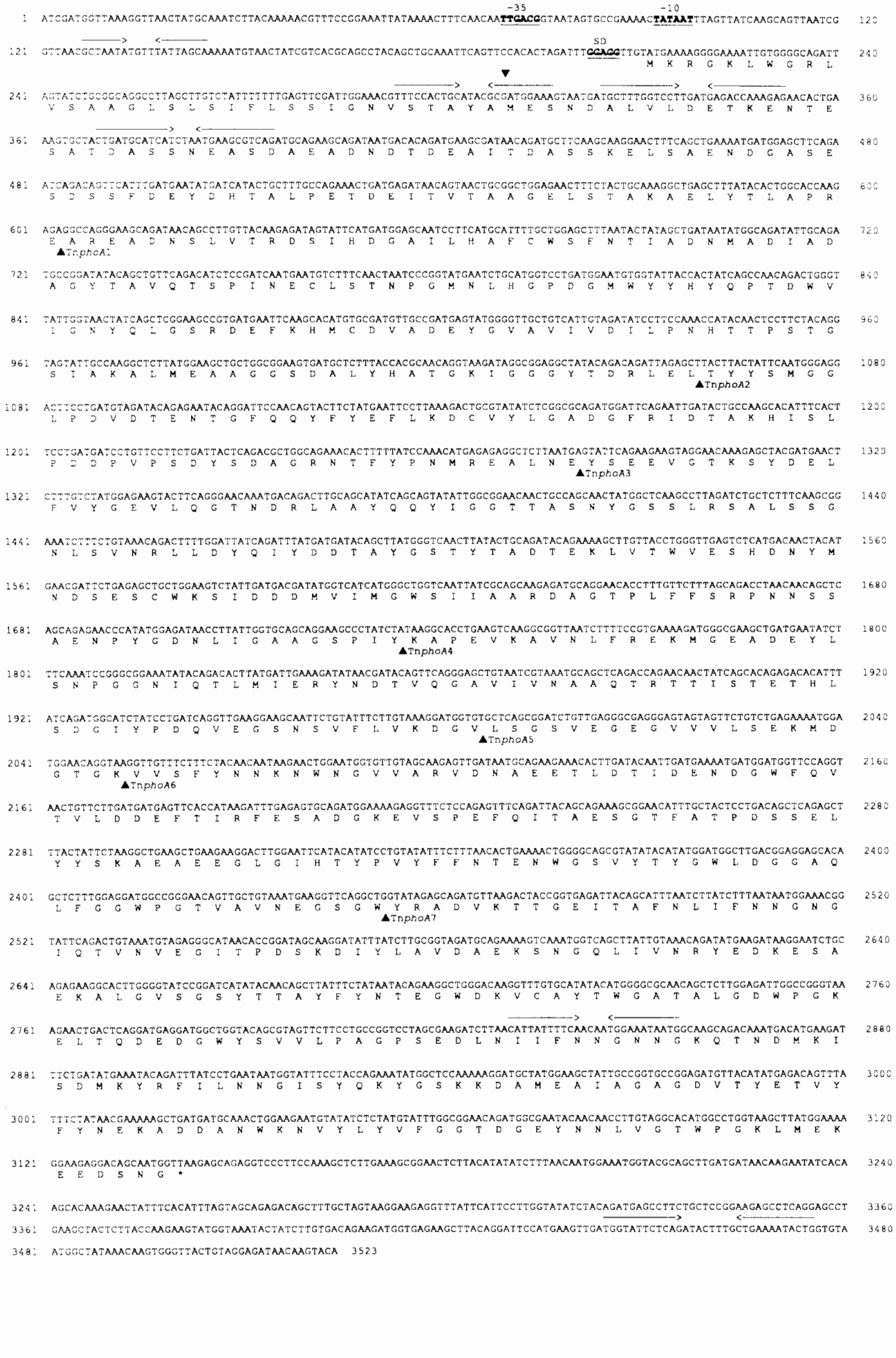
3.2.11 Protein assays. Protein concentration was measured by the dye-binding method of Bradford (1976). Bradfords reagent (2 ml; Appendix A) was added to 100 µl of sample, mixed well, and kept for 5 min at room temperature before reading the absorbance at 595 nm. Standard curves were prepared using bovine serum albumin (BSA; 0-200 µg/ml)

3.2.12 Nucleotide sequence accession number. The nucleotide sequence reported has been assigned the GenBank accession number M62507.

3.3 Results

3.3.1 Location of the *amyA* gene from *B. fibrisolvens*. A partial restriction endonuclease map of pBGB100 and the origination of the insert DNA from *B. fibrisolvens* H17c was described in Chapter 2. A more detailed restriction map of the insert DNA in pBAMY100 and derived subclones was constructed (Fig 3.1). The 3.52 kbp *Cla*I-*Sac*I fragment was subcloned in both orientations using the Bluescript SK and KS sequencing vectors (pBAMY200 and pBAMY300 respectively). Both subclones retained amylase activity. Construction of a 120 bp *Hpa*I-*Hpa*I deletion (pBAMY210) resulted in a loss of amylase activity. Exonuclease III deletions from the *Sac*I site (pBAMY220, 230 and 240) retained amylase activity, while pBAMY250 lost activity.

3.3.2 Nucleotide sequence of the *B.fibrisolvens amyA* gene. The nucleotide sequence of the 3 523-bp *Cla*I-*Sac*I fragment from pBAMY200 (Fig 3.2) revealed a single large open reading frame (ORF) which encoded a protein of 976 amino acids with a calculated M_r of 106,964. This protein is approximately double the molecular weight of the majority of prokaryote and eukaryote amylases (see reviews; Fischer and Stein, 1971; Vihinen and Mantsala, 1989), although a number of similarly sized enzymes have been described (see Discussion). A potential ribosome binding sequence (GGAGG) was present 4-bp upstream of the most likely ATG translational initiation codon at position 211. A putative


-35
-10
SD
TnphoA1
TnphoA2
TnphoA3
TnphoA4
TnphoA5
TnphoA6
TnphoA7

promoter region (TTGACG-N17-TATAAT) with strong homology to the *E. coli* (σ^{70}) consensus promoter (Hawley and Maclure, 1983), was located 113-bp upstream of the ATG start codon (Fig 3.2). A 10-bp inverted repeat sequence, which could form a Rho-dependent terminator (ΔG= -11.0 kcal(-46 kJ)/mol) (Salser *et al.*, 1977), was located 301-bp downstream of the putative TAA stop codon. Several inverted repeat sequences of unknown function were present upstream and within the ORF.

3.3.3 Putative signal sequence and Tn*phoA* fusions. In *B. fibrisolvens* the amylase enzyme is either cell associated or secreted (Cotta 1988) and thus the corresponding gene would be expected to contain a leader sequence. Also a large number of amylases have been reported to contain signal peptides (Vihinen and Mantsala, 1989). Sequence analysis of the N-terminal region of the *B. fibrisolvens* amylase revealed a putative 33-amino acid signal sequence that showed several features characteristic of signal peptides (von Heijne, 1983; 1985). A cluster of positively charged amino acids adjacent to the N-fMet (two Lys and two Arg) was followed by a hydrophobic core (Fig 3.2). There were two Ala residues at positions -3 and -1 with respect to the most likely cleavage site calculated according to the rules of von Heijne (1983). The ability of this region to function as a signal sequence in *E. coli* was confirmed by Tn*phoA* analysis.

Seven different Tn*phoA* insertions along the length of the *amyA* gene carried on pBAMY100 (Fig 3.2) conferred alkaline phosphatase activity on the PhoA⁻ *E. coli* CC118 recipient strain. The exact positions of the Tn*phoA* insertions were confirmed by nucleotide sequencing using a primer complementary to the start of the *phoA* gene. As the PhoA protein encoded by Tn*phoA* lacks its own signal peptide and is not active unless exported from the cytoplasm (Manoil and Beckwith, 1985), it was concluded that the AmyA protein provided the signal sequence for protein export. Alkaline phosphatase activity was determined from overnight *E. coli* CC118 cells containing the Tn*phoA-amyA* recombinant plasmids. All the clones with Tn*phoA* insertions in the correct frame and orientation expressed alkaline phosphatase activity. A trend towards a decrease of activity with the distance of the insert from the signal sequence was noted (Table 3.1).

Table 3.1. Levels of alkaline phosphatase activity expressed by *E. coli* CC118 cells carrying Tn*phoA-amyA* fusion plasmids.

Tn*phoA* fusion plasmid	Alkaline phosphatase activity Units/A_{600}
Tn*phoA*1	3311
Tn*phoA*2	2496
Tn*phoA*3	2224
Tn*phoA*4	1746
Tn*phoA*5	1327
Tn*phoA*6	1666
Tn*phoA*7	1326

The amylolytic activity of all the fusion proteins was tested; two fusion proteins, PhoA6 and PhoA7 (resulting from insertions at nt 2052 and 2452 respectively), retained amylolytic activity.

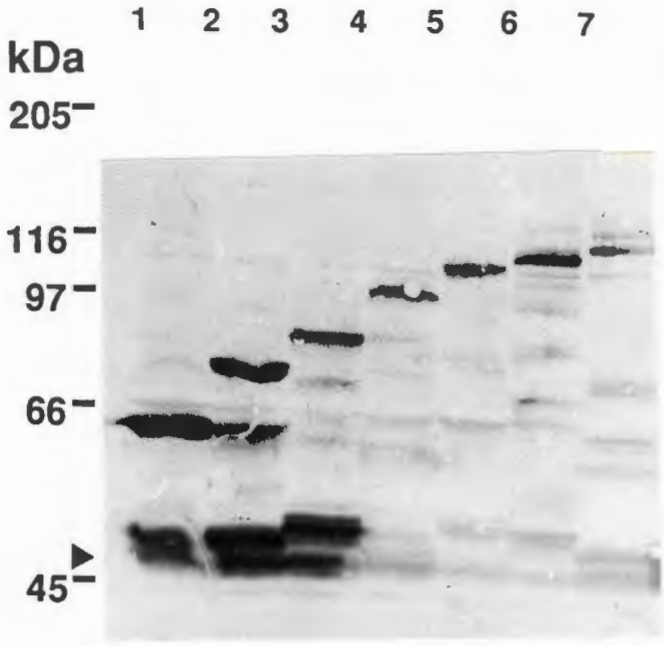


Fig. 3.3. Western blot of amylase-phosphatase fusion proteins using antibodies to alkaline phosphatase as the probe. Lanes 1-7 represent Tn*phoA* insertions 1-7 respectively. The 48 kDa protein common to all insertions is indicated by an arrow head. The molecular weight (Mr) standards indicated were: rabbit muscle myosin (205 kDa); β-galactosidase (116 kDa); phosphorylase *b* (97.4 kDa); BSA (66 kDa); ovalbumin (45 kDa); carbonic anhydrase (29 kDa).

3.3.4 Analysis of the expressed amylase polypeptides. A western blot of the amylase-phosphatase-fusion proteins using antibodies to alkaline phosphatase is shown in Fig 3.3. The predicted increase in M_r of the fusion proteins from PhoA1 to PhoA7 was 57, 77, 82, 99, 107, 112 and 127-kDa respectively. Polypeptides with apparent M_r values that corresponded closely to these predicted values were detected (Fig. 3.3). An additional protein with an apparent M_r of approximately 48,000 was observed in all lanes of the western blot. This protein corresponded to the size of the phosphatase moiety and might possibly be due to instability of the fusion proteins. These results indicated the presence of a single signal peptide and single ORF signal up to the last Tn*phoA* insertion. *In vitro* protein synthesis and analysis were carried out using a procaryotic DNA-directed translation kit (Amersham) using pBAMY100 DNA without success suggesting that additional components or membrane bound factors could be required for correct transcription or translation (data not shown).

The results of an amylase activity gel of the crude enzyme extracts obtained from *E. coli* G6MD3 containing pBAMY100 or deletion plasmids pBAMY220, 230, 240 are shown in Fig. 3.4.

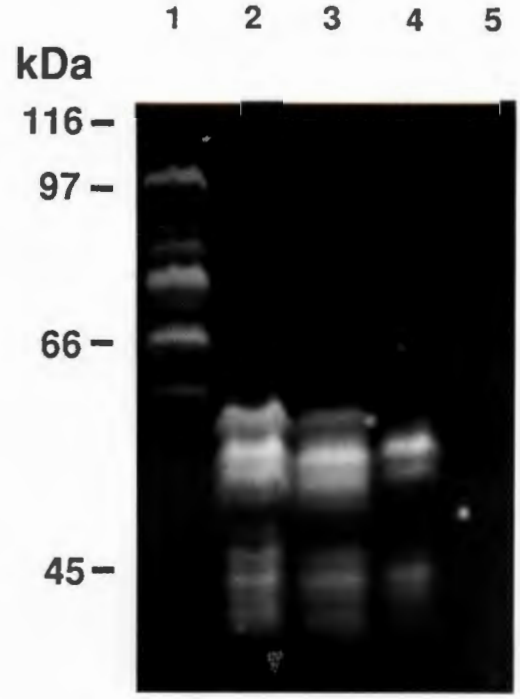


Fig. 3.4. SDS-PAGE of amylolytic activity of extracts from *E. coli* G6MD3 containing pBAMY100(lane 1), pBAMY220 (lane 2), pBAMY230 (lane 3), pBAMY240 (lane 4), and vector pBluescript.SK (lane 5). Molecular weight standards indicated were as for Fig. 3.3.

E. coli G6MD3 (pBAMY100) produced an amylase active protein with an apparent M_r of approximately 100,000 (Fig 3.4; lane 1), in close agreement with the calculated M_r of 103,257 for the amylase enzyme after signal peptide cleavage. *E. coli* G6MD3 containing plasmids pBAMY220, 230 and 240, encoding amylase proteins with predicted C-terminal deletions of 197, 260 and 315 amino acids respectively (Fig 3.4; lanes 2, 3 and 4), produced correspondingly smaller active amylase polypeptides all of which retained amylolytic activity even though up to approximately 40% of the C terminus had been deleted. The *E. coli* G6MD3 (pBluescript) control did not show any amylolytic activity (Fig. 3.4; lane 5). Additional active amylase bands of lower molecular size were also detected

in all the extracts. These may have been either due to proteolysis in the crude extract or to post-translational modification in *E. coli*. This latter mechanism has been suggested for the endo-β-1,4-glucanase of *B. fibrisolvens* (Hazlewood *et al.*, 1990).

3.3.5 Localization of amylolytic activity in *E.coli*. The cellular location of the *B. fibrisolvens* amylase enzyme in *E. coli* C600 cells was determined using full length plasmid pBAMY100 and the exonuclease III shortened plasmid pBAMY220. The results using both plasmids were very similar with the periplasmic fraction containing approximatley 86 % of the amylolytic activity (Table 3.2) indicating that the C terminus was not involved in the secretory process in *E. coli*.

Table 3.2. Localization of amylolytic activity in *E. coli* C600(pBAMY100) and *E. coli* C600(pBAMY220).

		Total activity units[a]		
Plasmid	Fraction	Amylase	β-galactosidase	β-lactamase
pBAMY100	Supernatant	12.8 (7)	55.2 (1)	59.6 (4)
	Periplasmic	159.0 (87)	584.3 (15)	1410.0 (94)
	Cytoplasmic	11.8 (6)	3198.4 (84)	22.0 (2)
pBAMY220	Supernatant	9.3 (11)	56.0 (2)	66.0 (4)
	Periplasmic	75.2 (86)	479.0 (14)	1369.0 (93)
	Cytoplasmic	3.2 (3)	2876.0 (84)	41.0 (3)

[a] 1 Unit of activity was determined as the release/hydrolysis of 1 μmol substrate per min. Numbers in parentheses represent the percentage of enzymatic activity in the respective fractions.

Only low levels of amylase activity were detected in cytoplasmic and supernatant fractions. The location of the β-galactosidase (a cytoplasmic

enzyme) and β-lactamase (a periplasmic enzyme) activity confirmed the correct fractionation of the cell components.

3.3.6 Characterization of the *B. fibrisolvens* amylase expressed in *E. coli*. *B. fibrisolvens* amylase activity was dependent on the presence of $CaCl_2$ both in the growth medium and in the assays. By following the growth (the absorbance at 600 nm (A_{600}) and corresponding amylolytic activity of *E. coli* LK111(pBAMY100) cultures, the synthesis of the amylase was found to be constitutive (Fig 3.5). The specific activity of the amylase enzyme was constant during the entire growth cycle.

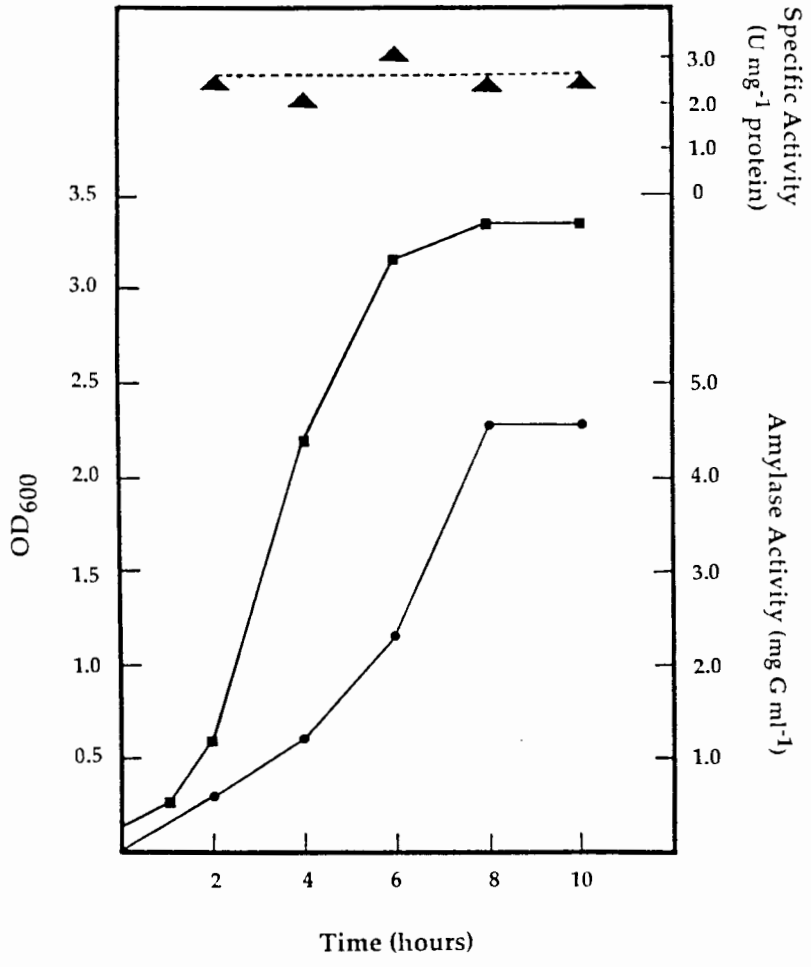


Fig. 3.5. The growth cycle and associated amylolytic activity of *E. coli* LK111(pBAMY100) grown in LB plus Ap (100 ug/ml) and 5mM $CaCl_2$. Growth was measured by absorbance at 600 nm (A_{600}) (■), amylolytic activity measured as mg reducing sugars (G) released ml^{-1} culture in 20 min, (●), and specific activity as units (μmol G min^{-1}) mg^{-1} protein, (▲).

To determine whether the expression and localization of the amylase was subject to metabolite regulation, *E. coli* C600(pBAMY100) was grown in LB containing either glucose (0.1% w/v), maltose (0.1% w/v), or starch (0.1% w/v). The activity and location of the enzyme was similar to that shown in Table 3.2 irrespective of whether maltose, glucose or starch was included in the growth medium. In *B. fibrisolvens* the growth medium is thought to influence both the expression and localization of amylolytic activity (Cotta, 1988). The expression of the *E. coli* C600(pBAMY100) amylase showed none of these regulatory influences.

The effects of pH on *the B. fibrisolvens* amylase produced by *E. coli* C600(pBAMY100) was investigated in different buffers with pH values ranging from 3.0 to 10.0 (Fig. 3.6). The enzyme showed maximal activity between pH 5.5 and pH 7.5 with a peak at pH 6.8. At pH 7.5 the enzyme showed higher activity in phosphate buffer than Tris-HCl buffer.

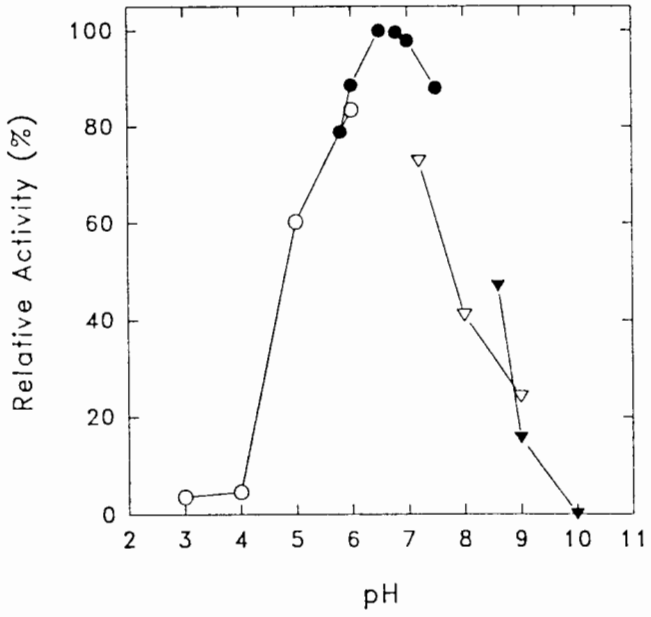


Fig. 3.6. pH activity profile of the amylase from cell extract of *E. coli* LK111(pBAMY100). Activity was assayed using soluble starch (0.1%) in the different buffers and the enzyme solutions were diluted in buffer at the particular pH. Buffers utilized were: citrate, pH 3.0-6.0 (○); phosphate, pH 5.8-7.5 (●); Tris-HCl, pH 7.2-9.0 (▽); glycine, pH 8.5-10 (▼).

The optimum temperature for amylolytic activity was 50°C (Fig. 3.7A). Temperature stability was determined by holding the enzyme at various

temperatures for 20 min and then assaying for residual activity at the optimum temperature (Fig. 3.7B) in phosphate buffer, (pH 6.8). The amylase enzyme was relatively stable up to 45°C (approximately 80% activity remaining) but showed a sharp decreased thereafter and was completely inactivared by incubation at 54°C. The relative instability of the enzyme at the optimum temperature (50°C) could be due to the absence of the substrate during the holding time. It has been shown for other amylases (Kainuma *et al.*, 1975; Brown *et al.*, 1990) that the substrate gives the enzyme greater protection against thermal inactivation.

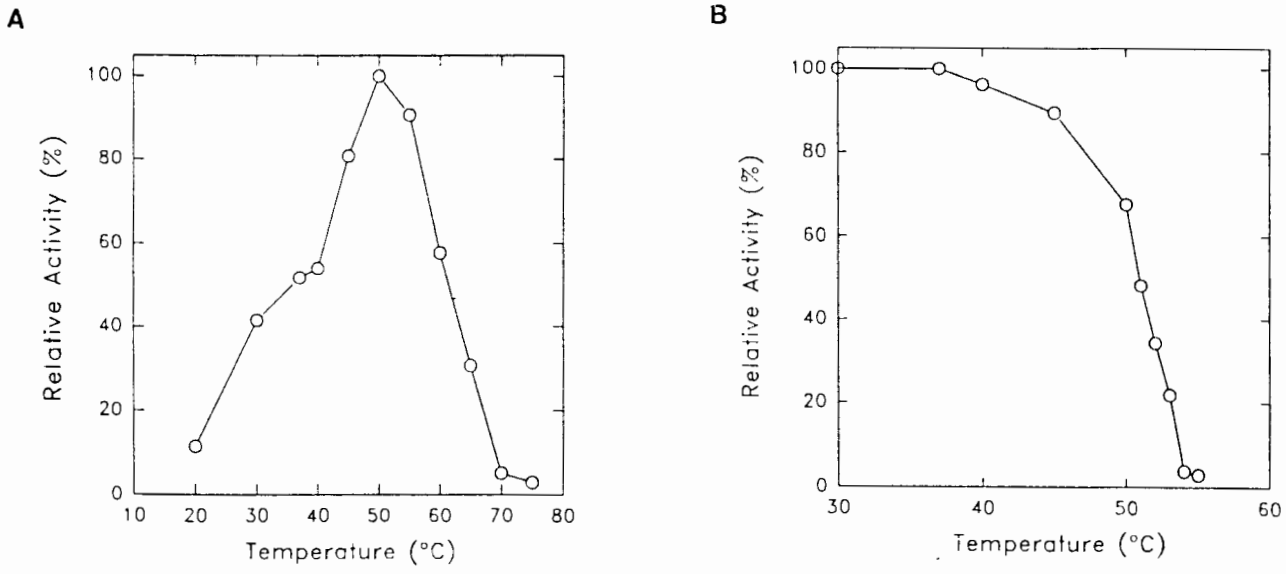


Fig. 3.7. **(A)** Temperature activity profile of the amylase from the cell extract of *E. coli* LK111(pBAMY100). Enzyme solutions were added to substrate that had been prewarmed at various temperatures, and assayed at a particular temperature. **(B)** Temperature stability of the amylase from cell extracts of *E. coli* LK111(pBAMY100). The amylase was held at various temperatures for 20 min and the activity then assayed at 50°C.

The relative rates of hydrolysis of soluble starch, amylose, amylopectin, and glycogen were determined by the release of reducing sugars and were found to be 100, 65, 58, and 14 % respectively. Pullulan, α- and β-cyclodextrins were not hydrolysed but γ-cyclodextrin was, indicating that the enzyme had endo-amylolytic activity. This was confirmed by hydrolysis of the Phadebas substrate (Ceska *et al.*, 1969). A decrease in amylase reactivity against the Phadebas substrate was detected with subclones containing deletions of the amylase C terminus (Table 3.3). The complete enzyme showed no ability to hydrolyse or

adsorb to raw corn starch granules (Walker and Hope, 1964) (data not shown). These latter results are in contrast to those found by McAllister *et al.* (1990) who reported the digestion of starch in barley and maize by *B. fibrisolvens* A38. Differences in the source of substrate and experimental conditions could account for this.

Table 3.3. α-Amylolytic activity against the Phadebas substrate of cell extracts of *E. coli* G6MD3 transformants.

Plasmid	Amino acids deleted[a]	Specific Activity Units[b]
pBAMY100	0	70
pBAMY220	197	22
pBAMY230	260	20

[a] The number of amino acids deleted from the C terminus are indicated.
[b] Specific activity was determined as the hydrolysis of 1 μmol glucosidic linkage per min, per mg protein at 45°C.

3.3.7 Analysis of the amylolytic hydrolysis products. *E. coli* has been reported to contain an α-amylase (MalS) which has been shown to hydrolyze linear and cyclic maltodextrins (Freundlieb and Boos, 1986). Whereas the cell extract of *E. coli* C600 containing the Bluescript plasmid vector degraded starch and amylose very slowly (<0.1% of the activity of the *B. fibrisolvens* amylase), G6 was hydrolysed at an appreciable rate with a similar pattern of polysaccharide products to that described previously for the α-amylase (MalS) of *E. coli* (Freundlieb and Boos, 1986)(Fig. 3.8A). The appearance of G7 and G8 was probably due to polymerization catalysed by amylomaltase (MalQ). The cell free extract of *E. coli* G6MD3 containing the Bluescript plasmid vector did not hydrolyse starch, amylose or G6 (Fig. 3.8B). This strain has a deletion in the *malA* region encoding *malQ* and *malT*, the latter coding for a positive regulator protein for maltose operons including *malS*. The *B. fibrisolvens* amylase was therefore

expressed in this strain of *E. coli* for analysis of the hydrolysis products. Initially, the hydrolysis of G6 using a crude enzyme extract of *E. coli* G6MD3(pBAMY200) was investigated; G6 was hydrolysed into mainly G3, G2 and a little G1 and G4 (Fig. 3.8C).

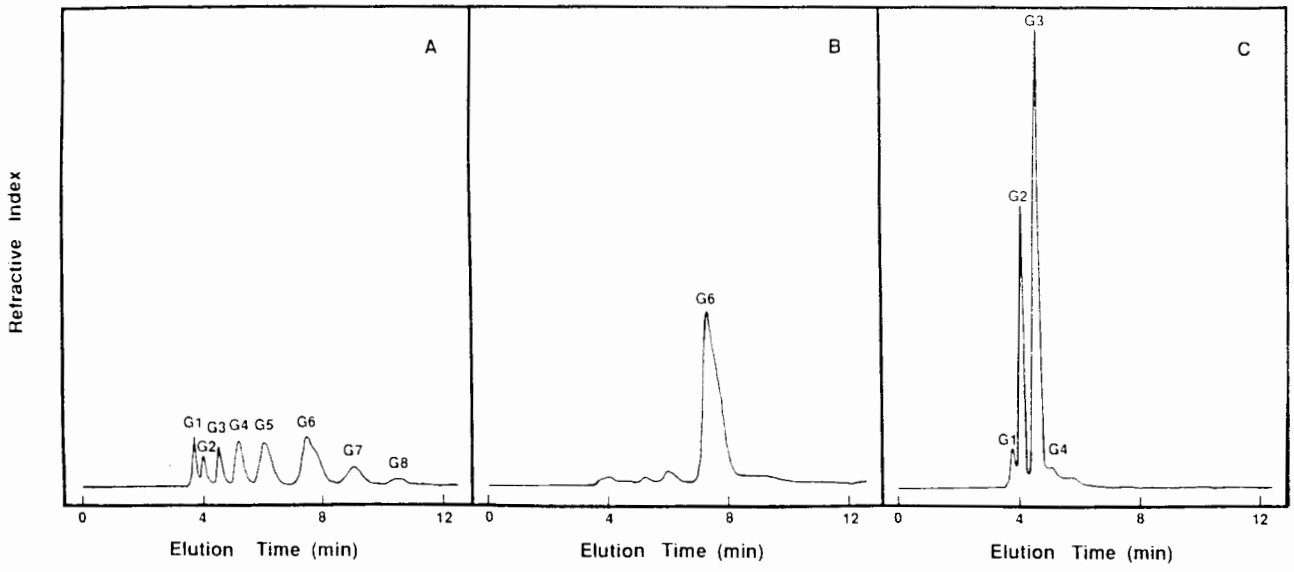


Fig. 3.8. HPLC analysis of hydrolysis products of maltohexaose (G6) using extracts of *E. coli* C600(pBluescript.SK) (A), *E. coli* G6MD3(pBluescript.SK) (B), and *E. coli* G6MD3(pBAMY200) (C). Incubation with 1% G6 was for 2 h at 45°C.

Starch degradation was followed by measuring the reduction in blue value and the production of reducing sugars as a function of time (Fig. 3.9). The shape of the blue value and reducing sugar curves confirmed that the *B. fibrisolvens* amylase could be classified as an endo-amylase (Hobson and Macphearson, 1952; Boyer and Ingle, 1972). At the point where the blue value decreased to almost zero, the proportion of reducing sugars in the reaction mixture was 27%. HPLC analysis showed that the initial hydrolysis product was maltotriose (G3), with smaller amounts of maltose (G2) and maltotetraose (G4) (Fig. 3.10). Very little glucose (G1) was detected; trace amounts of maltopentaose and maltohexaose (G5 and G6) were also initially produced but disappeared rapidly. After extended incubation (24 h) the amount of G3 decreased with a concomittant increase in G1 and G2 . As the amylase seemed to be active against G3, the hydrolysis of G3 was examined. This was found to be slow with 32 % of the G3

remaining after 24 h and with G1 and G2 as the digestion products. No amylolytic activity could be detected using G2 as the substrate.

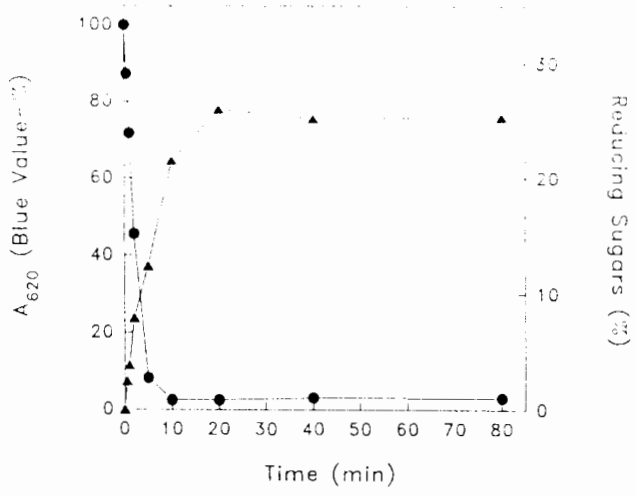


Fig. 3.9. Kinetics of starch hydrolysis using an extract of *E. coli* G6MD3(pBAMY200). The blue colour values (●) and the amount of reducing sugars (▲) were measured by the I_2/KI method and by the 3,5 dinitrosalicylic method respectively.

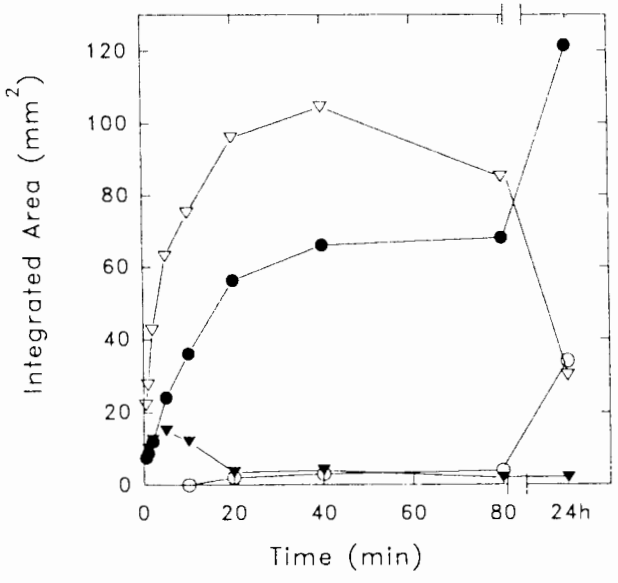


Fig 3.10. HPLC analysis of the starch hydolysis products. Glucose (○), maltose (●), maltotriose (▽) and maltotetraose (▼) are illustrated but maltopentaose and maltohexaose were not included as they were only present in trace amounts. The break in the time axis indicates the incubation period extended to 24 h.

Hydrolysis of amylose and amylopectin yielded products qualitatively similar to those from starch except that with amylopectin and starch, two unidentified compounds (probably branched oligosaccharides) were also detected eluting between G3 and G4 and between G4 and G5. This suggested that the enzyme was unable to hydrolyse but could by-pass the α-1,6 branch points which is characteristic of endo-amylases (Robyt and Whelan, 1968a; Vihnen and Mantsala, 1989).

3.3.8 Comparison with other amylases. The TFASTA subroutine, based on the Pearson and Lipman alogarithm, was used to screen nucleotide sequence databases for amylases with amino acid sequence similarity to the *B. fibrisolvens* amylase. The greatest similarity was found with a limited number of α-amylases from both prokaryotes and eukaryotes. Alignment of the amino acid sequence of the *B. fibrisolvens* enzyme with other α-amylases for greatest homology (Fig. 3.11A) showed the presence of the three highly conserved domains previously reported (Regions I, II and IV)(Rogers, 1985). A fourth conserved domain (Region III), reported by Nakajima *et al.* (1986) from a comparison of 11 α-amylases, was less clear but could be present at one of two positions (353-356 or 374-377). Region IV is characterized by a highly conserved Phe residue as the first amino acid of the region; the *B. fibrisolvens* and *B. subtilis* amylases, however, are exceptions in that they have a Trp residue in this position. Close inspection of the amino acid sequences of α-amylases from diverse genera indicated the conservation of four additional regions A, B, C and D situated between the N terminus and region I and one additional region, E between regions I and II (Fig. 3.11B). Region C of the *B. fibrisolvens* amylase included a W-Y-X-X-Y-Q-P amino acid sequence which is similar to the W-W-X-R-Y-Q-P motif shown to be involved in substrate binding in porcine pancreatic amylase (Buisson *et al.*, 1987). No discernable homology with any amylases was detected in the C terminal region of the *B. fibrisolvens* amylase.

From the three dimensional structure of porcine pancreatic amylase, Buisson *et al.* (1987) proposed two important catalytic residues, Asp 197 and Asp 300 in

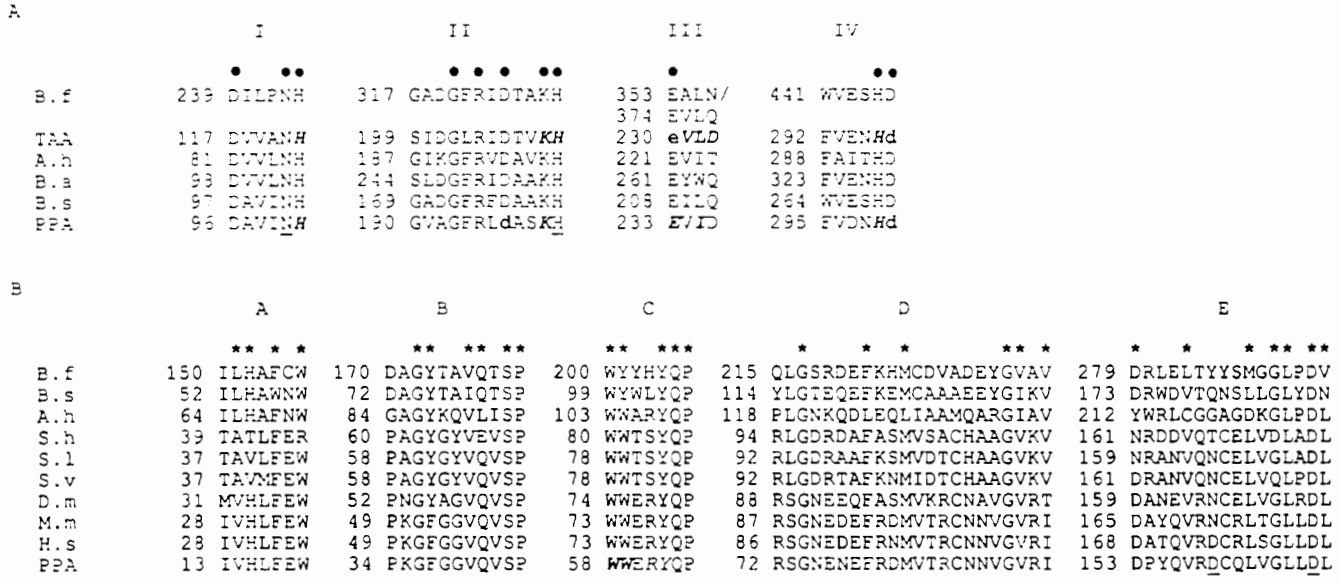


Fig. 3.11. Amino acid sequence alignment using the TFASTA program of prokaryotic and eukaryotic amylases with that of tha *B. fibrisolvens* amylase. Catalytic residues and substrate binding residues (Buisson *et al.*, 1987; Matsuura *et al.*, 1984) are denoted in bold lower case and bold italic upper case respectively. Calcium binding residues (Buisson *et al.*, 1987) are underlined. Abbreviations used are: TAA, *Aspergillis oryzea* TAKA amylase A (Toda *et al.*, 1982); B.f, *B. fibrisolvens* (this study); A.h, *Aeromonas hydrophila* (Gobius and Pemberton, 1988); B.a, *Bacillis amyloliquefaciens* (Takkinen *et al.*, 1983); B.s, *Bacillis subtilis* (Yang *et al.*, 1983); PPA, *sus scrofta domestica* (porcine pancreatic amylase, Pasero *et al.*, 1986); S.h, *Streptomyces hygroscopicus* (Hoshiko *et al.*, 1987); S.l, *S. limosus* (Long *et al.*, 1987); S.v, *S. venezuela* (Virolle *et al.*, 1988); D.m, *Drosophila melanogaster* (Boer and Hickey, 1986); M.m, *Mus muscularis* (mouse pancreatic amylase, Tosi *et al.*, 1984); H.s, *Homo sapiens* (pancreatic amylase, Nakamura *et al.*, 1984).
A: Comparison of the previously recognized conserved regions I-IV. Residues identical in all sequences are indicated by a solid circle above the sequences.
B: Additional conserved regions A-E reported in this Chapter. Regions A-D are situated between the N terminus and region I (Fig. 3.12A) and region E is between regions I and II. Asterices indicate the location of residues which are the same or have undergone conservative changes in seven of the ten amylase sequences.

regions II and IV respectively and four calcium binding residues, Asn 100 in region I, Asp 159 and Asp 167 in region E, and His 201 in region II. Similarly, Matsuura *et al.* (1984) suggested for the *Aspergillis oryzea* (Taka amylase A) amylase that the Glu 230 of region III and Asp 297 of region IV were the catalytic residues. Residues corresponding to these catalytic residues were clearly present in the *B. fibrisolvens* amylase (Fig. 3.11A). Three of the four calcium binding residues corresponding to those identified for porcine pancreatic amylase (Buisson *et al.*, 1987) were present in Regions I, II and E and were conserved. The residue which could not be identified was Asp 159 which has often been found

in mammalian but not always in bacterial amylases (Fig. 3.11B). As well as the catalytic and calcium binding residues, possible additional substrate binding residues of porcine pancreatic and Taka A amylases were present in regions I, II, III, IV, and C (Fig. 3.11 A and B). Little similarity could be found with amylase in any regions other than those described.

3.4 Discussion

The gene cloned from *B. fibrisolvens* H17c was shown to code for a large polypeptide (M_r 106,694) that had α-amylolytic activity. This polypeptide was approximately twice the size of typical microbial α-amylases which are 50,000-60,000 daltons (Vihinen and Mantsala, 1989). Other large enzymes with α-amylolytic activity usually have additional properties. These include the G6-amylase of *Bacillus* sp. H-167 of 102,597 daltons (Shirokizawa *et al.*, 1990), the G4-amylase of a *Micrococcus* sp. of 118,000 daltons (Kimura and Horikoshi, 1990) and the amylase gene from *Bacillus polymyxa* of 127,314 daltons (Uozumi et al,. 1989). The *Bacillus* sp. H-167 and the *Micrococcus* sp. amylases have an associated specific exo-amylolytic activity whereas the *B. polymxa* amylase has both α- and β- amylolytic activities. Recently amylases were reported having unusually high molecular weights (>150,000 daltons) that could degrade starch-containing plastic films (Burgess-Cassler *et al.*, 1991). The finding that approximately 40% of the C-terminal region of the *B. fibrisolvens* amylase was not essential for amylolytic activity raised the question as to the function of this region. Deletion of this region resulted in a slower rate of hydrolysis of the insoluble cross-linked starch present in the Phadebas substrate (Table 3.3). It is therefore possible that this region is associated with efficient substrate binding although the enzyme did not bind to or hydrolyse raw corn starch. Cyclyomaltodextrin glucanotransferases usually also have an additional C-terminal domain. This region, originally thought to be an active centre separate from the N-terminal region, is now proposed to act as an additional substrate binding domain (Helman *et al.*, 1990).

The *B. fibrisolvens amyA* gene appeared to be expressed in *E. coli* from a promoter located on the cloned fragment. Expression of the *amyA* gene was independent of orientation with respect to the vector *lacZ* gene (pBAMY200 and pBAMY300). A putative *B. fibrisolvens amyA* gene promoter very similar to both the sequence and the spacing of the -35 and -10 regions of the *E. coli* consensus promoter was situated within a *Hpa*I fragment upstream of the 5' end of the gene. A *Hpa*I deletion of this sequence did not remove the ribosomal binding site nor the start codon, but resulted in a loss of expressed enzymatic activity indicating that the *B. fibrisolvens amyA* gene promoter was functional in *E. coli*. The first 33 amino acids of the amylase ORF had features characteristic of signal peptides (von Heijne 1983, Watson 1984). Although longer than signal peptides generally found in Gram-negative bacteria (Chang, 1987), this signal peptide was functional in *E. coli* as both the amylase and the AmyA-PhoA fusion proteins were exported to the periplasm. Leader sequences of between 31 and 44 residues have been reported previously for Gram-positive bacterial proteins (Watson, 1984), and the *B. fibrisolvens* amylase signal peptide is the same length as that from the xylanase gene of *B. fibrisolvens* (Mannerelli *et al.*, 1990a).

Most α-amylases studied in detail are calcium metalloenzymes and require calcium for optimal stability and activity (Fisher and Stein, 1966; Vinhinen and Mantsala, 1989). Calcium was a strong activator of the *B. fibrisolvens* amylase and had to be included in both the growth media and assay buffers for optimum enzymatic activity. The ability of the *B. fibrisolvens* amylase to hydrolyse the Phadebas substrate and the characteristic shape of the Blue value and reducing sugar curves enabled the enzyme to be identified as an endo-amylase. Hydrolysis of γ-cyclodextrin to maltotriose, maltose and glucose confirmed the endo- α-amylase character of the enzyme. α-, β- cyclodextrins and pullulan were not hydrolyzed by the *B. fibrisolvens* amylase but the reason why these substrates were not cleaved is unclear. The former substrates have, however, been shown to inhibit the *R. amylophilis* α-amylase (Mcwethy and Hartman, 1977) and the sweet potato β-amylase (Thoma and Koshland, 1960), and likewise may be

inhibitory to the *B. fibrisolvens* enzyme. Maltotriose was the main degradation product during the initial stages of substrate hydrolysis although after extended incubation the maltotriose was hydrolysed to maltose and glucose. This hydrolysis profile suggested that the *B.fibrisolvens* endo-amylase, like some exo-α-amylases, has a preference for removing maltotriose units. The action of the *B. fibrisolvens* amylase, however, appears to be different to that of the exo-α-amylases which produce specific malto-oligosaccharides from soluble starch (Nakakuki *et al.*, 1984; Fujita *et al.*, 1989; Shivokizawa *et al.*, 1989; Condussio *et al.*, 1990), in that it has no specific exo-activity. This is illustrated by the rapid disappearence of the blue colour in starch digests and the presence of oligosaccharides other than maltotriose after hydrolysis. An amylase isolated from a strain of *B. subtilis* (Takasaki, 1985) has very similar properties to that of *B. fibrisolvens*. This enzyme produces maltotriose as the main hydrolytic product by an endo-mechanism and cannot cleave α-, β- cyclodextrins and pullulan. The saccharifying-type activity of the *B. fibrisolvens* amylase has been described for α-amylases from *B. subtilis* (Yamane *et al.*, 1973; Matsuzaki *et al.*, 1974; Emori *et al.*, 1990) and for a few α-amylases from ruminal bacteria (McWethy and Hartman, 1977; Walker and Hope, 1964; Cotta, 1988). Ruminal amylases that have been characterized also have G1, G2 and G3 as their main α-amylolytic products (MacWethy and Hartman, 1964; Hobson and Macphearson, 1952; Walker and Hope, 1964; Cotta, 1988). The *B. fibrisolvens* amylolytic activity is also similar to the maltogenic α-amylase of *A. oryzea* which is used commercially in the production of high maltose content syrups (Norman, 1979).

Comparison of the amino acid sequence of the *B. fibrisolvens* H17c amylase with other amylases showed that the four conserved regions commonly found in α-amylases were present. Three putative calcium binding residues (Buisson *et al.*, 1987) were also observed which was not surprising since the *B. fibrisolvens* required calcium ions for activity. In addition, five regions of amino acid sequence similarity with prokaryotic and eukaryotic amylases were found of which three regions (A, C and E) are associated with substrate and calcium binding, and of which two regions (B and D) are associated with protein folding

(Buisson *et al.*, 1987). The overall amino acid homology between the *B. fibrisolvens* H17c amylase and other amylases was limited to the N terminal half of the enzyme with a 20-30% similarity to both prokaryotic and eukaryotic amylases in this region. No similarity in the C-terminal half was found with any previously determined amylase sequences. Buisson *et al.* (1987) have concluded from the crystal structure of porcine pncreatic α-amylase that domains A and B, comprising the first 407 amino acids, contain the calcium binding and catalytic residues. The function of domain C, a distinct globular region comprising the remaining 89 amino acids, is unclear.

Long *et al.* (1987) found that the amino acid sequence of the *Streptomyces limosis* amylase had a high degree of similarity with mammalian and invertebrate amylases and has suggested a common evolutionary origin. The similarity between the N-terminal region of the *B. fibrisolvens* amylase with that of amylases from both *S. limosis* and eukaryotes is interesting, as it supports this view.

Chapter 4

Characterization in *E. coli* of a *glgB* gene from *B. fibrisolvens* encoding a glycogen branching enzyme with starch clearing activity.

Chapter 4

Characterization in *E. coli* of a *glgB* gene from *B. fibrisolvens* encoding a glycogen branching enzyme with starch clearing activity.

4.0 Summary

A *B. fibrisolvens* H7c *glgB* gene, was isolated by direct selection for colonies that produced clearing on starch azure plates. The gene was expressed in *E. coli* from its own promoter. The *glgB* gene consisted of an open reading frame of 1920 bp encoding a protein of 639 amino acids (calculated M_r = 73,875) with 46-50% sequence homology with other branching enzymes. A limited region of 12 amino acids showed sequence similarity with amylases and glucanotransferases. The *B. fibrisolvens* branching enzyme was shown to have no hydrolytic activity towards starch and to stimulate the phosphorylase a mediated incorporation of glucose into α-1,4 glucan polymer 13.4 fold. The branching enzyme was purified to homogeneity by a simple two-step procedure; N terminal sequence and amino acid composition determinations confirmed the deduced translational start and amino acid sequence of the open reading frame. The enzymatic properties of the purified enzyme were investigated. The enzyme was shown to transfer chains of between 5 to 10 (optimum of 7) glucose units, using amylose and amylopectin as substrates, to produce a highly branched polymer. During "de novo" synthesis a polysaccharide with properties similar to glycogen was obtained. Optimal activity of the branching enzyme was at pH 7.2 and 37°C. The enzyme was inhibited by mercuric chloride but unaffected by 2 M sodium chloride.

4.1 Introduction

The *glgB* gene product catalyzes the synthesis of α-1,6-glucosidic linkages in glycogen. A number of reports have dealt with the properties and action of bacterial, mammalian and plant branching enzymes and have been reviewed in the General Introduction. The branching enzymes from *E. coli* (Boyer and Preiss, 1977), *Neurospora crassa* (Matsumoto *et al.*, 1983), potato (Borovsky *et al.*, 1985) and rabbit skeletal muscle (Cauldwell and Cohen, 1980) have been purified to apparent homogeneity.

Not many studies of bacterial branching enzymes at the molecular level have been reported, and most of the work has been done with *E. coli*. The synthesis of glycogen biosynthetic enzymes is under complex regulation influenced not only by nutrient depletion but also by the rate of bacterial growth (reviewed in Preiss, 1984). The *glg* genes have no selectable phenotype which is one of the reasons for the limited number of molecular studies. Two different strategies have been utilised for the isolation and cloning of branching enzymes. Indirect selection was used by Okita *et al.* (1981) to clone the *glg* genes by co-transformation with the selectable *asd* (aspartate semidehydrogenase) gene which is adjacent to the *glgB* gene. Kiel *et al.* (1989) isolated the *glgB* gene from the cyanobacterium *Synochococcus* sp. PCC7942 (*Anacystis nidulans)* by using the *glgB* gene from *E. coli* as a hybridizaton probe. The *A. nidulans* gene was sequenced and shown to have a 46% overall amino acid sequence similarity to the *E. coli* branching enzyme (Kiel *et al.*, 1990). It has been shown that branching enzymes from some bacteria (especially the enteric bacteria) cross-reacted with antibodies raised against *E. coli* branching enzyme (Holmes *et al.*, 1982). Similarly the branching enzymes from algae cross-react with branching enzymes from the cyanobacterium *A. nidulans* (Fredrick *et al.*, 1980). This suggests a similarity in the overall structure of branching enzymes.

In this chapter the nucleotide sequence of the *glgB* gene isolated from the *B. fibrisolvens* H17c genebank is described; the gene product was purified to homogeneity and the catalytic activity characterised. The amino acid sequence is compared with that of previously sequenced enzymes of glycogen metabolism. Reasons for the incidental isolation of the *glgB* gene using starch plates are discussed (see Chapter 2 for methods).

4.2 Materials and Methods

4.2.1 Media, buffers and enzymes. All media and buffers not described in the text are given in Appendix A. Potato amylose (amylopectin free), potato amylopectin (amylose free), rabbit muscle phosphorylase a, rabbit liver glycogen, pullulanase (from *Enterobacter aerogenes*) and isoamylase (from *Psuedomonas amyloderamosa*) were obtained from Sigma Chemical Co. [^{14}C] Glucose-1-phosphate was purchased from New England Nuclear, diethylaminoethyl (DEAE) - cellulose from Whatman, and Sephadex G 100 from Pharmacia. Chromatographic media were prepared according to the manufactures instructions. Restriction endonucleases, T4 DNA ligase and S1 nuclease were purchased from Boeringer Mannheim Biochemicals. Exonuclease III was obtained from Bethesda Research Laboratories. All other chemicals used were of analytical grade.

4.2.2 Bacterial strains and plasmids. The genotypes of *E. coli* strains are described in Appendix B. Genetic manipulations were carried out in *E. coli* LK111. Transformants of *E. coli* K12 G6MD3, the glycogen deletion strain (a gift from Dr. J. Preiss, Dept. Biochemistry, Michigan State University), were used for the purification and assays of the branching enzyme. The plasmid pBGB100 described in Chapter 2.3.5 was used as a primary source of DNA for plasmid subcloning. The M13-derived Bluescript SK plasmid (Stratagene, San Diego) was used for the preparation of nt sequencing templates (Appendix D). Plasmids

pBGB200, pBGB300, pBGB210, pBGB230 and pBGB240 were constructed during this study.

4.2.3 Growth conditions. *E. coli* strains were grown in Luria-Bertani (LB) medium and 0.1 mg ml^{-1} ampicillin was added for the selection of transformants. When *E. coli* G6MD3 was used, the medium was supplemented with 50 µg ml^{-1} diaminopimelic acid due to the *asd* deletion present in this strain. For the isolation of the branching enzyme 0.6 % glucose was added to the media.

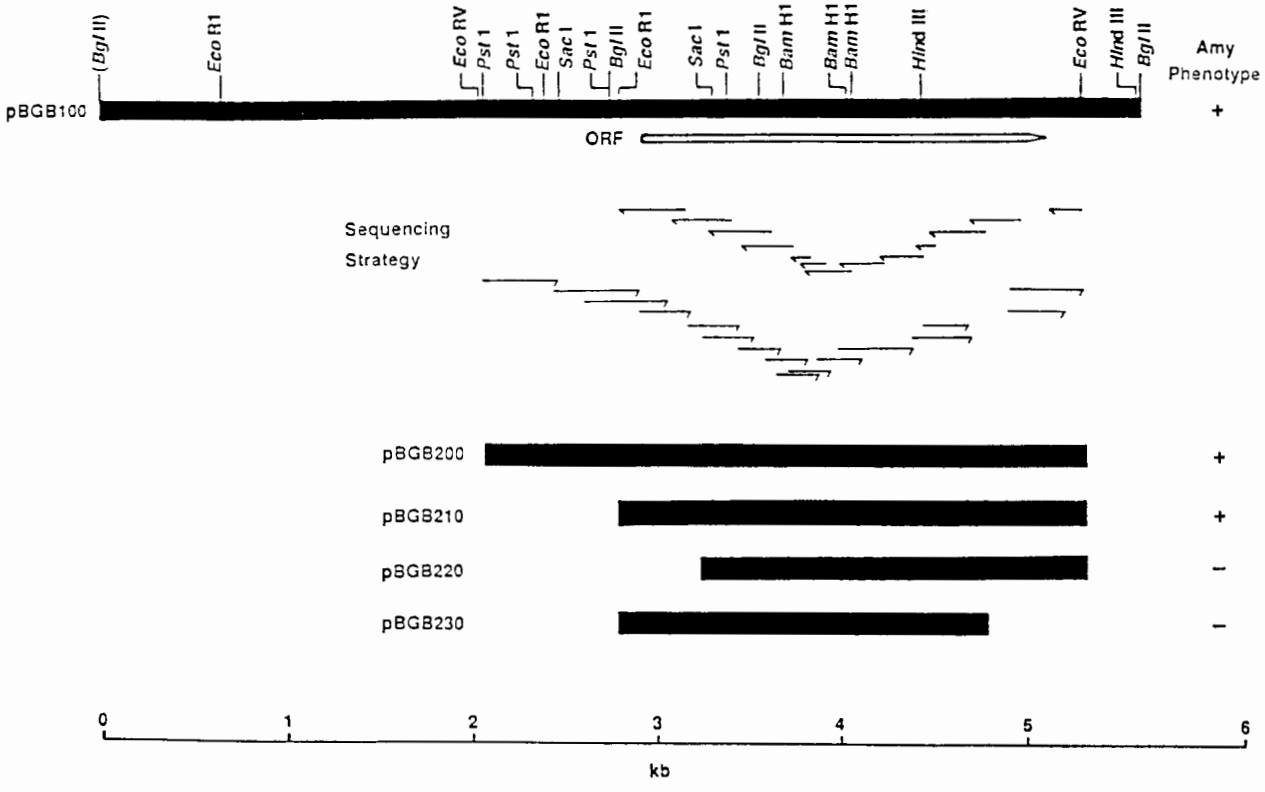


Figure 4.1 Restriction endonuclease map of the insert DNA on pBGB100, and derivatives thereof encoding the *B. fibrisolvens* H17c branching enzyme. The ORF and direction of transcription are indicated by an open arrow, amy phenotype indicates the presence /absence of starch clearing zones produced by clones. pBGB300 is not shown as it is identical to pBGB200 except in the opposite orientation. The sequencing strategy of pBGB200 is indicated above the insert restriction map. Thin arrows represent the extent and direction of sequencing of templates generated by exonuclease III digestion.

4.2.4 Sequencing strategy and exonuclease III digestion. The 3.25-kb *Eco*RV-*Eco*RV restriction endonuclease fragment from pBGB100 (section 2.3.5) was gel purified (Appendix A) and subcloned into *Eco*RV restriction endonuclease

digested pBluescript SK. The *B. fibrisolvens* *Eco*RV-*Eco*RV DNA fragment inserted in both orientations giving rise to pBGB200 and pBGB300 (Fig. 4.1). These plasmids were used to generate overlapping deletions from both ends of the *Eco*RV fragment by the exonuclease III shortening technique (Henikoff 1984; 1987). Progressive deletions from the 5' end of the insert were generated after digestion of pBGB200 and pBGB300 with *Apa*I and *Cla*I restriction endonucleases. Details of the exonuclease III digestion and selection of suitable DNA sequencing templates are described in Appendix A.

4.2.5 Nucleotide sequencing. The complete nt sequence of both strands of the DNA coding for the *glgB* gene was determined using the overlapping DNA fragments generated by exonuclease III digestion. DNA sequencing was carried out by the dideoxynucleotide triphosphate chain termination method of Sanger *et al.* (1977). This method is described in Appendix A. The nucleotide and deduced amino acid sequences were analysed as described in Chapter 3.2.5.

4.2.6 *In vitro* transcription and translation. A prokaryotic DNA-directed cell-free system (Amersham, Prokaryotic DNA-directed cell-free translation kit, Code N.380) was used to investigate the synthesis of proteins by plasmids pBGB200, pBGB300, pBGB210, pBGB220 and pBGB230. The protocol specified by the manufacturers was followed throughout using quarter quantities. The resulting polypeptides were labelled with L-[^{35}S]methionine (specific activity 1040 Ci/mmol) and resolved by 15% SDS-PAGE by the method of Laemmli (1970) (Appendix A). Translated polypeptides were visualized by autoradiography. Proteins expressed *in vivo* by *E. coli* G6MD3 from these plasmids were similarly resolved by 15% SDS-PAGE. Pharmacia molecular-mass standards were used as markers.

4.2.7 Preparation of cell extracts. Cell-free extracts were prepared as described in Chapter 3.2.7 except that 0.1 M sodium citrate buffer (pH 7.0) was used. The cell extracts of *E. coli* G6MD39(pBGB100) were used to assay for branching enzyme activity. Samples were stored at either 4°C or -20°C.

4.2.8 Large-scale preparation of cell extracts. Cell-free extracts were prepared from 2 X 200 ml overnight cultures of *E. coli* G6MD3(pBGB200) grown in LB medium with Ap (100 μg/ml), diaminopimelic acid (50 μg/ml) and 0.6% (w/v) glucose. The cells were harvested, washed with saline and resuspended in 20 ml 10 mM Tris-HCl 50 mM citrate (pH 7.4). The cells were passed through an Aminco French Pressure cell at 1.1 X 10^5 kPa and the extract clarified by centrifugation at 27 000 x g for 30 min at 4°C.

4.2.9 Enzyme assays.

Branching enzyme assay I: The basis of this assay is the stimulation caused by the branching enzyme preparation on the unprimed synthesis of α-1,4 glucan polymer from glucose-1-phosphate by rabbit muscle phosphorylase *a* (Brown and Brown, 1966a). The method used was essentially as described previously (Holmes et al, 1982). A reaction mixture of 0.1 ml contained 0.1 M sodium citrate (pH 7.0), 1 mM AMP, 370 nM [^{14}C] glucose-1-phosphate (313 mCi/mmol), 20 μg of rabbit muscle phosphorylase *a* and branching enzyme. The reaction was initiated by the addition of phosphorylase *a* rather than by glucose-1-phosphate (Holmes *et al.*, 1977) and incubated at 30°C for various time intervals. The incoporation of label into a methanol insoluble glucan was assayed as previously described (Hawker *et al.*, 1974). The incorporation of 1 μmol of glucose into glucan per minute under the above conditions is defined as a unit of enzyme activity. Controls containing no branching enzyme or heat denatured branching enzyme were included in the assays.

Branching enzyme assay II: The action of branching enzyme against α-1,4 glucans was determined by following both the decrease in absorption of the maximum wavelength, and the shift in absorbance spectrum of the α-glucan-iodine complex. The reaction mixtures contained 2 mg/ml of polysaccharide, 0.1 M sodium citrate buffer (pH 7.2) and branching enzyme. After incubation at 35°C for timed intervals, 0.05 ml samples were withdrawn, the reaction terminated by the addition of 0.1 ml of 0.1 N HCl, 0.05 ml of iodine reagent (2

g/L I_2 and 20 g/L KI) and diluted to 1 ml. The absorbance of the iodine complex was measured at the λmax for the natural α-glucan. One unit of branching enzyme activity was defined as the amount of enzyme that caused a decrease of 20 % in the absorbance of the α-glucan-iodine complex in 1 min at 35 ℃. The average degree of polymerization (DP), and the average length of the unit chains before and after the action of branching enzyme on amylopectin was determined according to the method of Boyer and Preiss (1977). The reaction products were debranched in 0.1 M sodium citrate buffer with isoamylase (50U/ml) at pH 3.5, and pullulanase (5U/ml) at pH 5.5 to enzymatically determine chain lengths. Total glucose equivalents were determined by the anthrone-sulphuric method (Ashwell, 1957) and reducing glucose equivalents by a copper reducing method of Nelson-Somogyi (Spiro, 1966). The unit chain lengths released in the above reactions were separated using a Beckman high pressure liquid chromatography (HPLC) system equiped with a model 156 refractive index detector and a Waters C18 column. Standards of glucose units, G1-G10, were used to calibrate the column.

Phadebas amylase test: This test, commercially available from Pharmacia (Sweden), is a quantitative assay of α-amylase activity (Ceska *et al.*, 1969). The manufacturers specifications were followed. One unit of activity is defined as the amount of enzyme required to catalyze the hydrolysis of 1 μmol glucosidic linkage per min at 37℃.

4.2.10 Protein assays. Protein concentration was measured by the dye-binding method of Bradford (1976) using BSA as the standard (Chapter 3.2.11).

4.2.11 Amino acid sequence and chemical analysis. Sequence analysis was performed on a gas-liquid solid phase sequencer (Brandt *et al.*, 1984). The amino acids were indentified by an isocratic HPLC system on a 3 x 250 mm 3μ Lichrospher C18 (Bishoff) column. The amino acid composition was determined by gas-phase hydrolysis of proteins (Bidlingmeyer *et al.*, 1984) and seperation on a HPLC C18 Waters column (Klapper, 1982).

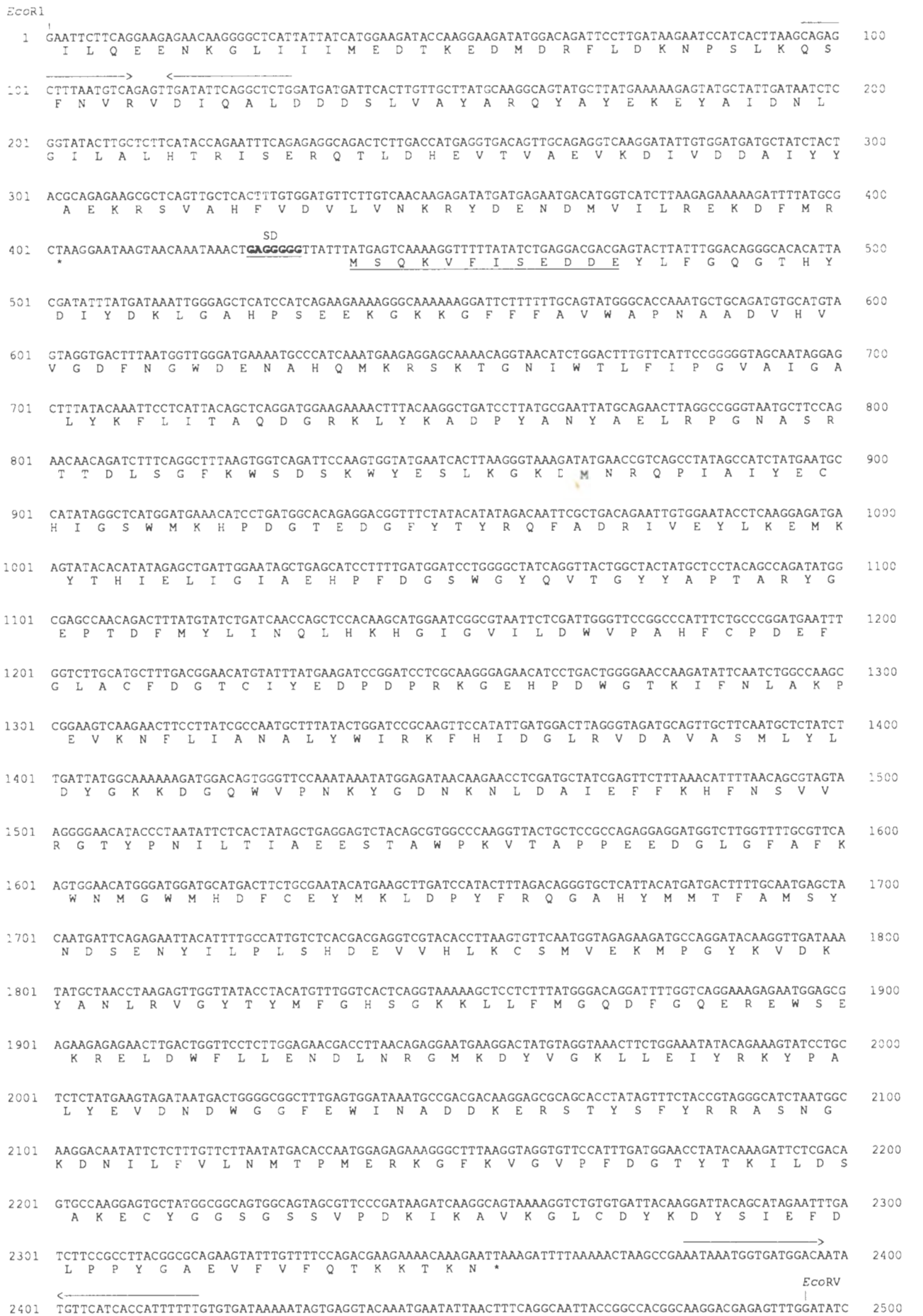
4.2.12 Nucleotide sequence accession number. The nucleotide sequence has been assigned the GenBank accession number M64980.

4.3 Results

4.3.1 Location of the *glgB* gene from *B. fibrisolvens*. The recombinant plasmid pBGB100, which when expressed in *E. coli* produced zones of clearing on starch azure plates, was chosen for further study. A preliminary restriction endonuclease map of pBGB100 and the confirmation of the origin of the insert DNA was described in Chapter 2.3.5. A restriction map of the 5.8 kbp insert fragment on pBGB100 is shown in Fig. 4.1. The 3.25 kbp *Eco*RV-*Eco*RV fragment was subcloned into the Bluescript SK sequencing vector in both orientations (pBGB200 and pBGB300), with the orientation of pBGB200 in the opposite direction to the vector *lac* promoter. Both subclones retained the ability to produce halos on starch plates suggesting that an endogenous promoter was present on the DNA insert fragment. A smaller 2.5-kbp *Eco*RI-*Eco*RV fragment, pBGB210, which retained starch clearing activity served to localize the position of the *glgB* gene. This plasmid contained the smallest fragment to code for an active gene as exonuclease III deletions from either the 5' or the 3' ends (pBGB220 and pBGB230 respectively) resulted in a loss of enzyme activity.

4.3.2 Nucleotide sequence of the *glgB gene*. The nucleotide sequence of the 2500 bp *Eco*RI-*Eco*RV fragment contained an ORF encoding a protein of 639 amino acids with a calculated M_r of 73 875 (Fig. 4.2). A potential ribosomal binding sequence (GAGGGGG) was situated 6 bp upstream of the ATG initiation codon at position 439. No sequence similar to either the σ^{70} or σ^{54} *E. coli* consensus promoter sequences (Harley and Reynolds, 1987) could be found upstream of the initiation codon. An incomplete unidentified ORF of 134 amino acid residues terminated at a TAA codon 35 bp upstream of the putative *glgB* start codon. An 18 bp inverted repeat sequence including a region of 6T residues was located 21 bp downstream of the putative *glgB* stop codon (Fig. 4.2). This

Fig. 4.2 Nucleotide sequence of the *B. fibrisolvens* H17c *glgB* structural gene. The predicted amino acid sequence is given below in single letter code. The putative Shine-Delgarno (SD) sequence is underlined and in bold, the amino acids determined by sequence analysis are underlined and the inverted repeat sequences are shown by converging arrows. The nucleotide and amino acid sequences of a second incomplete ORF are indicated upstream of the *glgB* gene.


EcoR1
SD
EcoRV

sequence has the potential to form a mRNA stem-loop structure with a ΔG = -16.55 kcal (-69.26 kJ)/mol (Salser, 1977) and could serve as a Rho-independent transcriptional terminator loop (Rosenberg and Court, 1979) in *E.coli*.

4.3.3 *In vitro* and *in vivo* detection of the translation product. An *in vitro E. coli* cell-free transcription-translation system was used to determine the *M*r of the proteins expressed by plasmid pBGB200 and its derivatives. SDS-PAGE of the *in vitro* translation products showed (Fig. 4.3.1) that a protein with an apparent M_r of approximately 71,000 as well as some degradation products were produced from both pBGB200 and pBGB300 (lanes 1 and 2).

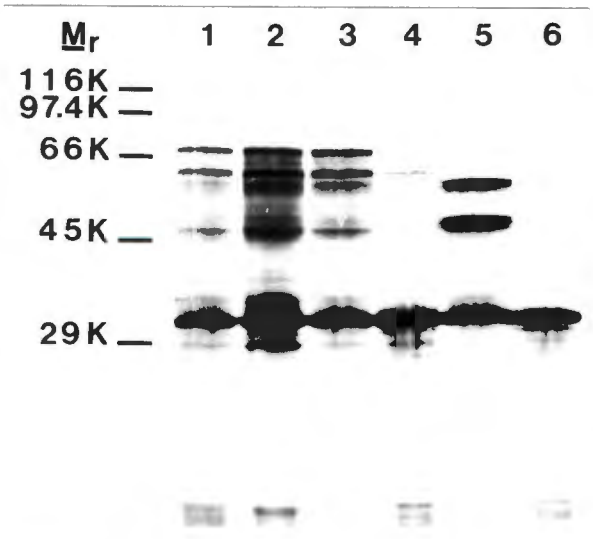


Fig. 4.3.1 SDS-PAGE analysis of *in vitro* expressed proteins encoded by the cloned *B. fibrisolvens glgB* gene. Lane 1, pBGB200; lane 2, pBGB300; lane 3, pBGB210; lane 4, pBGB220; lane 5, pBGB230; lane 6, pBluescript. The molecular mass (Mr) standards indicated were: rabbit muscle myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

The apparent M_r of this protein is in close agreement with the calculated M_r of 73,875 from the expected amino acid sequence of the *glgB* gene. A protein of a similar apparent M_r was also produced from pBGB210, the smallest insert coding for an active enzyme (lane 3). The higher concentration of protein produced when pBGB300 was used (lane 2) may reflect an increased level of transcription as both the *lacZ* promoter of the vector and the *glgB* promoter were

being transcribed in the same direction. The 71 kDa protein was absent when the Shine-Delgarno ribosomal binding site and the ATG initiation codon of *glgB* were deleted (pBGB220, lane 4) and the apparent M_r of the protein was reduced when a C terminal deletion of the *glgB* polypeptide was used (pBGB230, lane 5). The protein with an apparent M_r of 30 000 present in all lanes including that of the Bluescript vector (lane 6) corresponded to the β-lactamase polypeptide. A dominant protein with an apparent M_r of 71,000 was present in the *in vivo* translation products of *E. coli* G6MD3 carrying plasmids pBGB100,pBGB200, pBGB300 and pBGB210 (Fig. 4.3.2, lanes 1, 2, 3 and 4 respectively).

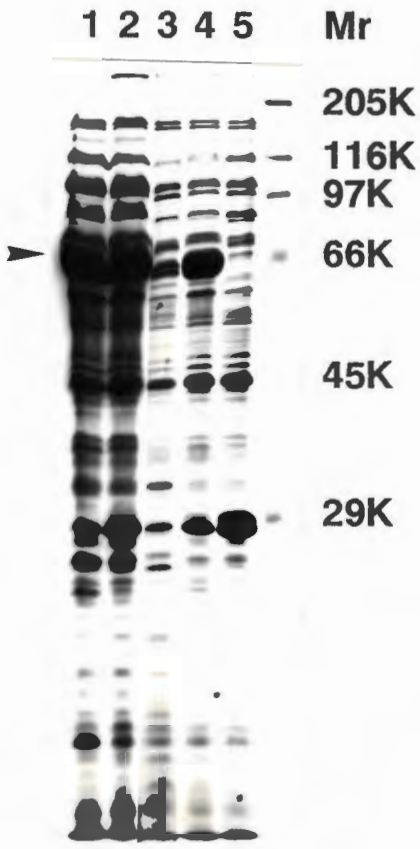


Fig. 4.3.2 SDS-PAGE analysis of *in vivo* expressed proteins encoded by the cloned *B. fibrisolvens glgB* gene. Lane 1, *E. coli*(pBGB100); lane 2, *E. coli*(pBGB200); lane 3, *E. coli*(pBGB300); lane 4, *E. coli*(pBGB210); lane 5, *E. coli*(pBluescript). Maps of the pBGB subclones are shown in Fig. 4.1. The molecular mass (Mr) standards indicated were as described in Fig. 4.3.1

This protein was not present in *E.coli* G6MD3 (pBluescript) (lane 5). High levels of expression of the *glgB* gene product appeared to be lethal. Although pBGB300

had the highest protein level *in vitro*, only low yields of protein were observed *in vivo* (Fig. 4.3.1, lane 2 and Fig. 4.3.2 lane 3)

.4.3.4 Amino acid sequence alignments of the *B. fibrisolvens* branching enzyme with other bacterial branching enzymes. The TFASTA subroutine based on the Pearson and Lipman algorithm was used to compare the deduced amino acid sequence of the *B. fibrisolvens glgB* polypeptide with sequences present in several data bases. Alignment of the amino acid sequence of branching enzymes from *A. nidulans* (Kiel *et al.*, 1990), *Bacillis stearothermophilis* (Kiel *et al.*, 1990, GenBank accession number M35089) and *E. coli* (Baecker *et al.*, 1986) with the *B. fibrisolvens* enzyme revealed an identity of 50%, 46% and 46% respectively (Fig. 4.4.1). The *E.coli* and *A. nidulans* branching enzymes had approximately 100 additional amino acid residues at the N terminus. Some similarity to one of the conserved regions of amylases and glucanotransferases (Nakajima *et al.*, 1986) was detected in a region of the N terminus (Fig. 4.4.2), a region known to have contacts with the α-1,4 glucan substrate (Matsuura *et al.*, 1984). Similarity in this region to the *glgX* gene product from the *E. coli* glycogen gene cluster was also found and this region is particularly well conserved amongst the branching enzymes (Fig. 4.4.1).

4.3.5 Branching enzyme activity in crude cell extracts. Branching enzyme activity expressed by the *B. fibrisolvens glgB* gene in the glycogen deletion strain *E.coli* G6MD3 was determined (Assay I, Fig. 4.5). Plasmid pBGB200 was chosen for study since the *glgB* gene is expressed from its own promoter (section 4.3.1). Whereas cell-free extracts of *E. coli* G6MD3 (pBluescript) were unable to stimulate the phosphorylase *a* mediated incorporation of [^{14}C] glucose into an α-1,4 glucan polymer, *E. coli*(pBGB100) extracts containing the glgB gene product caused a 13.4 fold increase in this incorporation. This is similar to the 11-14 fold increase reported for the spinach branching enzyme (Hawker *et al.*, 1974). Heat denaturation of the *E. coli* G6MD3 (pBGB100) cell free extract by incubation for 2 min at 100 °C did not totally abolish the stimulated incorporation of glucose and approximately 30 % of the original activity was retained. This residual activity

Fig. 4.4.1 Sequence alignment of the branching enzymes of *B. fibrisolvens* (Bf), *E. coli* (Ec)(Baecker *et al.*, 1986), *A. nidulans* (*Synechococcus* sp.)(An)(Kiel *et al.*, 1990) and *Bacillus stearothermophilus* (Bs)(Kiel *et al.*, 1990). Similarity was maximized by introducing gaps denoted by a dash (-), identical amino acids in three or four of the above sequences are boxed. Numbers on the left refer to the first amino acid in each line.


Ec 1 MSDRIDRDV-INALIA-GHFADPFSVLG----MHKTTAGLEVRALL
An 1 TGTTPLPSSSLSVEQVNRIASNQEQNPFDILGPHPYEHEGQAGWVIRAYL
Ec 41 PDATDVWVIEPKTGRKLAKLECLDSRGFFSGVIPRRKNFFRYQLAVVWHGQQNLI
An 51 PEAQEAAVICPALRREFA-MHPVHHPHFFETWVP-EETLEIYQLRITEGERERII
Bf 1 MSQ-KVFISEDDEYLFGQGTHYDIYDKLGAHPSEEKGKKGFFFAVWAPNAAD
Ec 96 DDPYRF-GPLIQEMDAWLLSEGTHLRPYETLGAHADTMDGVTGTRFSVWAPNARR
An 105 YDPYAFRSPLLTDYDIHLFAEGNHHRIYEKLGAHPCELENVAGVNFAVWAPSARN
Bs 1 MRVRLI-AVGPTDLEIYLFHEGSLYKSYELFGAHVIKKNGMVGTRFCVWAPHARE
Bf 52 VHVVGDFNGWDENAHQMKRSKTGNIWTLFIPGVAIGALYKFLITAQDGRKLYKAD
Ec 150 VSVVGQFNYWDGRRHPMRLRKESGIWELFIPGAHNGQLYKYEMIDANGNLRLKSD
An 160 VSILGDFNSWDGRKHQM-ARRSNGIWELFIPELTVGAAYKYEIKNYDGHIYEKSD
Bs 55 VRLVGSFNEWNGTNFNLMKVSNQGVWMIFIPENLEGHLYKYEITTNDGNVLLKSD
Bf 107 PYANYAELRPGNASRTTDLSGFK-W-SDSKWYESLK-GKDMNRQPIAIYECHIGS
Ec 205 PYAFEAQMRPETASLICGLPE-K-V-VQTE--ERKK-ANQFD-APISIYEVHLGS
An 213 PYGFQQEVRPKTASIVADL-D-RYTWGDADWLERRRHQEPLR-QPISVYEVHLGS
Bs 110 PYAFYSELRPHTASIVYNIKGYQ-W-NDQTWRRKKQ-RKRIYDQPLFIYELHFGS
Bf 159 WMKHPD----------GTEDGFYTYRQFADRIVEYLKEMKYTHIE
Ec 253 WRRHTD----------NNFWLSYRELADQLVPYAKWMGFTHLE
An 265 WMHASSDAIATDAQGKPLPPVPVADLKPGARFLTYRELADRLIPYVLDLGYSHIE
Bs 162 WKKKED----------G---SFYTYQEMAEELIPYVLEHGFTHIE
Bf 194 LIGIAEHPFDGSWGYQVTGYYAPTARYGEPTDFMYLINQLHKHGIGVILDWVPAH
Ec 286 LLPINEHPFDGSWGYQPTGLYAPTRRFGTRDDFRYFIDAAHAAGLNVILDWVPGH
An 320 LLPIAEHPFDGSWGYQVTGYYAATSRYGSPEDFMYFVDRCHQNGIGVILDWVPGH
Bs 194 LLPLVEHPFDRSWGYQGIGYYSATSRYGTPHDLMYFIDRCHQAGIGVILDWVPGH
Bf 249 FCPDEFGLACFDGTCIYEDPDPRKGEHPDWGTKIFNLAKPEVKNFLIANALYWIR
Ec 341 FPTDDFALAEFDGTNLYEHSDPREGYHQDWNTLIYNYGRREVSNFLVGNALYWIE
An 375 FPKDGHGLAFFDGTHLYEHADSRQGEHREWGTLVFNYGRHEVRNFLAANALFWFD
Bs 249 FCKDSHGLYMFDGAPAYEYANMQDRENYVWGTANFDLGKPEVRSFLISNALFWME
Bf 304 KFHIDGLRVDAVASMLYLDYGKKDGQWVPNKYGDNKNLDAIEFFKHFNSVVRGTY
Ec 396 RFGIDALRVDAVASMIYRDYSRKEGEWIPNEFGGRENLEAIEFLRNTNRILGEQV
An 430 KYHIDGIRVDAVASMLYLDYNRKEGEWIPNEYGGRENIEAADFLRQVNHLIFSYF
Bs 304 YFHVDGFRVDAVANMLY----------W-PNSDVLYKNTYAVEFLQKLNETVFAYD
Bf 359 PMILTIAEESTAWPKVTAPPEEDGLGFAFKWNMGWMHDFCEYMKLDPYFRQGAHY
Ec 450 SGAVTMAEESTDFPGVSRPQDMGGLGFWYKWNLGWMHDTLDYMKLDPVYRQYHHD
An 485 PGALSIAEESTSWPMVSWPTYVGGLGFNLKWNMGWMHDMLDYFSMDPWFRQFHQN
Bs 349 PMILMIAEDSTDWPRVTAPTYDGGLGFNYKWNMGWMNDILTYMETPPEHRKYVHN
Bf 414 MMTFAMSYNDSENYILPLSHDEVVHLKCSMVEKMPGYKVDKYANLRVGYTYMFGH
Ec 506 KLTFGILYNYTENFVLPLSHDEVVHGKKSILDRMPGDAWQKFANLRAYYGWMWAF
An 540 NVTFSIWYAFSENFMLALSHDEVVHGKSNLIGKMPGDEWQKFANLRCLLGYMFTH
Bs 409 KVTFSLLYAYSENFILPFSHDEVVHGKKSLLSKMPGTYEEKFAQLRLLYGYLLTH
Bf 469 SGKKLLFMGQDFGQEREWSEKRELDWFLLE--NDLNRGMKDYVGKLLEIYRKYPA
Ec 561 PGKKLLFMGNEFAQGREWNHDASLDWHLLEGGDNWHHGVQRLVRDLNLTYRHHKA
An 595 PGKKTLFMGMEFGQWAEWNVWGDLEWHLLQY-EP-HQGLKQFVKDLNHLYRNAPA
Bs 459 PGKKLLFMGGEFGQFDEWKDLEQLDWMLFD--FDMHRNMNMYVKELLKCYKRYKP
Bf 522 LYEVDNDWGGFEWINADDKERSTYSFYRRASNGKDNILFVLNMTPMERKGFKVGV
Ec 616 MHELDFDPYGFEWLVVDDKERSVLIFVRRDKEGNE-IIVASNFTPVPRHDYRFGI
An 648 LYSEDCNQAGFEWIDCSDNRHSIVSFIRRAHESDRFLVVVCNFTPQPHAHYRIGV
Bs 512 LYELDHSPDGFEWIDVHNAEQSIFSFIRRGKKEDDLLIVVCNFTNKVYHGYKVGV
Bf 577 PFDGTYTKILDSAKECYGGSGSSVPDKIKAVKGLCDYKDYSIEFDLPPYGAEVFV
Ec 670 NQPGKWREILNTDSMHYHGSNAGNGGTVHSDEIASHGRQHSLSLTLPPLATIWLV
An 703 PVAGFYREIFNSDARSYGGSNMGNLGGKWTDEWSCHNRPYSLDLCLPPLTT--LV
Bs 567 PLFTRYREVINSDAIQFGGFGNINPKPIAAMEGPFHGKPYHIQMTIPPFGISILR
Bf 632 FQTKKTKN*
Ec 725 RE-AE*
An 756 LELASGPESLSEAANSPL*
Bs 622 PVKKGSVKSFMKTPHPPSHGAS*

		** * * * *
B.fibrisolvens glgB	237	GIGVILDWVPAH
B.fibrisolvens amy	233	GVAVIVDILPNH
Bacillus subtilis amy	132	GIKVIVDAVINH
Dictyoglomus amy	231	GIRIILDFVPNH
Streptomyces amy	112	GVKVIADAVVNH
Aspergillus Taka amy	111	GMYLMVDVVANH
Psuedomonas iso	285	GIKVYMDVVYNH
Streptococcus gt	926	GLKVMADWVPDQ
Bacillus sp. cgt	156	NIKVIIDFAPNH
E.coli glgX	255	GIEVILDIVLNH

Fig 4.4.2 Alignment of the *B. fibrisolvens* branching enzyme with amylases over 12 amino acid residues. Amy (amylase); iso (isoamylase); gt (glucanotransferase);cgt (cyclodextrin glucanotransferase). Numbers on the left of each sequence refer to the amino acid position relative to the N terminus. Identical or conservative residue changes in 8 out of 10 sequences are indicated by an asterisk.

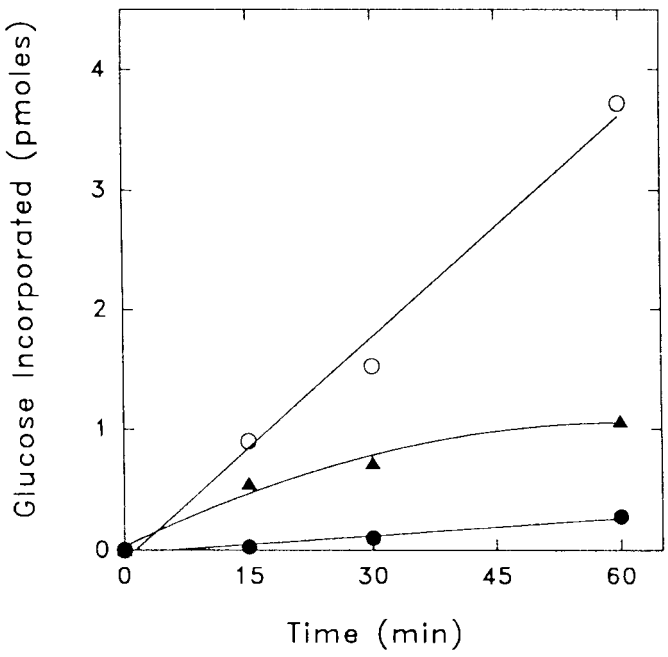


Fig. 4.5 Formation of [^{14}C] glucan catalyzed by the simultaneous action of the crude *B. fibrisolvens* branching enzyme and phosphorylase a (○); heat denatured branching enzyme (▲) and phosphorylase a only (●). Reactions are described in the text.

may have been due to the presence of a small amount of endogenous glucan primer present in the crude extract (Boyer and Preiss, 1977). Since the *glgB* gene containing clones were originally detected by their ability to clear starch plates, the cell free extract was incubated with soluble starch to determine the hydrolytic activity of the expressed enzyme. Measurement of the reducing sugars released (Miller, 1959; Appendix A) showed that the extract had no detectable amylolytic actvity against soluble starch.

4.3.6 Purification of the *B. fibrisolvens* branching enzyme from *E. coli* G6MD3(pBGB200) cell extracts. A simple two step procedure resulted in the purification of the branching enzyme to apparent homogeneity. All work was carried out at 4°C unless otherwise stated. Assay II (section 4.2.11) was used to determine the enzymatic activity at the various stages of the purification. A large scale preparation of an *E. coli* G6MD3(pBGB200) cell-free extract was absorbed onto a Whatman DE 52 column (2.5 X 15 cm) equilibrated in 10 mM Tris-HCl pH 8.0. After washing the column with 2 column volumes of this buffer, the branching enzyme was eluted with a linear gradient (0 - 0.3 M, 300 ml each) of NaCl in the same buffer. Fractions (2 ml) were collected, analysed by SDS-PAGE (Fig. 4.6 a,b) and those containing the branching enzyme were pooled. This pool was assayed for branching enzyme activity and then concentrated by ultrafiltration using an Amicon ultrafiltration cell (model 8050; Amicon Corp., Danvers, Mass.) fitted with an Amicon PM-10 membrane. The concentrated pool (4 ml) was then applied to a Sephadex G 100 column (2.5 X 90 cm) equilibrated in 10 mM Tris-HCl 20 mM NaCl pH 7.4. Fractions (0.5 ml) were collected and analysed as described above (Fig. 4.6 c,d). SDS-PAGE (Fig 4.6 d) showed that only one protein band was eluted in the major peak from the Sephadex G 100 column. Results of the purification procedure, resulted in a final recovery yield of approximately 33 % of the activity and a 5.4 fold increase in specific activity (Table 4.1). The enzyme was stable for several months at -20°C.

Fig. 4.6 Purification of the branching enzyme from *E. coli* G6MD3 (pBGB200). **(a)** DEAE-cellulose chromatography in 10 mM Tris-HCL pH 8 of the crude cell extract. Protein eluted from the column was determined by measuring the absorption at 280 nm of every 4^{th} fraction. **(b)** SDS-PAGE of every 8^{th} fraction of protein eluted from the DEAE column; lane 1, cell extract; lanes 2-13: fraction 6 to 94. Fractions 66-94 containing the branching enzyme were pooled and applied to the Sephadex G 100 column. **(c)** Sephadex G 100 chromatography in 10 mM Tris-HCL 20 mM NaCl pH 7.4 of the pooled fractions from the DEAE-cellulose column shown in (a). Protein eluted was determined as above. **(d)** SDS-PAGE of every 4^{th} fraction of protein eluted from the Sephadex G 100 column; lane 1, cell extract; lanes 2-19: fraction 202 to 270. Fractions 210-270 were pooled. The branching enzyme is indicated by the arrow-head.

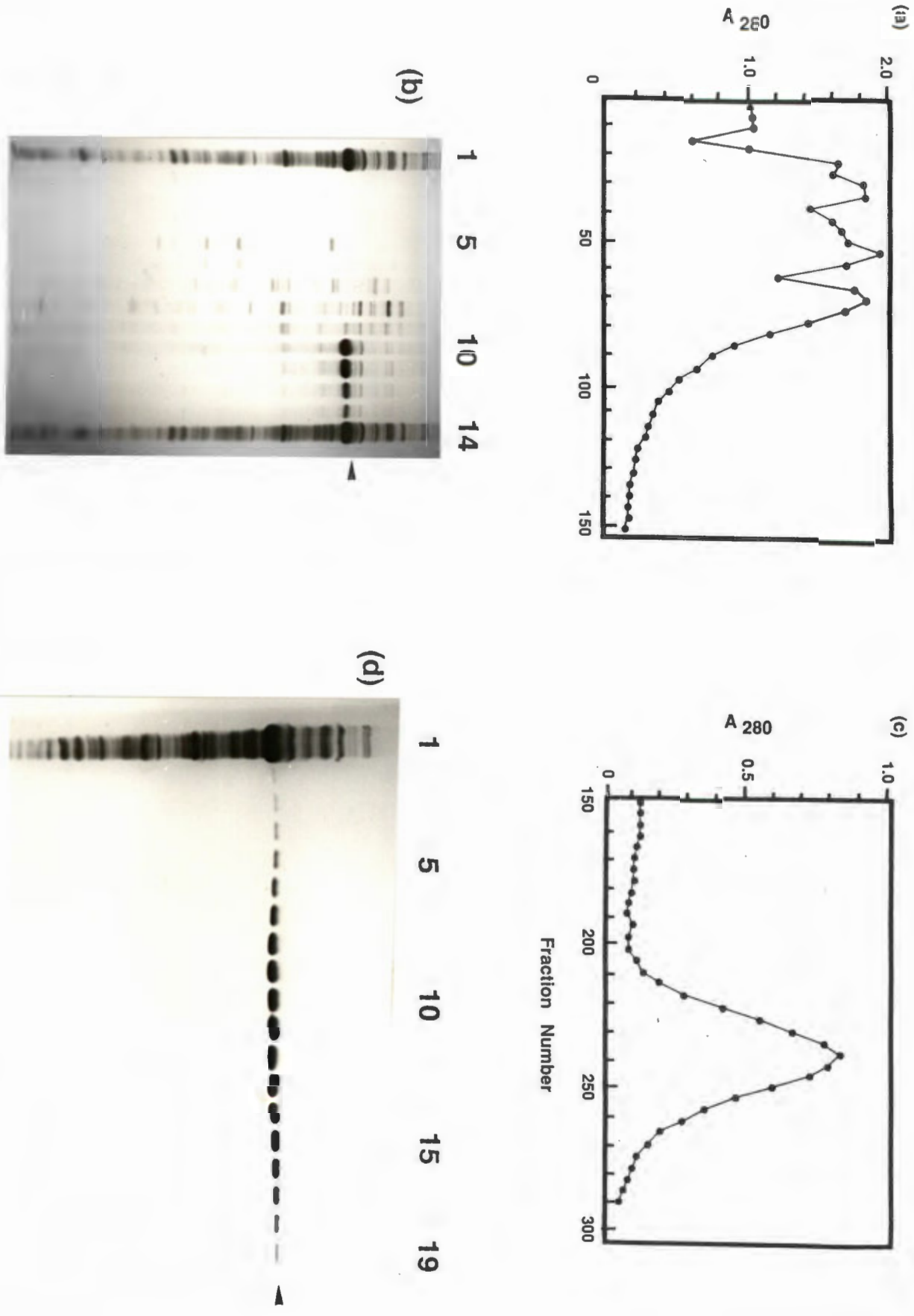

(a)
A 280
2.0
1.0
0
50
100
150
(b)
1
5
10
14
(c)
A 280
1.0
0.5
0
150
200
250
300
Fraction Number
(d)
1
5
10
15
19

Table 4.1 Purification of the *B. fibrisolvens* branching enzyme from *E. coli* G6MD3 (pBGB200).

Fraction	Volume (ml)	Protein (mg)	Activity (U)[a]	Sp. Act. (U/mg)	Yield (%)
1.Crude extract	20	215.69	53 900	249.89	100
2. DEAE-cellulose	50	45.42	43 575	761.22	64.2
3. Sephadex G 100	43	13.00	17 459	1343.03	32.4

[a] Units calculated using assay II (see methods).

4.3.7 Amino acid sequence analysis and hydrolysis. The amino acid sequence of the first 12 residues of the purified protein was determined. This showed that the N-terminal amino acid sequence was identical to that deduced from the nucleotide sequence (Fig. 4.2), confirming the translational start position of the *B.fibrisolvens glgB* gene. The initial methionine was present and was not formylated. The yield for sequencing the branching enzyme was approximately 80%, this value was based on 500 picomoles loaded on to the sequencer (Appendix C). The total amino acid composition agreed with the deduced composition (Appendix C).

4.3.8 Partial characterization of the purified branching enzyme. The effects of pH and temperature on the *B. fibrisolvens* branching enzyme purified from *E. coli* G6MD3(pBGB200) were determined. The branching enzyme was active between pH 5 and pH 9 with a pH optimum at pH 7.2 in 10 mM Tris-HCl 50 mM citrate buffer (Fig.4.7). The enzyme had a broad pH range for maximal activity with 90 % of the activity present between pH 6.8 and pH 8.0. The pH optimum is similar to those reported for other bacterial branching enzymes (Walker and Builder, 1971; Boyer and Preiss, 1977), yeast branching enzyme (Gunja *et al.*, 1960), and plant branching enzymes (Drummond *et al.*, 1972; Hawker *et al.*, 1974). Optimal branching activity was obtained in citrate buffer at 37 °C (Fig. 4.8), this value is in

the range of temperature optima reported for the rabbit skeletal muscle (Gibson *et al.*, 1971) and *Anacystis nidulans* (Kiel *et al.*, 1989) branching enzymes.

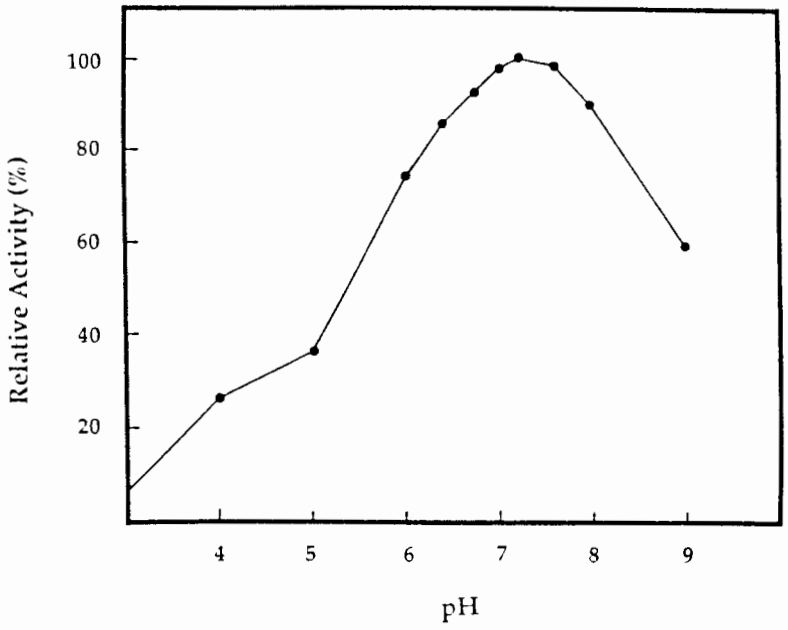


Fig. 4.7 pH activity profile of the branching enzyme purified from *E. coli* G6MD3(pBGB200). Activity was measured using amylopectin in 10 mM Tris-HCl 50 mm citrate at different pH values.

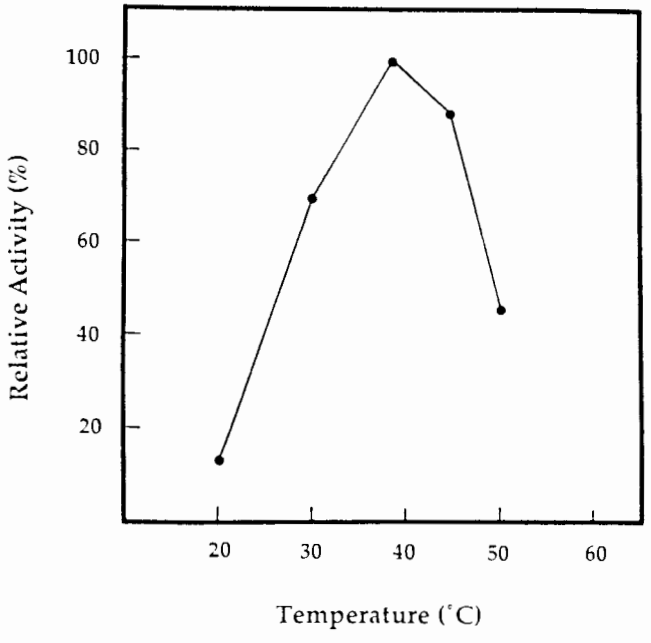


Fig. 4.8 Temperature activity of the branching enzyme purified from *E. coli* G6MD3(pBGB200). Amylopectin was prewarmed to various temperatures, enzyme was added and the activity assayed at the specific temperature.

The branching enzyme activity was unaffected by NaCl (up to 2 M) or dithiothreitol (1 mM) but the activity was completely inhibited by 0.1 mM mercuric chloride (Table 4.2). Inhibition by mercuric chloride has been reported for bacterial (Walker and Builder, 1971); plant (Drummond *et al.*, 1972) and yeast (Gunja *et al.*, 1960) branching enzymes.

Table 4.2 The effect of NaCl, DTT and $HgCl_2$ on the specific activity of the branching enzyme.

Additive	Concentration	Sp. Act. (U/mg)[a]
NaCl	0	1.87
	0.15 M	1.80
	0.5 M	1.82
	1.0 M	1.78
	2.0 M	1.70
DTT	0	1.72
	1.0 mM	1.85
$HgCl_2$	0	1.72
	0.1 mM	0.17

[a] Units defined as in assay II (section 4.2.11).

4.3.9 Purified branching enzyme activity;. The action of the *B. fibrisolvens* branching enzyme was tested on various natural α-glucans. Reactions were followed by monitoring the shift in the absorbance spectrum of the α-glucan-iodine complex and were only terminated when constant values were reached. A 98% decrease in the absorbance at the λmax of the α-glucan-iodine complex for both amylose (640 nm) and amylopectin (540 nm) was observed (Table 4.3). A shift in the maximum wavelength of the iodine complex to 420 nm indicated that a large increase in the number of branch points had occurred. Similarly, the branching enzyme caused a decrease of 59% in the λmax at 460 nm and a shift

from 460 nm to 430 nm of the maximum wavelength of absorption of the rabbit liver glycogen-iodine complex .

Table 4.3 Analysis of the effect of purified branching enzyme on various α-1,4 glucans.

Polysaccharide	Branching Enzyme	
	minus	plus
Amylopectin		
Unit chain length (DP)	22.1	7.1
% α-1,6 bonds	4.4	19.3
λ max (nm)[1]	540	420
% decrease in λ_{540}		98
Amylose		
λ max (nm)	640	420
% decrease in λ_{640}		98
Glycogen		
λ max (nm)	460	430
% decrease in λ_{460}		59
"Glycogen" synthesized *in vitro* [2]		
λ max (nm)		430

[1] λmax refers to the maximum wavelength of absorption spectra of the iodine-glucan complex.
[2] Branching enzyme and phosphorylase *a* (assay I) for "de novo" synthesis of glycogen.

To investigate the effect of the branching enzyme on amylopectin, the changes in the average chain length as well as the percentage of α-1,6 bonds were determined (Table 4.3). The degree of polymerization (as determined by the ratio of glucose to reducing glucose equivalents) of amylopectin changed from 22.7 to 7.1 and simultaneously the percent of α-1,6 bonds increased from 4.4 % to 19.3%. This average length for the chains was confirmed by HPLC analysis of

pullulanase or isoamylase treated amylopectin. Chain lengths in the range 5 to 10 (with a maximum peak at 7) were detected only after treatment with branching enzyme (Fig. 4.9). An identical set of experiments was performed using amylose as the the substrate. Similar results (not shown) were obtained.

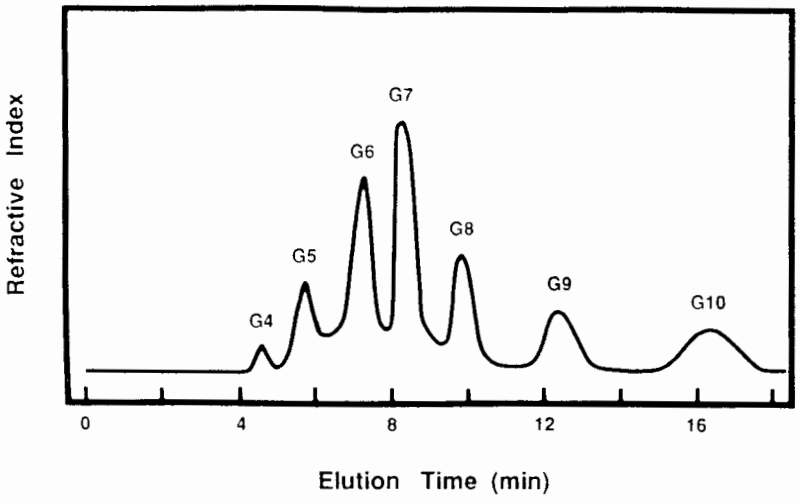


Fig. 4.9 HPLC analysis of unit chains debranched from amylopectin by isoamylase or pullulanase, after the action of branching enzyme. G4-G10 represent number of glucose residues present in the unit chains. The control, debranched native amylopectin, showed no glucose units released within the elution time measured.

The combined action of rabbit muscle phosphorylase a and *B. fibrisolvens* branching enzyme (1:1 weight ratio) was investigated using assay I (section 4.2.11) with 50 mM non-radioactive glucose-1-phosphate. The reaction yielded a branched product with a similar iodine complex spectrum to that of glycogen (Fig. 4.10). Although the initial λmax for the iodine complex was 490 nm, the λmax of the complex decreased to 430 nm as the reaction proceeded indicating a highly branched product. The shoulder present at 470 nm in the glucan-iodine spectrum after 30 min reaction may suggest a slight lag for the branching reaction compared with the elongation process. This synthetic glycogen was resistant to hydrolysis by pullulanase, whereas glucans, which had been branched by the branching enzyme, were hydrolyzed. Reducing the ratio of branching enzyme to phosphorylase *a* in the reaction mixture resulted in the

formation of a precipitate presumably due to the production of a more linear product with lower solubility (Whistler, 1973).

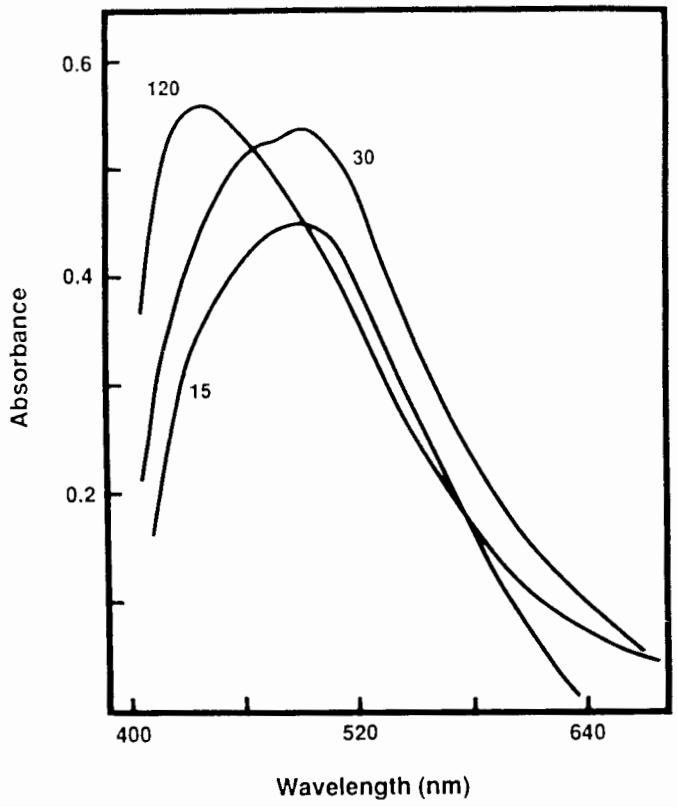


Fig. 4.10 Absorption spectra of the iodine-glucan complex from "de novo" biosynthesis of glycogen. Branching enzyme and phosphorylase *a* in a ratio of 1:1 were used (Assay 1, using 50 mM glucose-1-phosphate). Samples were measured after incubation for 15, 30 and 120 min, at 30°C.

The activity of the purified branching enzyme towards the Phadebas substrate in a liquid assay was investigated. A positive reaction was obtained, where according to the manufactures calibration curve, the enzyme had an "amylolytic" activity of 297 U/l.

4.4 Discussion

These results demonstrate that the gene isolated from a *B. fibrisolvens* H17c gene bank by selection for starch clearing activity codes for a polypeptide of M_r 73,875

with branching enzyme rather than amylolytic activity. Although this polypeptide is smaller than the *E. coli* branching enzyme (M_r = 84,231) (Baecker *et al.*, 1986), it is within the size range reported for branching enzymes from other sources (Hawker *et al.*, 1974; Matsumoto *et al.*, 1983; Kiel *et al.*, 1989).

The *B. fibrisolvens glgB* gene was expressed in *E. coli* from a promoter located on the cloned fragment as expression of the *glgB* gene was independent of orientation to the vector *lacZ* promoter (pBGB200 and pBGB300). No promoter sequences similar to the *E. coli* consensus sequences for promoters (Harley and Reynolds, 1987) were detected upstream of the *glgB* ORF. A similar lack of identifiable consensus promoter sequences has been reported for the *E. coli glg* genes (Preiss and Romeo, 1989) and it was suggested that either the required sigma factor has not yet been identified or that a combination of sigma factors is required. A possible candidate is the σ^S sigma factor which has recently been reported to be required for the expression of the *E. coli glg* genes (Lange and Hengge-Aronis, 1991). Alternatively, it is possible that the *glgB* gene may be expressed from promoter(s) internal to the upstream unidentified ORF as suggested for the *E. coli glgX* and *glgB* genes (Preiss and Romeo, 1989). It is not known if the *B. fibrisolvens glgB* gene is present in a glycogen operon as shown for the *E. coli glg* genes as no downstream nt sequence has been determined. The putative termination sequence found for the *B. fibrisolvens glgB* gene is different from the termination sequence found for the *E. coli glgB* gene (Baecker *et al.*, 1986), in that the *B. fibrisolvens* gene is followed by a T-rich region, a characteristic of Rho-independent terminators. No similarity between the amino acid residues encoded by the upstream ORF with any other sequence reported in the databases could be found. The amino acid sequence of the *B. fibrisolvens* branching enzyme showed similarity to those from *E. coli* (Baecker *et al.*, 1986), *Synechococcus* sp. (Kiel *et al.*, 1990) and *B. stearothermophilus* (Kiel *et al.*, 1990, GenBank accession no. M35089) over the entire length of the polypeptide.

The high degree of conservation between branching enzymes of bacteria that belong to vastly different groups is remarkable, particularly when it is

considered that glycogen storage is not essential for growth and as such is absent from many species of bacteria. Since *B. fibrisolvens* has been reported to be the most prevalent ruminal bacterium under adverse nutritional conditions (Margherita and Hungate 1963; Orpin *et al.*, 1985) and since the storage of carbohydrates is thought to play an important role in cell survival under such adverse conditions (Strange, 1968), it is tempting to speculate that *B. fibrisolvens* stores glycogen or a glycogen-like polysaccharide as a survival strategy. The storage of glycogen in *Butyrivibrio* has not been reported although several other ruminal bacteria are known to produce a glycogen-like polysaccharide (Cheng *et al.*, 1977; Stewart *et al.*, 1981; Wallace, 1980). Attempts to isolate glycogen from *B. fibrisolvens* were unsuccesful although an anthrone positive polysaccharide that could not be digested with α-amylase was isolated.

The simple two-step procedure for the purification of the *B. fibrisolvens* branching enzyme to homogeneity was largely due to the high level of expression of the *glgB* gene product in the *E. coli* cell free extract, 18,6% of total protein calculated from the relative specific activities. The abundance of the *B. fibrisolvens* branching enzyme in *E. coli* extracts is probably due to a combination of the high copy number plasmid vector as well as an efficient *glgB* promoter and ribosomal binding site. Due to the initial concentration of the branching enzyme in the crude extract, the increase in specific activity obtained on purifcation was not high (5.4 fold) compared to increases obtained for the purification of the *E.coli* B (754 fold, Boyer and Preiss, 1977) and the *Neurospora crassa* (226 fold, Matsumoto *et al.*, 1983) branching enzymes. The expression of a cloned gene on a high copy number plasmid vector may yield ample levels of the desired protein which allows for a simplified purification procedure, and sufficient amounts to investigate the biochemistry of the protein. This was also shown to be the case in the cloning and purification of the *E. coli asd* gene (Preiss *et al.*, 1982).

The *B. fibrisolvens* branching enzyme exhibited activity against a wide range of α-1,4 glucans as has been shown for other branching enzymes (Zevenhuizen, 1964; Walker and Builder, 1971; Boyer and Preiss, 1977). Using amylopectin as the

substrate, the *B. fibrisolvens* branching enzyme transferred a minimum size of oligosaccharide chains of between 5 and 10 glucose units in length. A peak obtained at 7 indicated this as the preferred unit size and agreed with the value calculated from the degree of polymerization. Branching enzymes from liver, muscle and *E.coli* B (Verhue and Hers, 1966, Brown and Brown, 1966b, and Boyer and Preiss, 1977 respectively) have been reported to exhibit a similar specificity for a 7 glucose chain unit transferred. The product obtained from branching amylopectin had a maximum wavelength of 420 nm for the iodine complex, characteristic of highly branched glycogen with comparitively short chain lengths (Archibald *et al.*, 1961). This λmax for the iodine complex is similar to that obtained from glycogen-like polysaccharide isolated from the rumen bacterium *Bacteroides succinogenes* (Stewart *et al.*, 1981). The percentage α-1,6 bonds introduced into amylopectin by the branching enzyme was 19.33%, which is higher than the 9.6% reported for *E.coli* B (Boyer and Preiss, 1977) and the 8% reported for *Arthrobacter globiformis* (Zevenhuizen, 1964) and is furthermore, greater than that of glycogen. The "de novo" biosynthesis of a glycogen-like polysaccharide using the *B.fibrisolvens* branching enzyme in conjunction with rabbit muscle phosphorylase α resulted in the production of a branched glucan that resembled glycogen in its iodine staining properties. This synthetic branched polysaccharide, like native glycogen, was resistant to pullulanase hydrolysis, although the branches formed by branching native α-1,4 glucans were readily hydrolyzed by pullulanase. This supports the hypothesis (Boyer and Preiss, 1977; Hawker *et al.*, 1974) that the enzymes involved in α-glucan polysaccharide synthesis act in a co-ordinate rather than a successive fashion.

The ability of the *B. fibrisolvens* branching enzyme to produce halos on starch azure and Phadebas substrates, can be explained by the ability of the *B. fibrisolvens* branching enzyme to produce highly branched structures, which are more soluble than the relatively unbranched substrates. Although the Phadebas substrate, an insoluble starch incorporating a covalently cross-linked blue dye, is considered to be specific for starch hydrolysis (Ceska *et al.*, 1969), increased branching of the Phadebas substrate would result in an increased solubility

(Whistler, 1973) without hydrolysis of the dye-substrate linkage. The soluble substrate would then diffuse to equilibrium over the entire plate thereby producing a halo (Chapter 2, Fig.2.1). An increase in absorbance was also obtained with Phadebas substrate in liquid assays giving units of α-amylase activity. The *B. fibrisolvens* branching enzyme did not display any hydrolytic activity towards soluble starch as no release of reducing sugars could be detected during assays. The branching enzyme of starch (Q-enzyme) has in fact been reported to have liquefying properties not attributable to either α- or β-amylase, as an increase in reducing sugars are not detected; the solution does not become achroic; and the polysaccharide formed is indistinguishable from the natural glucan (Barker *et al.*, 1950). Caution must therefore be exercised in using substrates utilising the solubility of dye-linked starch for the assay of amylolytic activity.

Chapter 5

General Conclusions

Chapter 5

General Conclusions

This study aimed to obtain further insight into the role of *B. fibrisolvens* H17c in the rumen through the characterization of genes and enzymes involved in the breakdown of the major plant polysaccharides.

A library of chromosomal DNA fragments from *B. fibrisolvens* H17c was established in the *E. coli-B. subtilis* shuttle vector pEB1. This library was screened in *E. coli* for genes expressing cellulolytic and amylolytic activities. Two clones expressing cellulolytic activity were isolated. The one clone was found to contain a cellodextrinase gene that had already been characterized (Berger *et al.*, 1990). The other clone expressing β-glucosidase activity was further characterized by Lin *et al.* (1990). A gene expressing xylanase activity has also since been isolated (Lin and Thomson, 1991). The library was also screened for genes involved in nitrogen regulation and clones expressing glutamine synthetase activity were isolated. One of these clones pGS400 is presently being characterized by Dr. H. Goodman (Dept. of Microbiology, UCT). This work is proving to be most interesting since it appears that the glutamine synthetase of *B. fibrisolvens* is not a conventional GSI or GSII type, but is rather similar to the novel GSIII type reported from *Bacteroides fragilis* by Hill and Woods (1989). This thesis reports the isolation and characterization of two different clones expressing amylolytic-type activity on starch azure plates. The genebank has therefore provided a useful basis for the molecular genetic study of *B. fibrisolvens* H17c. Because of the versatility of the cloning vector used, genes which may be lethal (eg. proteases) or not expressed in *E. coli* can now being investigated for expression in *B. subtilis*.

The α-amylase gene, *amyA*, isolated from the *B. fibrisolvens* library encoded an enzymatically active 1,4-α-D-glucan-4-glucanhydrolase which was produced in *E. coli*. Most of the amylolytic activity was detected in the periplasm of the *E. coli*

host indicating that protein export from the cytoplasm had occurred. A signal peptide with characteristics typical of prokaryotic signal sequences (Watson, 1984) was identified and found through Tn*phoA* analysis to function in *E. coli*. The exact mechanism of secretion of the α-amylase to the periplasm is intriguing. This protein seems to need some factor present in the cell membrane for correct transcription and/or translation. A mechanism similar to that suggested for exoenzyme synthesis and secretion in *B. subtilis* (Ingle and Erickson, 1978) might occur in *B. fibrisolvens*. This involves the association of the *Bacillus* amylase-specific mRNA with the membrane-bound 50S ribosomal subunit prior to translation and coordinated secretion of the amylase. This type of mechanism has also been suggested for the translation of the membrane bound amylase of *Clostridium acetobutylicum* (Annous and Blaschek, 1990)

The *B. fibrisolvens amyA* gene was expressed from its own promoter in *E. coli* and typical -35 and -10 consensus promoter sequences were present upstream of the gene. Synthesis of the amylase was constitutive in *E. coli* and did not seem to be regulated by the presence of a carbohydrate source in the growth medium in either the magnitude or the location of the amylolytic activity. This is unlike the amylolytic activity of *B. fibrisolvens* 49 which has been found to be regulated in both respects (Cotta, 1988). *B. fibrisolvens* strain 49 has been shown in a number of studies to be very similar to strain H17c in EPS composition, enzymatic activities and levels of DNA relatedness (Cotta and Hespell, 1986; Mannarelli, 1988; Stack, 1988; Mannerrelli *et al.*, 1990a). In addition, both strains have similar end products of starch hydrolysis and thus it is not unlikely that both strains may have similar amylolytic activities. The absence of regulatory regions on the cloned *amyA* gene or possible dissimilar catabolic repression systems in *B. fibrisolvens* are factors which may explain the lack of regulation of *amyA* expression in *E. coli*. Dissimilar regulatory systems were found to occur when the *B. subtilis* amylase was expressed in *E. coli* (Nicholsen and Chambliss, 1985). Alternatively, the regulation of *B. fibrisolvens* H17c may be different to that of *B. fibrisolvens* 49. Further work investigating this regulatory response in *B. fibrisolvens* H17c as well as isolation of further upstream possible regulatory

regions of *amyA* through DNA "walking" could elucidate regulatory mechanisms in *B. fibrisolvens*.

The action pattern of the *B. fibrisolvens* H17c amylase on α-1,4-glucans suggests both a multiple and random mode of attack. Should only random fragmentation occur, then substantial amounts of maltotetraose and maltose would be produced concurrently with maltotriose. Maltotriose, however was the dominant product during the initial stages of substrate hydrolysis. The initial high production of maltotriose could be explained if, after random attachment of the α-amylase to the polysaccharide chain, the enzyme continues to hydrolyze the same chain releasing maltotriose. This kind of multiple attack has been described for *S. bovis* and *B. subtilis* α-amylases (Robyt and French, 1963; Walker, 1965).

The other clone isolated from the gene bank that had amylolytic-type activity on starch azure plates was unexpectedly found to contain a *glgB* gene encoding a glycogen branching enzyme. The amylolytic-type activity observed on starch azure plates was due to an increase in starch solubility brought about by increased substrate branching. No hydrolytic action against starch or any α-1,4-glucans could be demonstrated. This enzyme showed similar glycosyltransferase properties to those reported previously for *E. coli* and mammalian branching enzymes (Preiss and Walsh, 1981). A high degree of deduced amino acid homology between the *B. fibrisolvens* and *E. coli* branching enzymes was found over the length of the polypeptide. The *glgB* gene was expressed from its own promoter in *E. coli* as demonstrated by deletion and reversal of orientation of restriction fragments. The high level of *glgB* expression obtained in *E. coli* could be attributed in part to an efficient *glgB* promoter. No consensus promoter sequences were found in the region upstream of the *glgB* gene although N-terminal amino acid sequencing of the purified protein confirmed the translational start codon. The promoter activity of this region can be tested by fusion of the putative promoter region to a *lacZ* gene lacking a promoter and assaying for β-glucosidase activity. mRNA transcript mapping

could determine the actual start of transcription in both *B. fibrisolvens* and *E. coli*. The identification of the promoter could prove interesting. It has recently been found (Lange and Hengge-Aronis, 1991) that a novel sigma factor, involved in the early regulation of a large starvation/stationary phase regulon in *E. coli*, may be absolutely required for expression of the *glg* genes. It is therefore possible that the *B. fibrisolvens glgB* gene requires a similar sigma factor for expression. The sequence of the promoter could contribute towards the development of a consensus sequence for this factor.

Investigation of the four other clones obtained from independant cloning events but with some overlapping regions to the *glgB* gene may yield additional *glg* genes. Both the *E. coli* and *Salmonella typhimurium* *glg* genes are located in glycogen operons (Steiner and Preiss, 1977; Okita *et al.*, 1981). Thus it would be of interest to discover whether the *B. fibrisolvens glgB* gene also resides in an operon.

Some microbes may be intrinsically better equipped to survive under starvation conditions since they may be able to more efficiently turn over cellular constituents, scavenge needed materials both intracellulary and extracellulary, and possess different ATP requirements to maintain essential cellular functions. Russel and Baldwin (1979) in a study on the energy maintenance coefficients of ruminal bacteria, found that *B. fibrisolvens* had relatively low maintenance requirements. The low maintenance energy coefficients may indicate an energetic adaptation of these strict anaerobes to the low molar ATP yields found in anaerobic environments.

Since *B. fibrisolvens* is known to be one of the most dominant bacteria under adverse nutritional conditions (Margherita and Hungate, 1963; Orpin *et al.*, 1985), it is surprising that no work on the survival strategies of this bacterium has been carried out. The importance of the amylase in ruminal digestion is difficult to evaluate from the information presently available. One can however hypothesise that the highly maltogenic nature of the *B. fibrisolvens* amylase, which efficiently

breaks down soluble starch into readily absorbable substances (maltose and glucose), may be a factor that aids *B. fibrisolvens* survival. To my knowledge no work has yet been carried out on either storage substrates or starvation in *B. fibrisolvens*. The presence of a gene involved in glycogen biosynthesis is an indication that *B. fibrisolvens* may store a glycogen-like polysaccharide as an endogenous substrate. The presence of this storage polysaccharide combined with the low maintenance energy levels of *B. fibrisolvens* may contribute together with a number of other factors towards the strategies of this rumen bacterium which lead to its relative success during low nutrient starvation conditions.

Appendix A

Standard Methods, Media, Buffers and Solutions

Appendix A

Standard Methods, Media, Buffers and Solutions

A.1 Standard Methods

A.1.1 Small scale isolation of plamid DNA (miniprep)

Plasmid was isolated from a 5 ml overnight culture (LB + Ap, 100 µg/ml) as described by Ish-Horowicz and Burke (1981). Cells from a 1.5 ml sample of the culture were harvested by centrifugation in an Eppendorf microfuge tube for 1 min. The pellet was resuspended in 100 µl Solution I (50 mM glucose; 25 mM Tris-HCl, pH 8.0), incubated for 5 min at room temperature, and then 200 µl of Solution II (0.2 M NaOH, 1% (w/v) SDS) was added. The sample was vortexed briefly and placed on ice for 5 min, before the addition of 150 µl ice-cold Solution III (5 M KOAc, pH 4.8). The sample was gently mixed, and, after 5 min on ice, cellular debris and denatured chromosomal DNA were pelleted by centrifugation for 5 min. The supernatant (450 µl) was removed to a fresh tube and sedimented by centrifugation with two volumes of 95% ethanol for 5 min. The pellet was resuspended in TE (150 µl), sodium acetate (15 µl, 3 M, pH 4.8) and two volumes 95% ethanol were added and the sample held at -70°C for 5 min. The DNA was pelleted by centrifugation for 15 min, washed with 70% ethanol, dried and resuspended in 50 µl TE buffer.

A.1.2 Large scale isolation of plasmid DNA (maxiprep)

A 200 ml culture was grown overnight at 37°C in the presence of the appropriate antibiotic. The cells were harvested by centrifugation at 6 000 *g* for 5 min and then resuspended in 4 ml Solution I. After 5 min at room temperature 8 ml Solution II was added, and the mixture was kept on ice for 5 min, before the addition of 6 ml ice cold Solution III. After a further 5 min on ice the cellular debris was removed by centrifugation at 12 000 *g* for 10 min. An equal volume of isopropanol was added to

the supernatant and the DNA was precipitated by centrifugation at 27 000 *g* for 15 min. The pellet was washed with 70% ethanol and resuspended in 4.2 ml TE buffer, and purified by isopicnic CsCl-EtBr ultracentrifugation (Maniatis et al. 1982). The plasmid preparation was prepared for ultracentrifugation by the addition of CsCl (1 mg/ml) and EtBr (0.5 ml of a 10 mg/ml stock). The solution was centrifuged at 27 000 *g* for 15 min to precipitate any remaining protein debris. The refractive index of the supernatant was adjusted to 1.395, the sample sealed in Beckman Quickseal ultracentrifuge tubes and centrifuged for 12 h at 55 000 rpm at 15°C in a Beckman Vti 65.2 rotor. The plasmid DNA band was visualized by long wave UV light (350 nm), and removed in the smallest volume possible. The EtBr was removed by extraction (3 times) with equal volumes of NaCl-saturated isopropanol. The DNA was precipitated from the CsCl solution by the addition of two volumes of water followed by an equal volume of isopropanol, and centrifugation in an Eppendorf microfuge for 15 min. The pellet was resuspended in 200 µl TE buffer and the concentration was determined spectrophotometrically by measuring the absorbance of 10 µl (diluted in TE) between 220 and 310 nm. The concentration was determined by using the relationship A_{260} = 1 for 50 µg/ml double-stranded DNA.

A.1.3 Extraction of chromosomal DNA from *B. fibrisolvens*

The method described by Strydom et al. (1986), a modification of the method of Marmur (1961) was used. *B. fibrisolvens* chromosomal DNA was prepared from 500 ml overnight (16h) cultures which were harvested by centrifugation (10 000g for 10 min), resuspended in 4 ml Solution A (10 mM TRIS_HCl, pH 8.0, 25% w/v sucrose) containing 5 mg/ml lysozyme, and mixed gently at 37°C for 30 min. Two ml of ice-cold Solution B (0.5 M EDTA, pH 8.0) were added and the mixture kept on ice for 5 min. Four ml Solution C (10 mM TRIS-HCl, pH 7.5, 1 mM Na_2EDTA, 2% w/v SDS), containing 5 mg/ml proteinase K were added. The mixture was kept at 20°C for 10 min. CsCl (1 g/ml) and EtBr (250 µg/ml) were added, and the mixture centrifuged at 15 000 *g* for 30 min. The refractive index of the supernatant was adjusted to 1.396, and the DNA purified by isopycnic CsCl/EtBr ultracentrifugation. The DNA band visualized under UV light (350 nm) was removed by puncturing the the bottom of

the Quickseal tube and allowing the fluid to drip through slowly. The DNA was then processed as described in section A.1.2.

A.1.4 Exonuclease III digestion

Exonuclease III digestion was carried out by a modification of the method of Henikoff (1984). Ten sample tubes containing S1 mixture (25 µl) were prepared immediately before starting the digestion, and were kept on ice. Plasmid DNA (10 µg) previously digested with appropriate restriction endonucleases and resuspended in 100 µl exobuffer, was equilibrated at 37°C for 5 min. A sample (9 µl) was first removed to a tube containing S1 mixture before any exonuclease III was added. Exonuclease III (500 units, Boehringer Mannheim) was added to the equilibrated DNA, the mixture briefly vortexed, and after a lag period of 30s 9 samples (9 µl) were removed at regular time intervals. Each sample was immediately transferred to a tube containing S1 mixture. Once all the samples had been taken, the tubes were incubated at room temperature for 30 min, before the S1 nuclease reaction was terminated by the addition of S1 stop solution (3.4 µl). Tubes were then incubated at 70°C for 10 min, and the exonuclease III generated ends filled in by the addition of 1 unit per tube of Klenow enzyme (Boehringer Mannheim) in Klenow buffer (3.4 µl). Samples were incubated at room temperature for 3 min, followed by a further incubation for 5 min in the presence of a mixture of each dNTP (0.125 mM each). A sample of the shortened DNA (10 µl) from each tube was blunt end ligated in a large volume (50 µl final volume). The deletions were transformed into *E. coli* LK111 and the transformants were selected on LB agar containing Ap (100 µg/ml).

A.1.5 Restriction endonuclease digestion

Restriction digests were carried out as described by Maniatus et al. (1982), using one of the four restriction buffers (Appendix A) according to the salt requirements of the particular enzyme. Digestion volumes were routinely 20 µl containing 300-500 ng DNA and one unit of restriction enzyme. Digestions were incubated at recommended temperatures for 1 h. For electrophoretic analysis, the digestions were terminated by the addition of 5 µl DNA loading solution (Appendix A) to the

20 µl digestions. If the digestion products were to be ligated, or filled in before ligation, they were purified by a phenol-ether extraction. The DNA solution was diluted with sterile distilled water (380 µl) and phenol was added (1/10 volume, TE-saturated). The mixture was vortexed briefly, the two phases were separated by centrifugation, and the aqueous phase was extracted several times with water-saturated ether. The DNA was precipitated by the addition of 3 M sodium acetate (1/10 volume, pH 4.8), and 2 volumes of 95% ethanol, cooled to -70°C for 5 min, and centrifuged for 30 min in a microfuge at 4°C. If the DNA concentration was less than 2 µg/100µl *E. coli* tRNA was added (2 µg) before precipitation. After centrifugation the pellet was washed with 70% ethanol, dried and resuspended in TE buffer.

A.1.6 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using a horizontal submerged gel system. Tris-acetate or Tris-borate buffers (Appendix A) were used routinely. Sigma type II agarose was used at varying concentrations (0.5%-1.2%). The amount of DNA loaded/lane varied with the sizes and number of fragments, but usually about 300 ng of plasmid DNA was used. The gels were electrophoresed at 2 V/cm for 16 h. Gels were stained in electrophoresis buffer containing EtBr (0.5 µg/ml) for 15-30 min. DNA bands were visualised using a 254 nm transilluminator. A 310 nm transilluminator was used if the DNA was to be recovered from the gel.

Gels were photographed using a Polaroid CU-5 Land camera fitted with a red filter and a fixed focal length attachment. Polaroid type 667 film (ASA 3 000) was used with an exposure time of 1-2 sec at f4.7. If a negative was required then a Polaroid type 665 film (ASA 64) with an exposure of 120-140 sec at f 4.7 was used.

A.1.7 DNA ligation reactions

DNA ligation reactions were of two basic types: recircularization of plasmids for the isolation of deletion clones (use low DNA concentrations, 1 pmole DNA/ml) and recombination reactions, for example in subcloning (use 5 pmole DNA/ml). DNA

concentration was calculated using the formula 1 pmole = (0.662 x kb)μg. Vector and insert DNA were added to the ligation reactions at a molar ratio of 1:2. Ligation reactions containing DNA, ligation buffer (Appendix A) and water to the required volume, were performed in sterile microfuge tubes. Sticky-end ligations were performed at room temperature for 3 h or at 15°C overnight using 0.1-0.25 U of ligase, whereas blunt-end ligations were performed at room temperature for 3-20 h using 20-100 x more ligase.

A.1.8 Subcloning protocol

The rapid subcloning protocol of Struhl (1985) was used. The DNA fragments were separated by electrophoresis through low melting point (LMP) agarose (0.8%) (Seaplaque^{R}) in Tris-acetate buffer (50 mM, pH 8.2, no EDTA, no EtBr). The gel was stained with EtBr after electrophoresis and the DNA bands were viewed under UV light (310 nm), as briefly as possible. The desired bands were excised using sterile scalpel blades, in as small a volume as possible. The gel slices were melted at 70°C for 5 min in a microfuge tube and the required amounts (2 μl vector DNA, 8 μl insert DNA) were added hot to the prepared ligation mixture containing ligation buffer, ligase and water (10 μl). The ligation was incubated at room temperature for 3 h. Before transformation of *E. coli* competent cells, the gelled ligation reactions were melted at 70°C for 5 min, and then diluted with 4 volumes of TSB solution (Appendix A).

A.1.9 The preparation and transformation of competent *E. coli* cells

E. coli cells were made competent for DNA uptake according to the method of Chung and Miller (1988). A 1/100 dilution of an overnight *E. coli* culture in LB was inoculated into 25 ml prewarmed LB and incubated at 37°C, with shaking, until the culture had reached early exponential phase (OD_{600} = 0.3-0.6) (2-4 h). The cell culture was poured into a pre-cooled sterile SS34 tube and the cells were harvested at 1000 *g* for 5 min at 4°C. The cell pellet was resuspended in 2.5 ml (1/10 volume) ice-cold transformation and storage buffer (TSB) (Appendix A) and held on ice for 10 minutes. The *E. coli* cells (100 μl) were then mixed with DNA (routinely 50 ng)

and held on ice for a further 30-60 min. TSB solution (0.9 ml) containing glucose (20 mM) was added to each transformation mixture and incubated at 37°C for 60 min, to allow expression of the plasmid borne antibiotic marker.

Unused cells could be stored at -70°C after rapid freezing in a dry ice/ethanol bath or liquid nitrogen and retained viability provided that the cells are thawed slowly on ice when needed

A.1.10 Nucleotide sequencing

Primer annealing reaction. The supercoiled DNA (6-10 µg, in TE buffer) was diluted to a final volume of 20 µl in distilled water. Alkaline denaturation in 0.2 N NaOH (5 min at room temperature) was followed by the addition of 5 µl of 3 M sodium acetate (pH 5.2), 25 µl of distilled water and 150 µl of ethanol. This mixture was chilled to -70°C, centrifuged at 4°C for 20 min in a microfuge and washed with 200 µl of ethanol (70%). The DNA pellet was dried and resuspended in a final volume of 10 µl of sequencing buffer (40 mM Tris-HCl, pH 7.5; 20 mM $MgCl_2$; 50 mM NaCl) and 12 ng of primer. This mixture was annealed for 15 min at 40°C immediately prior to sequencing. The forward sequencing primer as supplied in the Sequenase DNA sequencing kit (US Biochemical Corp., Cleveland, Ohio) and the M13 reverse sequencing primer (Amersham) were used.

Sequencing reactions. DNA sequencing was done by the dideoxy chain termination method of Sanger et al. (1977) according to the protocol of Tabor and Richardson (1987), using T7 DNA polymerase and a "Sequanase" sequencing kit supplied by the US Biochemical Corporation, Cleveland, Ohio. The DNA chain was radiolabelled with [α-^{35}S]dATP (1200 Ci/mmol; Amersham).

Gel electrophoresis and autoradiography. The sequencing reactions were analyzed on standard 6% denaturing acrylamide urea sequencing gels. The composition and running conditions of the gels were as described in the Amersham M13 Sequencing Handbook. After electrophoresis the gels (0.2mm thick) were dried onto Whatman No. 3 filter paper using a Dual Temperature Slab Gel Dryer (Model 1125B; Hoefer Scientific Instruments, San Francisco). Gels containing ^{35}S-labelled DNA were

placed under XAR-5 autoradiographic film and exposed for 1-2 days. The autoradiographs were developed using Kodak GBX X-ray developer and fixer.

A.1.11 DNA/RNA alkali blotting procedure

DNA fragments resolved by agarose gel electrophoresis were transferred to a Hybond N+ hybridization membrane (Amersham) by the protocol of Reed and Mann (1985). After electrophoresis the gel was rinsed in 2 volumes of HCl (0.25 M) for 20 min at room temperature with gentle agitation, followed by a brief rinse in distilled water. The gel was then placed onto 2 sheets of Whatman 3 MM filter paper (wetted with 0.4 N NaOH, and placed on top of a small platform in a plastic box, so that the filter paper formed a wick), and was flooded with 50-100 ml of 0.4 N NaOH. A sheet of Hybond N+ (prewetted with water) was placed on top of the gel, and any air bubbles were removed. Three sheets of Whatman 3 MM filter paper, wetted in 0.4 N NaOH, were laid onto the membrane, followed by a 4 cm thick layer of absorbant paper. A light weight was placed on top of this, and left overnight. After transfer, the membrane was rinsed briefly with gentle agitation in 2 x SSC (Appendix A). The membrane was then ready for hybridization or could be wrapped in saran wrap and stored at 4°C.

A.1.12 SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE procedure. Discontinuous SDS-PAGE was done according to the method of Laemmli (1970), using a Hoefer SE600 vertical slab electrophoresis unit (Hoefer Scientific Instruments, San Fransisco, CA, USA). The 1.5 mm thick gel spacers were used. The resolving gel was prepared and degassed before pouring. Water was layered on the gel to promote a sharp interface. After the gel had polymerized (30 min), the water was removed by rinsing with the stacking gel buffer, and the stacking gel was cast. Samples were prepared in sample treatment buffer (Appendix A) and in some instances placed in a boiling waterbath for 2 min before being loaded onto the gel. Electrophoresis was continued at 35 mA (constant current)/gel until the dye front migrated to the end of the gel (four to five hours).

After electrophoresis the gels were stained in Page G-90 staining solution with gentle agitation, destained and dried.

The acrylamide gels (10% and 15%) were prepared as follows:

Solution	Resolving gel		Stacking gel
	10%	15%	
Acrylamide solution	12.5 ml	18.7 5ml	2.66 ml
1.125M Tris-HCl pH 8.8	8.35 ml	8.35 ml	-
0.375M Tris-HCl pH 6.8	-	-	4 ml
10% SDS	0.25 ml	0.25 ml	0.125 ml
Distilled water	28.65 ml	18.75 ml	5.55 ml
Ammonium persulphate	0.25 ml	0.25 ml	0.3 ml
TEMED	25 µl	25 µl	20 µl

SDS-PAGE solutions:

Acrylamide solution

Acrylamide	40.0 g
Bis-acrylamide	0.2 g
Distilled water	to 100 ml

Filter through Whatman's paper (No. 1) and store in dark bottle at 4°C.

Ammonium persulphate (10% w/v)
A fresh solution was made immediately before use.

Reservoir buffer (10x)

Tris base	8 g
Glycine	30.0 g
SDS (0.1% w/v)	0.2 g
Distilled water	to 2000 ml

The pH should be approximately 8.5.

SDS (10%)

SDS	50 g
Distilled water	to 500 ml

Page G-90 staining solution

Page G-90 (0.1% w/v)	1 g
Phosphoric acid	final 3%
Distilled water	1000 ml

The solution was stirred vigorously to dissolve the dye and then filtered through Whatman's paper (No. 1).

Destain solution

Acetic acid (7%)	70 ml
Distilled water	1000 ml

Sample treatment buffer

0.375 M Tris-HCl pH 6.8	2 ml
SDS (10%)	2 ml
Glycerol	2 ml
2-mercaptoethanol	0.5 ml
Distilled water	3.5 ml

The solution was stored in aliquots at -20°C.

A.2 Media, Buffers, and Solutions

All media, buffers, and solutions were sterilized by autoclaving at 121°C for 20 min unless otherwise indicated. Heat labile substances were sterilized by filtration through 0.22 µm membrane filters (Millipore). Polyacrylamide gel solutions and buffers are given in Appendix A.1.12.

A.2.1 Media

B. fibrisolvens non-rumen fluid medium (M10)

Ingredient	Amount/l
Glucose	10.0 g
Cellobiose	0.5 g
Mineral solution 1[a]	38.0 ml
Mineral solution 2[b]	38.0 ml
Cysteine HCl (5% w/v)	10.0 ml
Na_2CO_3 (5% w/v)	50.0 ml
Resazurin (0.1% w/v)	1.0 ml
Trypticase	2.0 g
Yeast extract	0.5 g
Volatile fatty acid[c]	3.1 ml
Hemin[d]	10.0 ml
Distilled water	850.0 ml

[a]Mineral solution 1 contained/l:

K_2HPO_4	6.0 g

[b]Mineral solution 2 contained/l:

KH_2PO_4	6.0 g
NaCl	12.0 g
$(NH_4)_2SO_4$	6.0 g
$CaCl_2 \cdot 2H_2O$	1.6 g
$MgSO_4 \cdot 7H_2O$	2.5 g

[c]Volatile fatty acid solution contained:

acetic acid	17 ml
propionic acid	6 ml
butyric acid	4 ml
isobutyric acid	1 ml
n-valeric acid	1 ml
iso-valeric acid	1 ml
2-methylbutyric acid	1 ml

Solution is stable at 4°C.

[d]Hemin solution:

KOH	0.28 g
ethanol (95%)	25.0 ml
hemin	0.10 g
distilled water	75 ml

Solution is stable at 4°C

All the ingredients were added and the pH adjusted to 6.8 prior to autoclaving.

Luria-Bertani medium (LB)

Bacto tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Distilled water	1000 ml

Solid media contained 1.5% (w/v) agar.

In starch plates, 0.4% (w/v) starch azure was added to LB agar

A.2.2 Media additives

Media were cooled to 50°C before addition of antibiotics, XGal, XP, or IPTG. Plates containing these additives were stored for no longer than one week at 4°C.

Antibiotics:

Antibiotic stock solutions were as follows:

Ampicillin	100 mg/ml water
Chloramphenicol	20 mg/ml ethanol (96%)
Kanamycin	62.5 mg/ml water
Tetracycline	12.5 mg/ml ethanol (50%)

All antibiotics were filter sterilized and stored at -20°C, except for Tc which was always made fresh

IPTG (isopropyl-β-D-thio-galactopyranoside)

IPTG (100mM)	23.4 mg
Distilled water	1 ml

The solution was stored in aliquots at -70°C.

XGal (5-bromo-4-chloro-3-indolyl-β-galactoside)

XGal (2% w/v)	0.2 g
Dimethylformamide	10 ml

The solution was stored at -70°C.

XP (5-bromo-4-chloro-3-indolyl phosphate)

XP	80 mg
DMSO	1 ml

The solution was stored at -70°C.

A.2.3 Buffers and solutions

ATP (10x) (Maniatis et al. 1982)

Adenosine triphosphate	30 mg
Distilled water	5 ml

Adjust pH to 7.0 with 0.1 N NaOH before making up to 5 ml. Store in 100 µl aliquots at -70°C. Discard remainder once defrosted.

Bradford solution (Bradford 1976)

Coomassie Brilliant Blue (G-250)	100 mg
Ethanol (95%)	50 ml

Dissolve, then add 100 ml phosphoric acid (85%). Dilute to final volume of 1 l. Filter through Whatman GF/C filter paper. Store in dark bottle.

Dinitrosalicylic acid solution (DNS) (Miller 1959)

3,5 Dinitrosalicylic acid	10.6 g
NaOH	19.8 g
Rochelle salts (Na K Tartrate)	306 g
Phenol	7.6 ml
Na-meta bisulphite	8.3 g
Distilled water	1416 ml

The dinitrosalicylic acid, NaOH, and Rochelle salts were dissolved completely in the water before adding the other constituents and dissolving each in turn. The phenol was melted at 50°C. A 3 ml sample was titrated to the end-point with 5-6 ml HCl (0.1 M) using phenolphthalein as an end-point indicator. However, if less HCl was required then solid NaOH was added to the DNS solution at the rate of 2 g/ml of HCl less than five ml, and the titration repeated. The DNS solution was stored in a dark bottle under N_2.

DNA loading solution (6x)

Bromophenol blue	0.25 g
Sucrose	40 g
Distilled water	to 100 ml

The solution was stored at 4°C.

DTT (1M)

DTT	0.618 g
Sodium acetate (0.01 M, pH 5.2)	4 ml

Filter sterilize

EDTA (0.5 M, pH 8.0) (Maniatis et al. 1982)

EDTA·$2H_2O$	168.1 g
Distilled water	to 1000 ml

EDTA will only dissolve when pH has been adjusted to 8.0.
(Use approximately 20 g NaOH pellets for this purpose)

Exo-nuclease III shortening solutions (Henikoff 1987)

Exo buffer.	
Tris/HCl (1 M, pH 8.0)	660 µl
$MgCl_2$ (0.1 M)	66.4 µl
Distilled water	9.27 ml

Klenow mixture.	
Tris/HCl buffer (0.1 M, pH 8.0)	3 µl
$MgCl_2$ (1 M)	6 µl
Distilled water	20 µl

Ligase mixture.	
Ligase buffer	144 µl
Distilled water	1440 ml

S_1 buffer (10x).	
KOAc (3 M)	1.1 ml
NaCl (5 M)	5 ml
Glycerol	5 ml
$ZnSO_4$	30 mg

S_1 mixture.	
S_1 buffer (10x)	41 µl
Distilled water	259 µl
S_1 nuclease (60 U)	1.5 µl

S_1 stop.	
Trisma Base (no HCl)	0.3 M
EDTA (pH 8.0)	0.05 M

Isopropanol (salt saturated)
Isopropanol was saturated with aqueous 5 M NaCl, 10 mM Tris-HCl and 1 mM EDTA (pH 8.0).

Klenow (DNA polymerase I) buffer
The buffer was made according to the following table and stored at -20°C.

Stock solution	Final conc.	/10 ml
Tris-Cl (1 M, pH 7.6)	0.1 M	1 ml
$MgCl_2$ (1 M)	0.1 M	1 ml
NaCl (5 M)	0.5 M	1 ml
2-mercaptoethanol	0.7 M	50 µl
Distilled water		6.95 ml

Ligation buffer (10x)
The buffer was made according to the following table and stored in aliquots at -70°C.

Stock solution	Final conc.	/ml
Tris-Cl (1 M, pH 7.6)	66 mM	0.66 ml
$MgCl_2$ (1 M)	6 mM	66 µl
ATP (0.1 M)	1 mM	0.1 ml
DTT	0.1 M	15.4 mg
Distilled water		0.174 ml

Phenol (TE saturated)
Phenol (200 g, Merck) was melted at 65°C and 0.3 g of 8-hydroxyquinoline was added. The phenol was extracted three times with TE (10x) or until the pH of the aqueous phase was approximately pH 7.6. The phenol was stored under TE (1x) at -20°C.

Restriction enzyme buffers (10x)

Stock solution	Final conc.
Tris-Cl (1 M, pH 7.9)	0.1 M
$MgCl_2$ (1 M)	0.1 M
DTT (0.5 M)	10 mM
BSA (10 mg/ml)	1 mg/ml
Glycerol	44% (v/v)
NaCl (5 M)	0, 50, 100, or 150 mM

The buffers were made using the following table and stored at -20°C.

	Salt concentration (mM)			
Stock solution	0	50	100	150
Tris-Cl	1 ml	1 ml	1 ml	1 ml
$MgCl_2$	1 ml	1 ml	1 ml	1 ml
DTT	0.2 ml	0.2 ml	0.2 ml	0.2 ml
BSA	1 ml	1 ml	1 ml	1 ml
Glycerol	4.4 ml	4.4 ml	4.4 ml	4.4 ml
NaCl	-	1 ml	2 ml	87.7 mg
H_2O	2.4 ml	1.4 ml	0.4 ml	2.4 ml

Sodium acetate (3 M, pH 5.2)

Sodium acetate·$3H_2O$	4.08 g
Distilled water	to 10 ml

Adjust pH with glacial acetic acid. Autoclave.

SSC (20x)

NaCl (3 M)	175.3 g
Sodium citrate (0.3 M)	88.2 g
Distilled water	to 1000 ml

Adjust pH to 7.0 with NaOH (10 N). Autoclave.

T4 DNA Polymerase buffer (10x)

This buffer was prepared using restriction endonuclease buffer A (Boehringer Mannheim) and adding bovine serum albumin (BSA Pentax Fraction V) to a final concentration of 1 mg/ml. Buffer A has the following composition:

Tris-acetate (pH 7.9)	0.33 M
K-acetate	0.66 M
Mg-acetate	0.1 M
Dithiolthreitol	5 mM

After addition of the BSA the buffer was divided into 100 µl aliquots and stored at -20°C.

Tris acetate buffer (50x)

Tris base	242 g
Acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0)	100 ml
Distilled water	to 1000 ml

Tris borate buffer (TBE, pH 8.0, 5x)

Tris base	54 g
Boric acid	27.5 g
EDTA (0.5 M, pH 8.0)	20 ml
Distilled water	to 1000 ml

TE (Tris-EDTA) buffer (100x)

Tris-Cl (pH 7.6)	121 g
EDTA (0.5 M, pH 8.0)	200 ml
Distilled water	to 1000 ml

Autoclave and dilute with sterile water before use.

TSB solution

LB	150 ml

pH to 6.1 with 2 drops conc. HCl.

PEG 4000	15 g
$MgSO_4$ (1 M)	1.5 ml
$MgCl_2$ (1 M)	1.5 ml

Dispense in 20 ml aliquots and autoclave. Add DMSO (1 ml) and glucose (0.5 M, 400 µl when necessary) immediately before use.

Western blotting buffers, reagents and solutions

Blocking buffer stock solution

Tris-HCl (pH 7.4)	1.21 g
NaCl	9 g
Distilled water	to 1000 ml

Blocking buffer

Blocking buffer stock solution	500 ml
Fat-free milk powder	10 g
Tween-20	0.25 ml
Sodium azide	0.1 g

New transfer buffer

Tris-HCl (0.025 mM, pH 8.8)	0.03 g
Methanol	200 ml
Distilled water	to 1000 ml

Substrate buffer

Tris-HCl (0.1 M, pH 7.5)	0.3 g
NaCl (0.1 M)	0.55 g
$MgCl_2$ (5 mM)	
Distilled water	to 100 ml

Washing buffer

NaCl (0.9%, w/v)	9 g
Tween-20 (0.05%, w/v)	0.5 ml
Distilled water	to 1000 ml

Z-buffer (pH 7.0)

Na_2HPO_4 (60 mM)	16.1 g
$NaH_2PO_4 \cdot 2H_2O$ (40 mM)	5.5 g
KCl (10 mM)	0.75 g
$MgSO_4 \cdot 7H_2O$ (1 mM)	0.246 g
2-mercaptoethanol (0.05 M)	2.7 ml
Distilled water	to 1000 ml

Appendix B

E. coli strains, genotypes, and references

E. coli strain	Genotype/description	Reference/origin
C600	*supE*44 *hsdR thi*-1 *thr*-1 *leuB*6 *lacY*1 *tonA*21	Appleyard (1954)
CC118	*araD*139 Δ(*ara, leu*)7697 Δ*lacX*74 *phoA*Δ20 *galE galK thi rpsE rpoB* $argE_{am}$ *recA*1	Manoil and Beckwith (1985)
K12 G6MD3	Hfr *his thi* Str^S Δ (*malA-asd*)	Schwartz (1966)
K514	*thr*-1 *leuB*6 *thi*-1 *supE*44 *lacY*1 *ton*A21 r_k^-,m_k^+ (C600 derivative)	Wood (1966)
LK111	*lacI*^{q} *lacZ*Δ*M15* *lacY*$^+$ (K514 derivative)	Zabeau and Stanley (1982)
YMC11	*glnA*$^-$ *ntrB*$^-$ *ntrC*$^-$ Ap^S	Backman et al. (1981)

Appendix C

C.1 One- and three-letter codes used for amino acids

Amino acid	Codes		Code	Amino acid
Alanine	Ala	A	A	Alanine
Arginine	Arg	R	C	Cysteine
Asparagine	Asn	N	D	Aspartic acid
Aspartic acid	Asp	D	E	Glutamic acid
Cysteine	Cys	C	F	Phenylalanine
Glutamine	Gln	Q	G	Glycine
Glutamic acid	Glu	E	H	Histidine
Glycine	Gly	G	I	Isoleucine
Histidine	His	H	K	Lysine
Isoleucine	Ile	I	L	Leucine
Leucine	Leu	L	M	Methionine
Lysine	Lys	K	N	Asparagine
Methionine	Met	M	P	Proline
Phenylalanine	Phe	F	Q	Glutamine
Proline	Pro	P	R	Arginine
Serine	Ser	S	S	Serine
Threonine	Thr	T	T	Threonine
Tryptophan	Trp	W	V	Valine
Tyrosine	Tyr	Y	W	Tryptophan
Valine	Val	V	Y	Tyrosine

C.2 Table of N-terminal amino acid sequencing

Degradation cycle	Amino acid	Picomoles
1	Met	391
2	Ser	268
3	Gln	380
4	Lys	330
5	Val	289
6	Phe	329
7	Ile	240
8	Ser	131
9	Glu	214
10	Asp	150
11	Asp	180
12	Glu	255

Input = 500 picomoles (by amino acid analysis)

Initial yield approx. = 80%

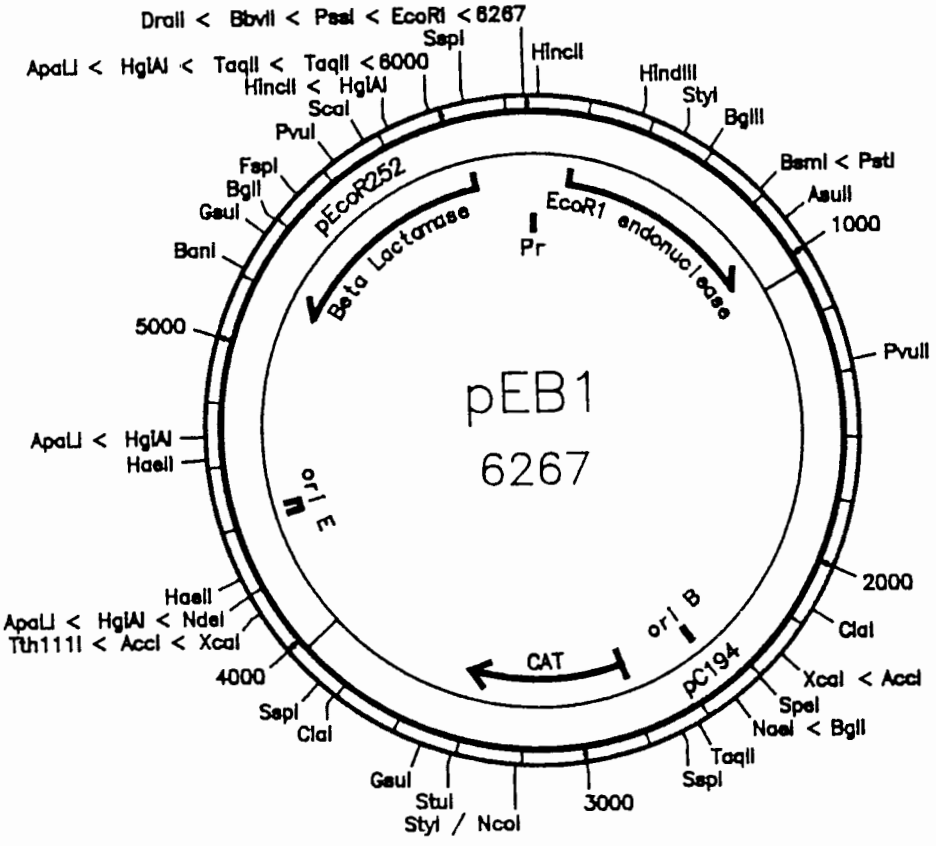
C.3 Comparison of amino acid composition of *B. fibrisolvens* branching enzyme from data obtained via amino acid analysis and from DNA sequence data.

Amino acid	Amino acid analysis			
	Deduced from DNA sequence		Observed from analysis	
	Residues	mole (%)	Residues	mole (%)
Asp	78	12.21	79	12.86
Thr	26	4.07	25	3.96
Ser	28	4.38	27	4.37
Glu	53	8.29	58	9.43
Pro	30	4.69	30	4.63
Gly	56	8.76	62	10.38
Ala	39	6.10	42	6.89
Cys	8	1.25	7	1.14
Val	31	4.85	30	4.78
Met	20	3.13	18	2.81
Ile	31	4.85	29	4.64
Leu	43	6.73	43	6.82
Tyr	44	6.89	41	6.44
Phe	39	6.10	37	5.84
Trp	18	2.82	ND[a]	ND[a]
His	19	2.97	18	2.88
Lys	54	8.45	56	9.08
Arg	22	3.44	19	3.02

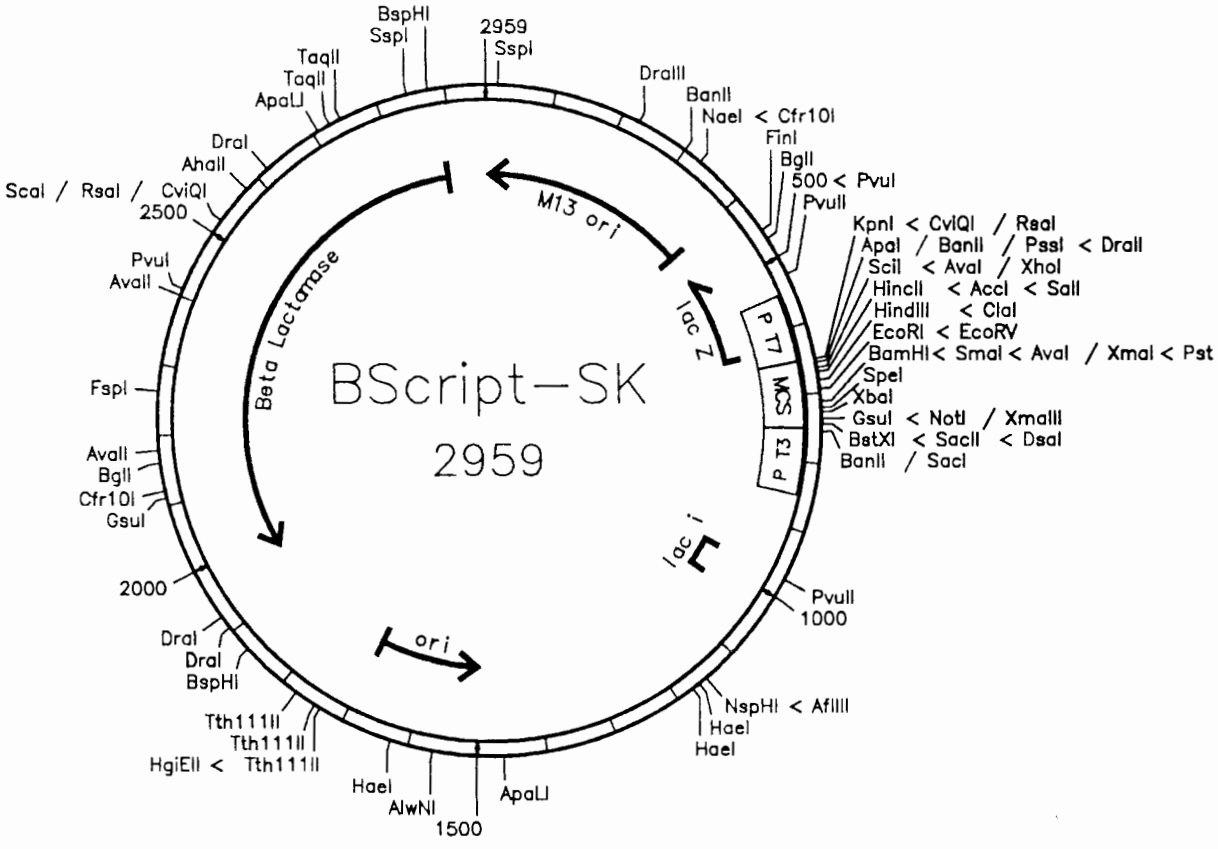
[a] ND is data not determined.

Appendix D

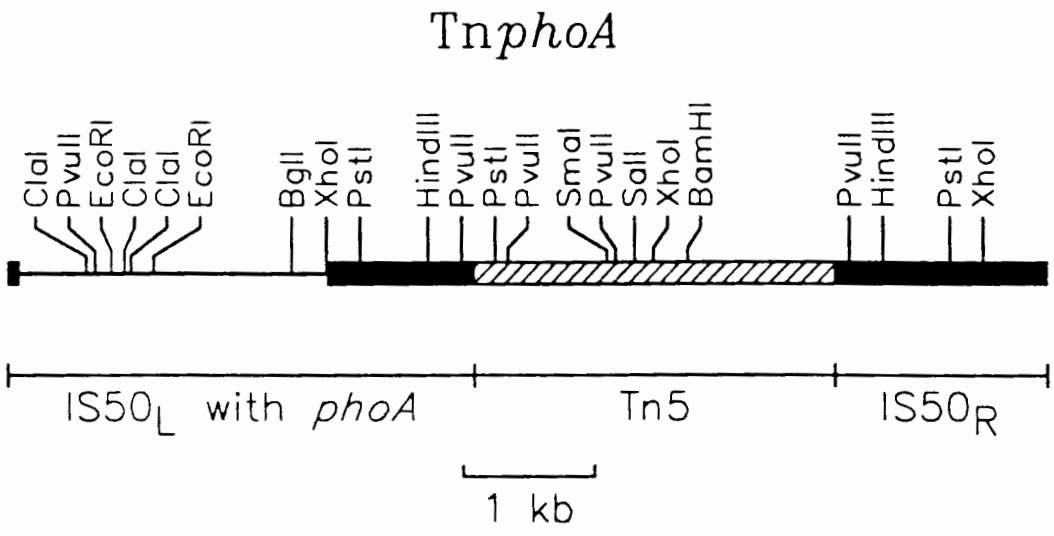
Plasmid vector and phage maps



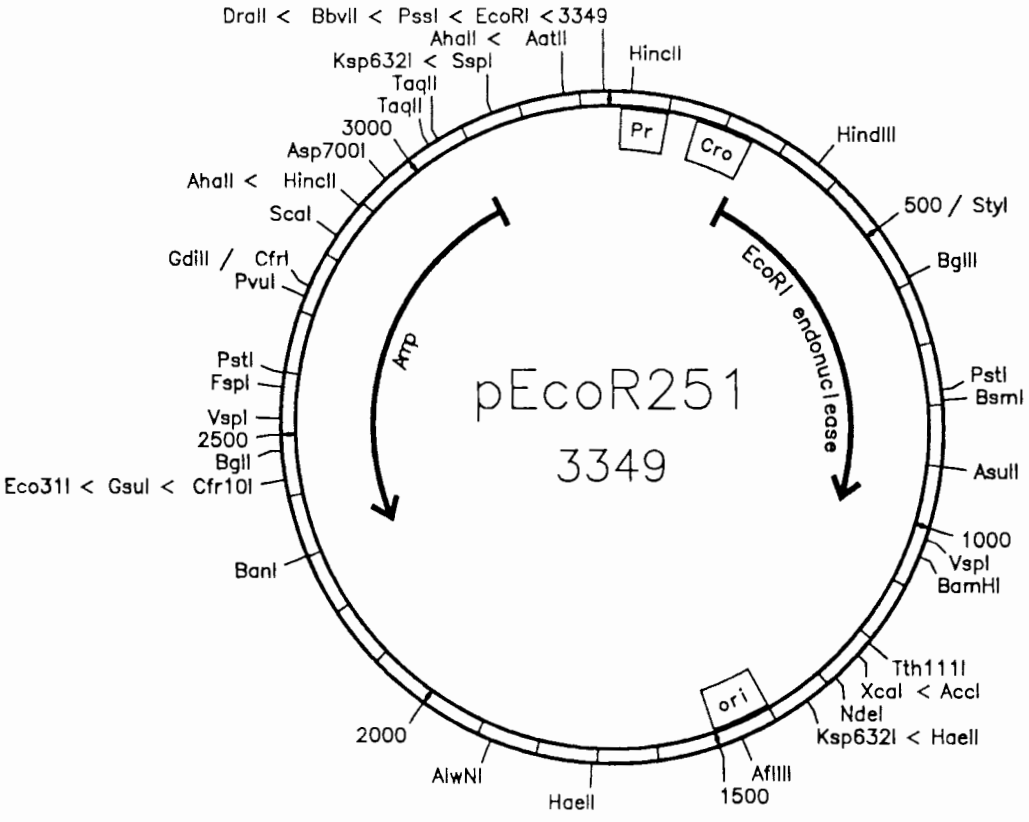
Restriction map of **pEBI** an *E. coli-B. subtillus* shuttle vector (Lin *et al.*, 1990).



Restriction map of **Bluescript SK** (Stratagene, San Diego, CA). This is a high copy number cloning vector designed for shortening and sequencing techniques. **Bluescript KS** differs in the orientation of the multiple cloning site polylinker.



A partial restriction map of **Tn*phoA*** showing the position of Tn5 relative to the leftward ($IS50_L$) and rightward ($IS50_R$) insertion sequences (Manoil and Beckwith, 1985; Gutierrez *et al.*, 1987).



A restriction map of **pEcoR251** (Zabeau and Stanley, 1982). This is a positive selection vector using insertional inactivation of the lethal *Eco*RI gene. **pEcoR252** does not contain the *Pst*I site in the β-lactamase gene.

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