COMPARATIVE ANALYSIS OF TWO ATTACHMENT VARIANTS OF BUTYRIVIBRIO FIBRISOLVENS

Djarot Sasongko Hami Seno

Thesis submitted for the degree of

Doctor of Philosophy

in the Department of Animal Sciences

The University of Adelaide

November 1, 2002

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

One of the major limitations to production-efficiency in the livestock industries is the low rate of plant-material digestion, in which carbohydrate breakdown is the fundamental digestive process and the primary rate-limiting step. It has been suggested that bacterial attachment to plant carbohydrate may be both a precondition and a trigger for fibre digestion in the rumen (Lee 1980, Kudo *et al.* 1987). An understanding of the control and regulation of rumen bacterial attachment to surfaces, particularly to carbohydrate commonly found in plant fibre, is essential for improving plant fibre digestion within the rumen. This approach may be more effective in enhancing plant polysaccharide degradation than introducing additional cellulase genes (Kudo *et al*. 1987).

Among ruminal bacteria, *Butyrivibrio fibrisolvens* is one of the most metabolically versatile species, as almost all strains of this species are able to grow on simple or complex sugars. A large number of isolates have been obtained from both ruminal and caecal portions of the gastrointestinal tracts of a variety of animals. Some of the *B. fibrisolvens* strains have been shown to adhere to the epithelium of the roof of the dorsal rumen (Bauchop *et al*. 1975, Dehority 1975, Dehority and Grubb 1981) and cellulosic substrates (Rasmussen *et al*. 1989).

Extracellular polymer production of various *B. fibrisolvens* strains have been reported, comprising teichoic acids (Sharpe *et al.* 1975, Hewett *et al.* 1976) and extracellular polysaccharides (Stack 1988; Stack and Ericsson 1988; Stack *et al.* 1988a,b; Stack and Weisleder 1990). Various enzymatic activities related to plant fibre degradation have been identified in most *B. fibrisolvens* strains and the corresponding genes have been cloned in *Escherichia coli* (Romaniec *et al*. 1987a, Sewell *et al*. 1989, Berger *et al* 1990, Hazlewood *et al*. 1990a, Mannarelli *et al*. 1990a, Rumbak *et al*. 1991, Utt *et al*. 1991, Dalrymple *et al*. 1996). However, no detailed study has been reported regarding the attachment of *B. fibrisolvens* to surfaces.

In this project the aim is to study the attachment of *B. fibrisolvens* to surfaces, using strain E14 (originally isolated from Alaskan Reindeer) (Orpin *et al*. 1985). For this purpose, two different variants, denoted sticky (S) and loose (L), discovered recently in our laboratory (Nili and Brooker 1995), were employed. The sticky variant sticks firmly on agar during the early stage of growth and can only be removed without by scraping the agar. On the other hand the L variant can be easily removed at all stages of growth.

1.2 Literature review

This literature review discusses i) rumen function and fibre digestion ii) the species concept of *Butyrivibrio fibrisolvens*, iii) the *Butyrivibrio fibrisolvens* strain to be used in the current project, iv) the mechanisms and the factors affecting bacterial attachment to surfaces, v) bacterial attachment within the rumen, vi) attachment in *Butyrivibrio fibrisolvens*, vii) possible ligands of *Butyrivibrio*

fibrisolvens which may be involved in its attachment to surfaces, viii) teichoic acids, ix) extracellular polysaccharides, x) genetic manipulation in *Butyrivibrio fibrisolvens*, and xi) aims of this project.

1.2.1. Rumen function and fibre digestion

1.2.1.1 The rumen and its microbial ecosystem

The ruminant digestive system (Figure 1.1) functions to transport, digest, absorb and to excrete undigested residues of endogenous and dietary origin (Kowalczyk 1989). The stomach of the ruminant is divided into the rumen, reticulum, omasum and abomasum (Kowalczyk 1989). General reviews of anatomy and function of the gastrointestinal (GI) tract of the ruminants can be found in (Kowalczyk 1989).

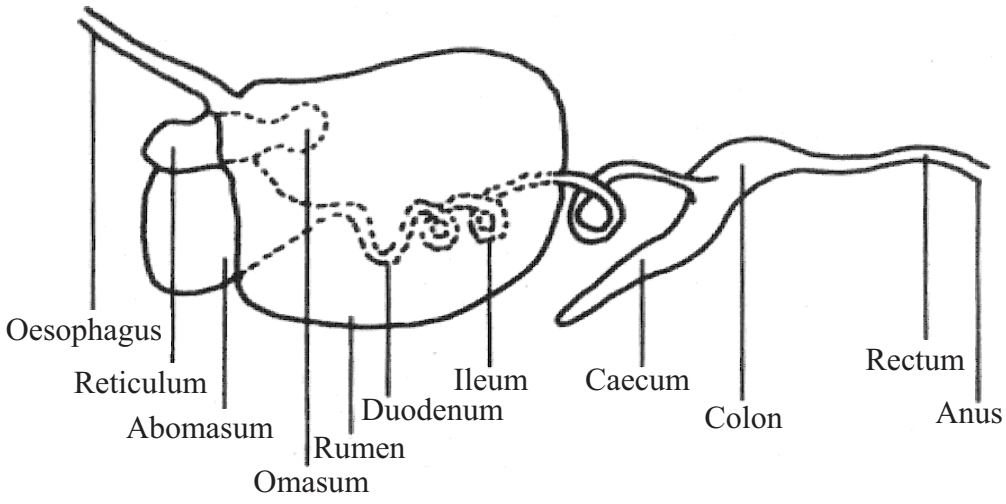


Figure 1.1 General anatomy of ruminant digestive system (Kowalczyk 1989).

The rumen, the first section of the GI tract, contains large numbers of bacteria (up to 10^{11} viable cells/ml comprising perhaps as many as 200 different species),

protozoa (10^4-10^6/ml spread over 25 genera), anaerobic rumen fungi (zoospore population densities of 10^3-10^5/ml divided into five genera), and bacteriophages (10^7-10^9 particles/ml) (Hobson 1988, Klieve and Swain 1993, Hespell *et al*. 1997, Orpin and Joblin 1997, Stewart *et al*. 1997, Williams and Coleman 1988, Kowalczyk 1989, Mackie *et al*. 2000). The rumen bacteria include the species listed in Table 1.1.

Table 1.1 Various rumen bacteria (Hobson 1988, Stewart and Byrant 1988, Stewart *et al*. 1997, Mackie *et al*. 2000).

Prevotella	*Streptococcus*
Selenomonas	*Syntrophococcus*
Butyrivibrio	*Ruminococcus*
Anaerovibrio	*Clostridium*
Spirochaetes	*Lactobacillus*
Wolinella/vibrio	*Lacnospira*
Succinomonas	*Eubacteria*
Succinivibrio	*Mycoplasma*
Megasphaera	Large bacteria
Veilonella	Methanogenic bacteria
Fibrobacter	

Details of other microorganisms and general reviews of rumen microorganisms can be found in “The Rumen Microbial Ecosystem” (Hobson, 1988). This study concentrates on the rumen bacteria *Butyrivibrio fibrisolvens*. This species is among the predominant species and may be isolated from 10^8 dilution of rumen content (Byrant 1984). The relative distribution of *B. fibrisolvens* compared to other major rumen bacteria is shown in Table 1.2 (Dehority and Orpin 1988). *B. fibrisolvens* is among the predominant hemicellulolytic (Hespell *et al*. 1987;

Sewell *et al*. 1988, 1989; Mannareli *et al*. 1990b; Lin and Thompson 1991; Utt *et al*. 1991; Hespell1992; Williams and Withers 1992; Strobel 1994; Hespell and Cotta 1995), proteolytic (Strydom *et al*. 1986, Cotta and Hespell 1986, Wachenheim and Patterson 1990), amylotic (Cotta 1988, Strydom *et al*. 1986, Rumbak *et al*. 1991) rumen bacteria and is weakly cellulolytic (Kopecny 1986; Romaniec *et al*. 1987a,b; Berger *et al*. 1989, 1990; Lin *et al* . 1990; Hazlewood *et al*. 1990a; Mohn and Teather 1995). Further details on the species concept (section 1.2.2), as well as research on *B. fibrisolvens* (sections 1.2.3, 1.2.6, 1.2.7, 1.2.12) are discussed separately.

Table 1.2 Distribution of major bacterial genera in rumen contents (Dehority and Orpin 1988)

Genera	Percentage of total isolates					
	*		**		***	
	HR	HC	HR	HC	HR	HC
Butyrivibrio	23	9	38	10	25	2
Selenomonas	0	16	5	12	7	19
Prevotella	22	26	8	9	11	4
Ruminicoccus	0	1	4	4	11	10
Peptostreptocccus	0	0	0	8	6	14
Streptocccus	0	0	3	6	4	8
Lactobacillus	0	4	2	17	6	20

HR = High-roughhage diet, HC = High-concentrate diet. * (Caldwell and Byrant 1966), ** (Latham *et al.* 1971), *** (Latham *et al.* 1972)

Despite the vast knowledge, there are still limitations in studying the GI ecosystem, such as the inevitable bias introduced by culture-based enumeration and characterization techniques and the lack of phylogenetically-based classification schemes (Amann *et al*. 1990, 1994; Stahl 1997, Stahl and Amann

1997, Mackie *et al*. 2000). New techniques using DNA, 16SrRNA, 18SrRNA, DGGE (denaturing gradient gel electrophoresis) or TGGE (temperature gradient gel electrophoresis) of PCR amplified DNA have been developed to study the molecular ecology and diversity in gut microbial ecosystems (Pace *et al*. 1986; Amann *et al*. 1990, 1995, 1996; Flint *et al*. 1990; Ning *et al*. 1991; Muyzer *et al*. 1993, 1998; Ludwig *et al*. 1994; Poulsen *et al*. 1994, 1995; Cann *et al*. 1996; Mackie and Dore 1997; Muyzer and Smalla 1998; Zoetendahl *et al*. 1998; Amann and Kuhl 1998; DeLong *et al*. 1989; Simpson *et al*. 1999; Ward 2000; Mackie *et al*. 2000). General reviews of these techniques can be found in Mackie *et al*. (2000).

1.2.1.2 Digestion, fermentation and metabolism of plant carbohydrates

The rumen represents a mobile self-sustaining fermentation system for plant materials, and ruminants gain their energy from the digestion of fibre due to the action of bacteria, fungi and protozoa in their rumen, allowing ruminants to subsist on fibrous, low protein feeds which are indigestible by most non-ruminant animals (Kowalczyk 1989, Mackie *et al*. 2000). Rumen microorganisms can also transform many toxic ingredients to harmless compounds, and therefore ruminants are able to digest many plants which are toxic to monogastric animals, including humans (Gregg *et al*.1994, Dawson and Allison 1988, Forsberg *et al*. 2000). However, some plants contain antinutritive or toxic compounds; such as alkaloids (pyrrolizidine, cyanogenic and coumarin glycosides), terpenes (saponins), simple acids (fluoroacetate and oxalate), amino acids (mimosine and indospicine), and polyphenols (tannins); which inhibit ruminant digestion or affect the nutritive value of forages, and therefore overall animal productivity (Chesson *et al*. 1982,

Hemken *et al*. 1884, Jones and Megarrity 1986, Dawson and Allison 1988). The toxic metabolites may be due to conversion of innocuous compounds by rumen bacteria (i.e. the production of cyanide from cyanogenic glycosides) or inability of the rumen microorganisms to metabolise the compounds responsible (Gregg *et al*.1994, Dawson and Allison 1988).

A number of studies have been carried out to investigate the microbial enzyme systems that degrade the relatively insoluble cellulose, hemicellulose, pectin and starch, which occur in plant forage, as well as protein (Cheng *et al*. 1989). The breakdown of cellulose and other resistant polysaccharides is the most important digestive process occurring in the rumen (Bauchop 1980; Cheng *et al*. 1983, 1989). The digestion and metabolism of plant carbohydrates may be classified into three stages (Figure 1.2). In the first stage, complex carbohydrates (cellulose, hemicellulose, starch) are broken down into simple sugars such as hexose or pentose by extracellular microbial enzymes (Beguin 1990, Ward 2000).

Cellulose is the main carbohydrate component of plant cell walls, comprising 40-59% of the dry mass of vascular plants (Cheng *et al*. 1989, Tomme *et al*. 1995, Forsber *et al*. 2000). The breakdown of cellulose is one of the most important digestive processes occurring in the rumen; *Ruminococcus flavefaciens, R. albus* and *Fibrobacter succinogenes* are the predominant cellulolytic bacteria in the rumen ecosystem and are extremely efficient in breaking down cellulose (Krause *et al*. 1999, Weimer *et al*. 1999, Forsberg *et al*. 2000). The major molecular paradigm of bacterial cellulolysis and adhesion to cellulose is based on the cellulosome concept of *Clostridium thermocellum* (Forsberg *et al*. 2000).

Cellulosome-like structures have been found in rumen cellulolytic microorganisms such as *R. albus*, *R. flavifaciens*, *F. succinogens* (Leatherwood 1973, Lamed *et al*. 1987, Miron *et al*. 1989, Forsberg *et al*. 2000). *B. fibrisolvens* H17C produces multi-enzyme aggregates, composed of several endoglucanases or xylanases, or both (Doerner and White 1990, Lin and Thompson 1991). Cellulose is degraded by one or more β-1,4-glucanases to the disaccharide cellobiose which is then converted either to glucose or glucose-1-phosphate (Doerner and White 1990, Beguin 1990, Lin and Thompson 1991, Forsberg *et al*. 2000).

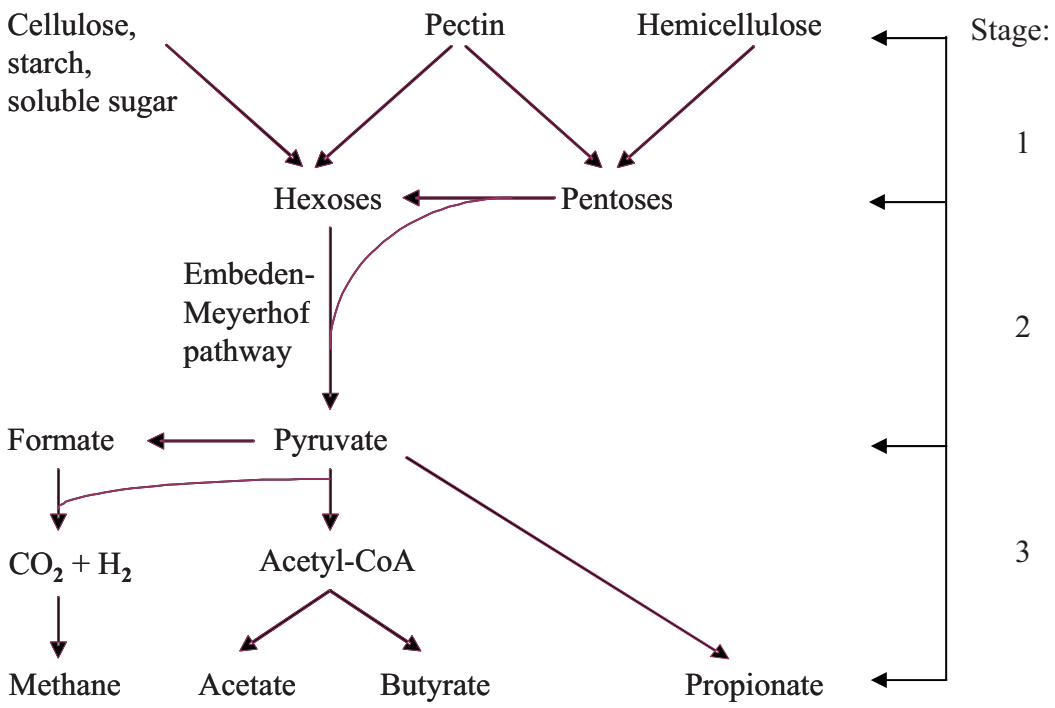


Figure 1.2. Pathways of carbohydrate metabolism in the rumen (Czerkawski 1986, Dijkstra *et al*. 1992, Sauvant and Van Migen 1995)

Stage 1, 2 and 3 are breakdown of complex carbohydrate to simple sugar, fermentation of simple sugar and pyruvate metabolism, respectively.

Hemicellulose is the second major plant polysaccharide (20-30%), and its predominant polymer is mainly a pentosan or xylan, which is composed of xylose with arabinose side chains (Hespell *et al*. 1987). Although representing a relatively high proportion of the total carbohydrate in forage, its contribution to animal dietary energy is often decreased due to its low overall digestion (Wedig *et al*. 1989, Hespell and Cotta 1995). The major xylanolytic bacteria in the rumen are *B. fibrisolvebs* and *Prevotella ruminicola* (Hespell *et al*.1987, Hespell 1992, Hespell and Cota 1995). The major products of hemicellulose breakdown are pentoses which are formed by enzymic action upon the β-1,4 linkages to produce xylose (Coen and Dehority 1970, Hespell and Cota 1995). Uronic acids may be produced from pectins, which are first hydrolysed to pectic acid and methanol by pectinesterase (Osborne 1989). The pectic acid is then attacked by polygalacturonidases to produce galacturonic acids, which in turn yield xylose (Dehority and Scott 1967, Osborne 1989).

Starch is one of the most important components of the ruminant diet, and a number of digestive disorders are often associated with high starch diets, such as lactic acid accumulation (lactate acidosis) (Styler 1976). Knowing the nature and regulation of amylolytic enzymes would therefore assist in improving starch digestion efficiency and in controlling these digestive disorders. Molecular studies and amino acid sequence of α-amylases have been reported for *S. bovis* and *C. butyricum* (Walker and Hope 1964); *R. amylophilis* or *B. amylophylus* (McWethy and Hartman 1977); and *B. fibrisolvens* (Cotta 1988). Starch and dextrans are first converted to maltose and isomaltose by the action of amylases, and then to

glucose by maltases and maltose phosphorylases (McWethy and Hartman 1977, Cotta 1988, Rumbak *et al*. 1991).

In the second stage, the simple sugars produced extracellularly from the first stage of carbohydrate digestion are then rapidly utilized by the microorganisms and subsequent metabolism of these sugars intracellularly (Yarlett *et al*. 1985). The primary path of sugar fermentation in rumen microbes is the Embden-Meyerhof-Parnas (EMP) pathway, and is the primary source of energy for the formation of the high energy phosphate bonds of ATP that are utilised by rumen microbes for maintenance and growth (Yarlett *et al*. 1985, Williams and Coleman 1988). Two phosphate bonds of ATP are formed during the conversion of hexoses to 2 pyruvate and 2 NADH (2 phosphate bonds of ATP are used to convert hexoses to triose-phosphates and four are formed to convert the triose-P's to pyruvate and NADH) (Yarlett *et al*. 1985, Williams and Coleman 1988, Russell and Wallace 1988). This yield is therefore substantially less than that of aerobic respirations, (28-38 moles ATP/ mole glucose), however the actual growth yield may differ to this theoretical energy yield (Williams and Coleman 1988, Russell and Wallace 1988).

In the third stage, the pyruvate and $NADH_2$ derived from hexose and pentose fermentation are further metabolized, and this process varies greatly depending on the microbes involved and incubation conditions (Dehority and Orpin 1988, Weimer *et al*. 1999). The main end products of pyruvate metabolism are the short-chain volatile fatty acids acetate, propionate and butyrate, as well as carbon dioxide and methane; while succinate and lactate are important intermediates

(Huhtanen and Gall1953, Roche *et al*. 1970, Styler 1976, Yarlett *et al*. 1985, Willem and Coleman 1988, Forster *et al*. 1996). Although in general, the predominant acid is acetic, the relative proportions of the volatile fatty acids in the rumen liquor vary with diet. High cellulose diets induce higher levels of acetate while diets high in concentrates result in a decrease in acetate or rise in propionate proportion (Latham *et al.* 1972, Roche *et al*. 1973). The molar ratios (moles acetate: propionate: butyrate) are usually near 65: 25: 10 with roughage diets and 50: 40: 10 for concentrate diets (Latham *et al.* 1972, Roche *et al*. 1973, Owens and Goetsch 1986, MacKenzie *et al*. 1987). Most of the acid produced is absorbed directly from the rumen, reticulum and omasum, although some may pass through the abomasum and be absorbed in the small intestine (Williams and Coleman 1988, Kowalczyk 1989). Some of the products of carbohydrate digestion are also used by bacteria and protozoa to form their own structural polysaccharides, especially under conditions of surplus carbohydrate (Czerkawski 1986, Owens and Goetsch 1986, Williams and Coleman 1988, Miron *et al*. 1994, Hespell *et al*. 1997).

1.2.1.3 The importance of microbial attachment to fibre digestion

Within the rumen, as much as 75% of microbial cells are associated with feed particles (Cheng *et*. 1989). Microbial adhesion to plant material is important to the process of digestion in ruminants (Kauri and Kushner 1985). Adhesion is the essential first step in the digestion of insoluble nutrients and enabling microorganisms to be retained within the rumen as well as avoiding washout with the fluid fraction of digesta (Kauri and Kushner 1985, Cheng *et al*. 1989). Once a microbial population has been established on the surface of insoluble nutrients, it

will then be followed by the secretion of specific enzymes within the complex digestive microbial consortia that form within adherent biofilms on the surfaces of plant materials (Kudo *et al*. 1987, Cheng *et al*. 1989). These degradative enzyme complexes function optimally in the multi-species consortia that develop within these adherent populations, and govern the rate of fibre digestion (Bayer 1986, Cheng *et al*. 1989; Bhat *et al*. 1990, 1994).

Direct examinations and *in vitro* studies have shown that rumen microorganisms must be attached to insoluble plant substrates to effect the digestion of the plant materials (Kauri and Kushner 1985, Kudo *et al*. 1987, Cheng *et al*. 1989). The primary colonizers are bacteria, while fungi are seen to colonize surfaces of plant tissue which are more resistant to digestion, and protozoa are associated with the plant tissues whose components they are digesting (Williams and Coleman 1988, Cheng *et al*. 1989, Tomme *et al*. 1995). The association of protozoa with readily digestible feeds such as cereal grains, as well as with tissues which resist digestion such as the parenchyma bundle sheath of forages have been reported (Bauchop 1975, Cheng *et al*. 1989, Hespell *et al*. 1997). Since most of the nutrient-rich tissues of plants are internal, the overall rates of plant digestion depend upon microbial access to these tissues (Dinsdale *et al*. 1978, Cheng *et al*. 1989, Bloomquist, *et al*. 1996). External plant tissues are only poorly colonized by rumen microorganisms and physical disruption (e.g., chewing) is necessary to allow optimal microbial access to inner tissues that are avidly colonised. Thus, it can be said that both grain and forage materials are digested inside out, with the bacterial attack being initiated against the innermost elements of the plant

materials, while the outermost components are much more slowly digested (Cheng *et al*. 1989, Tomme *et al*. 1995, Wilson and Mertens 1995).

The digestion of cellulose fibre is carried out by highly structured consortia of several different strains of bacteria, and cellulolytic fungi are always spatially associated with *Mycoplasma* (Cheng *et al*. 1989, Tomme *et al*. 1995, Orpin. and Joblin 1997). Similarly, rumen protozoa are often found associated with cellulolytic bacteria and methanogens (Williams and Coleman1988, Cheng *et al*. 1989, Hespell *et al*. 1997). *In vitro* studies of cellulolytic mechanisms and *in vivo* direct observations of rumen inocula showed that the process of cellulose digestion is key to the chemical nature of the glycocalyx of each species. Once established, the adherent microbial populations develop highly organized multispecies consortia within which the very high rates of cellulose digestion occur in the rumen (Kudo *et al*. 1987, Cheng *et al*. 1989, Tomme *et al*. 1995).

Microbial attachment, consortia formation (Stanton and Canale-Parola 1980, Kudo *et al*. l986, Wood *et al*. 1986) and sequestration are not accidental, and are integral and desirable characteristics in a multi-compartment system (Czerkawski and Cheng 1988). In fact there is a great deal of correspondence between the spatial distribution of microorganisms and their function in the rumen (Williams and Strachan 1984). Direct examination of cellulose surfaces undergoing rapid cellulolysis shows that a spatial hierarchy of microorganisms exists within the consortia (Chesson and Forsberg 1988, Czerkawski and Cheng 1988). Complex substrate transformations usually involve microbial consortia in which two or more species combine their enzymatic activities to degrade the substrates

(Czerkawski and Cheng 1988). The rate and extent of attack on cellulose in pure cultures of bacteria or fungi never achieve the rates of cellulose digestion as observed in the rumen; while in mixed cultures (e.g. *F. succinogenes* plus *Treponema bryantii* or *F. succinogenes* plus *Butyrivibrio* sp.), approaches that seen in the rumen, and combinations of rumen fungi and methanogenic bacteria are similarly effective (Wood *et al*. 1986, Czerkawski and Cheng 1988). Cellulose is rapidly colonized by primary cellulose-degrading organisms and subsequently colonized by secondary consortium organisms that do not degrade cellulose but 'drive' the digestive process by end-product removal (Chesson and Forsberg 1988, Czerkawski and Cheng 1988).

Although starch is more readily digested in the rumen than fibre, the development of microbial consortia is also pivotal for the digestion of starch in the rumen. Pure starch granules exposed to rumen fluid are colonized by a mixed bacterial population of which a large proportion of the organisms are amylolytic, and adherent starch digesting bacteria possess a greater amylolytic activity than those which remain free in the rumen fluid (Walker and Hope 1964, Cotta 1988, Cheng *et al*. 1989). The major factor which controls the rate of digestion in cereal grains is not the characteristics of the starch, but rather the properties of the protein matrix that surrounds the starch granule (Cheng *et al*. 1989). In maize, the protein matrix is extremely resistant to colonization and impedes the development of microbial consortia (Cheng *et al*. 1989, Forsberg *et al*. 2000). Therefore, microbial digestion of the protein matrix and starch occurs at a very slow rate. On the other hand, the protein matrix of barley is rapidly colonized by a variety of proteolytic and amylolytic rumen bacteria and thus the rate of digestion of the

protein matrix and starch proceeds at a rapid rate (Cheng *et al*. 1989). The development of microbial consortia differs and is dependent upon the type of cereal grain; in maize, starch granules were colonized by a consortia of which a Gram positive cocci resembling *Sarcina* was the major organism, whereas a Gram negative rods were the predominant bacteria in the consortia which colonized barley starch granules (Walker and Hope 1964, Cheng *et al*. 1989).

The mechanisms and factors affecting bacterial attachment to surfaces (section 1.2.4), bacterial attachment within the rumen (section 1.2.5), and attachment of *B. fibrisolvens* (section 1.2.6) are discussed separately.

1.2.2 The species concept of *Butyrivibrio fibrisolvens*

B. fibrisolvens is an anaerobic, curved, rod-shaped and butyric acid-forming bacterium (Hungate 1950, Huhtanen and Gall 1952, Byrant 1984, Stack 1988). Its cell walls stain Gram negative by standard techniques but contain typical compounds (lipoteichoic and glycerol teichoic acids) characteristic of Gram positive bacterial cell walls (Sharpe *et al*. 1975, Hewett *et al*. 1976). Furthermore, ultrastructural determinations of two representative strains of *Butyrivibrio*, using electron microscopy, showed that they were of the Gram positive morphological type, but very thin (12-18 nm), possibly accounting for the tendency to stain Gram negatively (Cheng and Costerton 1977). Cell shapes vary (between 0.4-0.8 μm in diameter and 0.3-3.0 μm in length) from one strain to another (Margherita *et al*. 1964) and the cells are motile using a monotrichous polar or subpolar flagella.

However, unflagellated (nonmotile) strains have also been reported (Shane *et al*.1969, Byrant 1984).

Moore (1976) has described and differentiated the genus into *B. fibrisolvens* and *B. crossotus* (the differences between these two strains are shown in Table 1.3). However the taxonomic criteria are not very precise and it has been suggested that they should be further revised, as there is tremendous diversity in morphology, nutritional requirements, fermentation substrates and products among various isolates of *B. fibrisolvens* (Stack 1988). Several researchers have attempted to classify isolates as to their relatedness based on one or more phenotypic characteristics. Examples are differences in the patterns of short-chain volatile fatty acids produced or consumed during growth (Shane *et al*. 1969), nutritional requirements (Roche *et al*. 1973), immunochemical properties (Margherita and Hungate 1963, Margherita et al. 1964, Sharpe et al. 1975, Hazlewood *et al*. 1986), esterase activity (Hespell and O'Bryan-Shah 1988), neutral sugar composition of the EPS produced (Stack 1988), and biochemical or genetic analysis (G + C content and hybridization level between DNA) or both (Mannarelli 1988, Mannarelli *et al*. 1990b). The mol % G + C of *B. fibrisolvens* DNA is 36-41 (Byrant 1984). Attempts to classify the genus using a phylogenetic analysis have also been reported (Forster *et al* 1996).

Most strains of *B. fibrisolvens* are able to grow on simple sugars as well as on cellulose, hemicellulose, starches, pectin, mannans, and even intact forages (Dehority and Scott 1967, Coen and Dehority 1970, Hespell 1991, Miron and Ben-Ghedalia 1993, Miron *et al*. 1994, Hespell and Cotta 1995). This species is

Table 1.3 Characteristic differences between *B. fibrisolvens* and *B. crossotus* (Moore 1976, Byrant 1984).

Characteristics	*B. fibrisolvens*	*B. crossotus*
Flagella	Monotrichous	lopotrichous
Antigenic structure	Diverse	not determined
Cell size	0.3-0.8 μm,1.0-5.0 μm	larger in diameter
H_2 production	+	-
CO_2 production	+	not determined
Fine structure of cells	thin cell wall (Gram positive)	not determined
Energy sources : - Maltose - galactose, inulin, salicin - lactose - starch - esculin, trehalose - glycerol, inositol, mannitol	 + + + not determined not determined -	 + - not determined + - -
Esculin hydrolysis	not determined	-
Fermentation substrates : - glucose and fructose - sucrose, cellobiose and xylose	 + +	 weak -
Acids produced from fermentation (from glucose or cellobiose in *B. fibrisolvens* and from maltose in *B. crossotus*) : - butyrate, formate - lactate, acetate	 + not determined	 + +

also one of the most common ruminal bacteria (Byrant 1959, Hungate 1988, Byrant 1974) and a large number of isolates have been obtained in both ruminal or cecal portions of the gastrointestinal tracts of sheep (Shane *et al*. 1969, van Glyswyk and Roche 1970, Dehority and Grubb 1981), goats (Dehority and Grubb 1977), cattle (Margherita and Hungate 1963), pigs (Stack 1988), Alaskan reindeer (Dehority 1975), other ruminant animals (Stack 1988), and human, rabbit or horse faeces (Moore and Holdeman 1974, Moore *et al*. 1976, Rumney *et al*. 1995).

A number of enzymatic activities have been reported from various strains of *B. fibrisolvens*. These include xylanolytic (Dehority 1966, Hespell *et al*. 1987, Sewell *et al*. 1988, Mannarelli *et al*. 1990a, Lin and Thompson 1991, Williams and Withers 1992, Hespell 1992, Hespell and Cotta 1995), pectinolytic (Dehority 1969), cellulolytic (Shane *et al*. 1969, Kopechny 1986, Romaniec 1987a), lipolytic (Hazlewood and Dawson 1979, Hazlewood *et al* 1983), proteolytic (Fulghum and Moore 1963, Strydom 1986, Cotta and Hespell 1986), esterase (Hespell and O'Bryan-Shah 1988), cinnamoyl esterase (Dalrymple *et al*. 1996, McSweeney *et al*. 1998), amylolytic (Cotta 1988, Rumbak *et al*. 1991) and branching enzyme (Rumbak *et al*. 1991) activities. However, none of these activities have been related to attachment of *B. fibrisolvens* to surfaces.

1.2.3 *Butyrivibrio fibrisolvens* strain used in this project

The strain to be used in the current project is *B. fibrisolvens* E14, which was originally isolated from Alaskan Reindeer. The sticky (S) and loose (L) variants were originally reported by Nili (1996), in studies on amino acid utilization by this strain. At early stages of growth, the S variant sticks firmly to agar surfaces and cannot be removed without scraping the agar. In contrast, the L variant can be easily removed from agar surfaces at all stages of growth. Previous comparative studies of chromosomal DNA (*EcoR* I and *Hae* III digested) and protein (cytoplasmic fraction) profiles, as well as other biochemical tests, showed no significant difference between the two variants. Biochemical tests that were carried out included volatile and non-volatile fatty acid analyses, semiquantitative (plate) assays of endoglucanase activity, capsular determination and identification

of various enzymes produced. The S variant was found to produce more EPS (based on the glucose content of a crude EPS preparation) (Nili 1996).

The current project, results of which are presented in this thesis, was carried out to further examine the two variants as a model for attaching and non attaching bacteria, and to study the attachment of *B. fibrisolvens* to surfaces.

1.2.4 Mechanisms and factors affecting bacterial attachment to surfaces

Studies on the mechanism and significance of microbial attachment have been carried out in a variety of ecological situations, such as roots and other plant surfaces (Dazzo 1980), oral surfaces (Newman 1980, Barber *et al*. 1993, Bloomquist *et al*. 1996), soil and sediments (Marshall 1980), viruses (Bitton 1980), fresh water systems (Paerl 1980), the marine environment (Fletcher 1980a,b) and as in the present study, the rumen (Costerton *et al*. 1978, Morris and Cole 1987, Rasmussen *et al*. 1989, Miron *et al.* 2001). In general, the factors that affect bacterial attachment to surfaces are intimate contact, short-range attractive forces, and the surface structures of both bacterium and the substratum.

Attachment of microbial cells to surfaces requires intimate contact. However both the cell surface and the substratum usually carry a net negative charge that results in an electrical repulsion barrier when these surfaces come close together (Verwey and Overbeek 1948). Short range attractive forces such as ionic bonding, van der Waals forces, and hydrophobic and hydrogen bonding are thought to be the balancing forces that overcome the repulsion between like charges (Marshall 1980). Although individually weak, these forces become effective when

quantitatively increased (Corpe 1980). Other mechanisms to overcome the repulsion barrier are dictated by the surface structures of substrata and bacteria, and have been reported in a number of bacterial species (Jones *et al*. 1969, Fletcher and Floodgate 1973, Marshall 1976, Newman 1980, Lee 1980) in various environments. However, extracellular-structure-bearing cells are not necessarily attached to surfaces.

The molecules involved in such attachment mechanisms are often called the binding molecules, those of bacterial surface, the ligands, while those of substrata are referred to as receptors (Ofek and Beachey 1980).

The possible bacterial structures (ligands) that may be involved in attachment to surfaces are flagella, fimbriae (pili), extracellular polymers, attachment proteins, or combinations of these types of bacterial surface structures. Very often, bacterial extracellular polymer (EP) is identical to extracellular polysaccharide (EPS), which is a term used to describe polymers secreted by bacteria, but not necessarily for attachment purposes. Although in many cases, these polymers are one and the same, some bacteria, including many strains of *B. fibrisolvens*, produce other polymers such as teichoic- and lipoteichoic acids (Sharpe *et al*.1975, Hewett *et al*. 1976, Ofek and Beachey 1980).

Fimbriae and EPS are among the bacterial surface structures that could conceivably participate in the attachment process to bridge the gap caused by like-charge repulsion forces, and allow anchoring of the bacterium to the surfaces (Jones *et al*. 1969, Corpe 1980, Lee 1980). For fimbriated bacteria, fimbriae (pili)

may be involved in primary adhesion. Attachment to surfaces, which is a primary step in substratum colonisation, is assisted by the secretion of EPS (Hultgren *et al*. 1993), and the formation of a biofilm leads to subsequent cell growth and EPS biosynthesis (Cheng *et al*. 1989). However, bacterial adhesion is not always associated with polymeric fimbriae. Monomers or simple oligomers attached to surfaces are also found in many adhesins such as in the marine prokaryote *Hyphomonas*, where EPS alone appears to be the primary adhesin and EPS^- variants are unable to perform attachment (Quintero and Weiner 1995). This phenotype (EPS^-), which often results from stable spontaneous gene deletions or rearrangements, has also been found in a number of bacterial species (*P. solanacearum*, *V. cholerae* and *H. influenzae*) (Jonnes 1980; Kuo *et al*. 1985; Hoiseth *et al*. 1985, 1986; Huang and Schell 1995). *Pseudomonas fluorescens* produces a compact polysaccharide that allows adhesion to almost any surface (Fletcher and Floodgate 1973; Fletcher 1980a, 1986; Gilbert *et al*. 1989, 1990), while dextran, produced in a similar manner by *Streptococci salivarius*, facilitates adhesion to a tooth surface (Newman 1980; Griffin *et al*. 1995, 1996). The extensions of filaments from *Lactobacillus fermenti* are thought to reinforce primary adhesion (Fuller 1975, Fuller and Brooker 1975). Polymer fibrils are also seen in many other examples of attachment within the gut or rumen, such as attachment of *Torulopsis pintolopessi* (Savage 1980), *Enterobacteriaceae* (Doig and Trust 1994) and *Streptococcus bovis* (Whitehead 1992).

The secretion and the conformation of the adhesive macromolecular surface materials may be influenced by physiological and environmental conditions (temperature, pH, Eh, specific ions, ionic strength, availability of nutrients and

surface active materials) which are known to affect growth and viability of organisms (Corpe 1980, Fletcher 1991).

In addition to cell-specific factors (intimate contact, short-range attractive forces, and the surface structures of both bacterium and the substratum), there are also other factors that must be considered in studying the effect of bacterial attachment to surfaces on nutrient utilization. These include culture age, nutrient diffusion, and cell mobility (Marshall *et al.* 1971; Costerton and Irvin 1981, 1985; Kjeleberg *et al.* 1982; Gordon *et al.* 1983; Morisaki 1983, Fletcher 1985,1986, 1991; Leigh and Coplin 1992).

Culture age-dependant attachment has been reported for *Pseudomonas* (Fletcher 1977, 1980a); *Streptococci* (Ørstavik 1977, Fletcher 1980b); and *Bacillus mycoides* as well as *Serratia marcescens* (Zvyagintsev *et al.*1977, Fetcher 1991). Although not specifically related to attachment, *B. fibrisolvens* OB156 has also been reported to have culture age-dependent characteristics (Beard *et al.* 1995).

The ability to attach to surfaces should enhance substrate utilization since this would provide a constantly renewed supply of nutrients (Costerton and Irvin 1981, Leigh and Coplin 1992). However, direct substrate transport and diffusion effects may reduce the advantage of attachment in soluble and simple substrates (Fletcher 1991). Low-molecular weight solutes in the medium are always in equilibrium, with both adsorption and desorption continuously occurring. Each time a substrate is taken up by the cell, a diffusion gradient would be established, which would continuously deliver fresh substrate to the cell (Marshall *et al.* 1971;

Fletcher 1980b, 1991; Leigh and Coplin 1992). Diffusion effects may or may not be eliminated at low concentrations of substrates. (Marshall *et al.* 1971; Fletcher 1980b, 1985, 1986; Kjeleberg *et al.* 1982; Gordon *et al.* 1983; Morisaki 1983). In this context, significant effects of microbial attachment on nutrient depletion have been reported (Doran and Bailey 1986, 1987; Galazzo *et al.* 1987)).

Mobility of attached cells would be less than unattached cells, and this may decrease the statistical probability of bacterial collision with substratum, and therefore could be a disadvantage for attached cells growing on soluble substrates (Fletcher 1980a,b).

1.2.5 Bacterial attachment within the rumen

Within the rumen, the possible substrata to which bacteria may adhere are the rumen epithelium, solid plant materials, or other organisms. Along with rumen bacterial ligands, the surface structures of these three components, which mostly carry a net negative charge, dictate the nature and mechanism of attachment within the rumen.

The epithelial cell surfaces are lined with polysaccharide and other materials that may be involved in a very specific mechanism of attachment determined by complementary molecular structures contributed by both the host and the microbial cells (Lee 1980). The outer surfaces of epithelial cells are composed of thousands of fine microvilli coated with a conspicuous layer of fine filaments radiating from the outer leaflet of the plasma membranes called the glycocalyx or the *mucus perimatrix*. This is rich in glycoproteins and polysaccharides (Ito 1965,

Bennett 1963, Swift and Makherjee 1976), as well as acid mucopolysaccharide (Arbuckle 1971, Hoskin and Zamcheck 1968). Observations of sections of intestinal surfaces by electron microscopy clearly showed thread-like structures between the epithelium and adsorbed bacteria, like those between the hydrophobic end of *Flexibacter* cells and araldite surfaces (Marshall and Cruickshank 1973). This adhesion is inhibited by periodate or proteolytic enzymes, (Fuller and Brooker 1974), Concanavalin A (Con A) (Fuller 1975, Sharon and Lis 1972), detergent treatment and heating (Lee 1980), and the addition of chondroitin sulfate A and gastric mucin (Suegara *et al*. 1975). These findings provide evidence for the involvement of mucopolysaccharides and/or glycoproteins with exposed glucose, mannose, fructose or arabinose moieties on the bacterial surface, in the specific attachment to epithelial cells in the gut (Lee 1980). In the colonization of lung tissue surfaces by *P. aeroginusa*, the preference for a specific strain of *P. aeroginusa* has been reported for rat lung tissue (Ceri *et al*. 1986) or human epithelial cell receptors (Doig *et al*. 1987).

The fact that different bacterial populations occur at different regions within the rumen or other animal tissues, and that this is due, at least in one part, to differences in surface structures, also provides evidence that epithelial cell surfaces are involved in determining the nature and the mechanism of attachment. Location specificities are also seen in various environments, such as in filamentous organisms in the mouse small bowel, where bacteria are found only in the lower duodenum or ileum and only attach to cells on the intervillous floor or the villus, but not deep in the intestinal crypts. Similarly, *spirochetes* in the monkey, cat, or guinea-pig large bowel only attach to the outer surface, not in the

crypts (Lee 1980). Differential reactivities of cell surfaces and secretory components have also been reported with various lectins, such as those of *Dolichos biflorus* (Etzler and Kabat 1970), wheat-germ agglutinin (Dazzo 1980), and *Ricirrus communis* (Nicholson and Blaustein 1972). Furthermore, sometimes there is even interspecies specificity, as seen in avian/rat *lactobacilli* which only attach to avian/rat epithelial cells (Fuller 1973, Suegara *et al.* 1975).

Adhesion of ruminal bacteria to solid plant material depends on the properties of the bacterial cell surfaces and the structurally complex substrates, which may consist of layers of lignin, hemicellulose and cellulose in plant cell walls or of protein coats surrounding starch grains. Complex structural barriers and resistance to digestion are often interposed between the microbes and their respective substrate. For successful access to preferred nutrient substrates, adhesive and degradative capacities must be used in combination by the rumen microorganisms in order to digest plant cell walls or protein coats. In addition, various enzyme activities are often required. For example, *Fibrobacter succinogenes* produces hemicellulase (xylanase) activity to digest complex cell walls and to expose the cellulose for digestion, and *S. bovis* expresses a protease to digest the starch grain protein coat and expose the readily fermentable starch. In addition, no single organism is capable of digesting all the available substrates within the rumen. Therefore a series of interrelated consortia that work together in fibre digestion is essential amongst the rumen microbial populations. Examples are the increased rate of cellulose digestion *in vitro* by *Treponema* and *Ruminococcus* species in coculture (Kudo *et al*. 1987), or the inhibition of cellulose digestion by ruminal fungi in the presence of *R. flavefaciens* (Bernalier *et al*. 1993).

Intimate contact between cell surfaces and insoluble substrates can bring cell-bound enzymes into contact with their substrates, ensuring effective digestion and the immediate availability of the degradation products to be used by the organism (Morris and Cole 1987). However the thickness of the EPS-protein glycocalyx secreted by each organism dictates the differences in spatial organisation. A cellulosome complex that comprises enzymic and non-enzymic moieties mediates the attachment of the highly cellulolytic non ruminal *Clostridia* to cellulose, whereas the equivalent process in ruminal *Clostridia* remains unknown. A complex EPS-protein matrix mediates starch digestion by amylolytic *S. bovis* (Bayer and Lamed 1986). The nature and mechanisms of each rumen microorganism's relationship with insoluble substrates varies from one strain to another. For example, *R. flavifaciens* adheres at a short distance from the cellulose fibril surface, whereas *F. succinogens* adheres very closely (Cheng *et al* . 1989).

The physicochemical properties of the substrate influences the affinity for cellulase (Lee *et al*. 1982). This enzyme-substrate system is heterogeneous, since native cellulosic materials are water-insoluble substrates, and several steps are involved in the hydrolysis reaction. One of the important parameters that governs the rate of enzymatic hydrolysis is the system of adsorption between the enzymes and substrates. For subsequent catalytic hydrolysis, direct physical contact or the adsorption of enzyme molecules on susceptible sites of the cellulose surface is a prerequisite (Lee *et al*. 1982). The distribution coefficient, the half-saturation and maximum adsorption constants are thought of as the adsorption parameters, which vary with the pretreatment and the source of cellulose, and are correlated with the

specific surface area (Lee *et al.* 1982) and the crystallinity (Ghose and Bisaria 1979) of the polysaccharides.

A specific binding reaction (adherence) will occur rapidly when cellulose is exposed to a cellulolytic microorganism and it can be competitively inhibited by specific functional group substitutions, as in methyl cellulose. This indicates that specific surface area modifications affect the adsorption parameters (Lee *et al.* 1982). The absence of a cellulose-cellulase adherence system will reduce or completely inhibit cellulose hydrolysis, as shown when adherence defective mutants of *F. succinogenes* were unable to digest cellulose, although the mutants retained their full complement of cellulase enzymes (β-1,4-endoglucanase, cellobiosidase, cellobiase). The breakdown did not even occur at elevated levels of cellulase, which again demonstrated the importance of adherence (Gong and Forsberg 1989).

R. albus numbers among the most predominant bacterial species involved in forage degradation in the reticulorumen of ruminants, and are known for their high cellulose binding capacity; the adhesion process may represent an important primary step for subsequent degradation of insoluble cellulosic substrates by *R. albus* (Kim *et al.* 1999, Miron *et al.* 2001, Morrison and Miron 2000). Complex mechanisms in the adhesion of *R. albus* SY3 to cellulose may involve glycocalyx exopolysaccharides, cellulosome-like structures, cell-surface glycanases/ glycoproteins, or fimbrial proteins (Miron *et al.* 2001).

The slime glycocalyx layer surrounding *R. albus* has been suggested to be involved in adhesion of these bacteria to cellulose (Cheng *et al.* 1977). This layer is composed of glycoproteins, and removal of glycocalyx carbohydrate by periodate oxidation significantly decreased adhesion of *R. albus* cells to cellulose (Pell and Schofield 1993), suggesting that surface carbohydrate components may also be involved in the adhesion of *R. albus* to cellulose (Pell and Schofield 1993). However, whether the glycocalyx exopolysaccharide moieties are directly involved in the adhesion process remains to be seen (Miron *et al.* 2001)

Electron microscopic observations (Miron *et al.* 1989, Kim *et al.* 1999) and comparative studies with adhesion-defective mutants (Miron *et al.* 1998, Reddy and Morrison 1998, Morrison and Miron 2000) revealed that protuberance-like structures were observed to be associated with cellulosomes on the surface of *R. albus*, similar to those in *Clostridium thermocellum* (Bayer and Lamed 1986) and other cellulolytic micro-organsims (Lamed *et al.* 1987), while adhesion-defective mutants lacked such structures on the surface.

Studies on the distribution of glycanase-related components in *R. albus* SY3 (Miron *et al.* 2001) revealed that most of the glycanases (cellulases and xylanases) were associated with capsular and cell-wall fractions, as those in *Fibrobacter succinogenes* (McGavin *et al.* 1990) and *F. intestinalis* (Miron and Forsberg 1998, 1999). Most of the bacterial enzyme activities were not integrated into cellulosome-like complexes, while in adhesion-defective mutants, the true cellulase activity appeared to be entirely confined to the cell membrane fractions, and overall glycanase activity was lower. Effective substrate degradation may be

facilitated by direct contact between the surface glycanases and the cellulosic substrate, And surface glycanases may directly or indirectly be involved in bacterial adhesion (Miron *et al.* 2001).

Cellulase activity of *R. albus* SY3 has been reported to be cell-associated, and appeared in an unstable high molecular mass 1500 kDa complex that could be disrupted by dissociating agents into discrete low molecular mass proteins (Wood *et al.* 1982). Phenylpropanoic acid or phenylacetic acid were reported as factors in the rumen fluid to stabilize *R. albus* 8 cellulases and to prevent dissociation of surface organelles (Stack and Hungate 1984; Pegden *et al.* 1998). In contrast, Miron *et al.* (2001) found that only a small portion of the cellulolytic and xylanolytic activity in *R. albus* SY3 envelopes and extracellular fluids were associated with cellulosome-like complexes. The glycanases may have been dissociated from a cellulosome-like complex during isolation of subcellular fractions, due to weak cohesin-dockerin affinity interaction. Genetic analysis, however, also support the presence of additional noncellulosomal glycanases, such as those of endoglucanases celA and celB of *R. albus* SY3, endoglucanases I, II, III and IV of *R. albus* F-40, as well as several xylanases; which lacked dockerin-like domains, and therefore would not be integrated as part of a cellulosome-like complex (Poole *et al.* 1990; Karita *et al.* 1997; Nagamine *et al.* 1997; White *et al.* 1997; Ohara *et al.* 2000). Whether the cellulosome-like complexes of the ruminococci contain a cellulose-binding domain on a scaffoldin subunit, or whether the molecular basis for delivering ruminococcal cellulosomes to the subunit involves an alternative mechanism(s), remains unknown (Miron *et al.* 2001).

Microscopic examination of *R. albus* strains suggested that fimbriae-like structures may be involved in cell adhesion to cellulose (Stack and Hungate 1984, Pegden *et al.* 1998, Kim *et al.* 1999). The gene sequence of one of the cellulose-bound proteins found in *R. albus* 8 (Pegden *et al.* 1998; Reddy and Morrison 1998; Larson *et al.* 1999), referred to as cellulose-binding protein type C (cbpC), belongs to the Pil family, and it was suggested that the binding of CbpC to cellulose may be mediated via a fimbrial-like mechanism. A number of other *R. albus* strains, including SY3, were also shown to possess the cbpC gene and fimbriae-like protein homologue(s) (Morrison and Miron 2000; Pegden *et al.* 1998). The finding that similarly-sized protein bands failed to react with adhesion-related antibodies suggests that the low molecular mass fimbrial proteins mediate cell adhesion of *R. albus* SY3 in a manner distinct from either a cellulosome-related adhesion mechanism or the involvement of cell-surface glycoproteins (Miron *et al.* 2001).

1.2.6 Attachment of *Butyrivibrio fibrisolvens*

Bauchop *et al*. (1975) have demonstrated the existence of a specific bacterial population associated with epithelial surfaces. The morphology and the density of the bacteria were found to vary with the sampling sites. The most dense bacteria were primarily rod-shaped cells, which covered the epithelium of the roof of the dorsal rumen. In estimating the magnitude of the adherent population in this region, Dehority and Grubb (1981) found that the very dense cover of rod-shaped bacteria is primarily comprised of the genera *Butyrivibrio* and *Prevotella*. Rasmussen *et al*. (1989) also reported the attachment of several *B. fibrisolvens* strains to cellulose substrates, although this was not necessarily followed by

cellulolysis. However, the type of attachment was not described in detail, ie. whether by an interaction between cell and cellulose surfaces or just simply adhesion interaction. Scanning Electron Microscopy (SEM) of several strains of *B. fibrisolvens* (Cheng *et al*. 1989) showed extracellular structures extending from the cell surface, which seemed to mediate cell-to-cell association. There has not been any other report concerning the attachment of *B. fibrisolvens* to surfaces. Thus the mechanism and control of attachment remains unclear.

1.2.7 Possible extracellular structures of *Butyrivibrio fibrisolvens* that may be involved in attachment

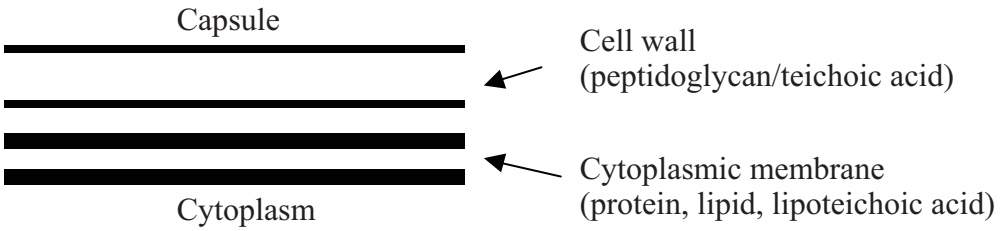
Amongst bacterial extracellular structures (flagella, fimbriae, extracellular polymer and attachment proteins), extracellular polymer and attachment proteins are the most likely ligands that may be involved in the attachment of *B. fibrisolvens* to surfaces. *B. fibrisolvens* variants S and L used in this project have no fimbriae or flagella. Therefore, only extracellular polymer and attachment proteins will be investigated in this study. Since no major differences were observed in the cytoplasmic protein profiles of S and L variants (Nili and Brooker 1995), the current studies will be more focused on the extracellular polymer.

The term extracellular polymer (EP) is often confused with extracellular polysaccharide (EPS). It is true that in many environments these two structures are almost equivalent. However, for Gram positive bacteria, which commonly produce other polymers such as teichoic acids, the terminology has become confused. Both EPS and teichoic acids have been reported to play roles in bacterial attachment in various environments. Usually the term bacterial

glycocalyx is used to describe the complex structure of the extracellular matrix. Many strains of *B. fibrisolvens* have been reported to produce both EPS and teichoic acids. It is so far unclear which of these two polymers, or both are involved in attachment. This will be discussed separately in the next section.

1.2.8 Teichoic acids

Teichoic acids are a group of polyolphosphate-containing polymers which are found in virtually all Gram positive bacteria (Duckworth 1977, Wicken 1980), and are divided into two broad classes depending on their cellular location, membrane and wall teichoic acids (Figure 1.3).



MEMBRANE TEICHOIC ACIDS
- Lipoteichoic Acids (LTA)

WALL TEICHOIC ACIDS
- Ribitol Teichoic Acids
- Glycerol Teichoic Acids
  - Polyol phosphate
  - Polymers in which glycosidically linked sugars are part of the chain
  - Glycerol polymers containing sugar 1-phosphate residues
- Polymers of Sugar 1-Phosphate Residues (Phosphorylated Polysaccharides)

Figure 1.3 Location and classification of teichoic acids (Duckworth 1977).

The membrane teichoic acid seem to be more important to the well-being of the cell than the wall teichoic acids, since membrane teichoic acids are more common, display a more uniform structure, and their presence is not so dependant on growth conditions (Duckworth 1977). Membrane teichoic acids (Figure 1.3

and 1.4A and B) are known as lipoteichoic acids (LTA). They always appear as polymers of glycerol phosphate units joined together by phosphodiester bonds and are covalently linked to lipid residues (Dukworth 1977, Wicken 1980). While wall teichoic acids are less common, they have considerable structural diversity and are subject to replacement by altered culturing conditions (Wicken and Knox 1975, Duckworth 1977). Wall teichoic acids display a full range of structural types containing glycerol phosphate, ribitol phosphate and sugar 1-phosphate (Figure 1.3 and 1.5), and are covalently linked to peptidoglycan (Duckworth 1977).

Lipoteichoic acids are not only located randomly around the cytoplasm membrane (Huff *et al.* 1974, Duckworth 1977). Their presence within the cell is also not static as seen in Figure 1.4C (Van Driel *et al.* 1973). At a later stage in their growth, the intracellular LTA are also exported to the cell wall and then released to the medium as acetylated (amphipathic and able to form micellar) or deacetylated extracellular LTA as can be seen in Figure 1.4D (Wicken 1980). LTA are found in either cell wall or extracellular fractions (Duckworth 1977, Wicken 1980).

Teichoic acids (Wall and LTA) and extracellular polysaccharides form very complex structures at the cell surface (Figure 1.6), and are difficult to separate or identify in terms of individual components. Usually in reports of extracellular polysaccharides, teichoic acids are regarded as being the non-carbohydrate fraction. However, the teichoic acids themselves also contain a carbohydrate fraction (Figure 1.4A, 1.4B and 1.5), which appears in carbohydrate or

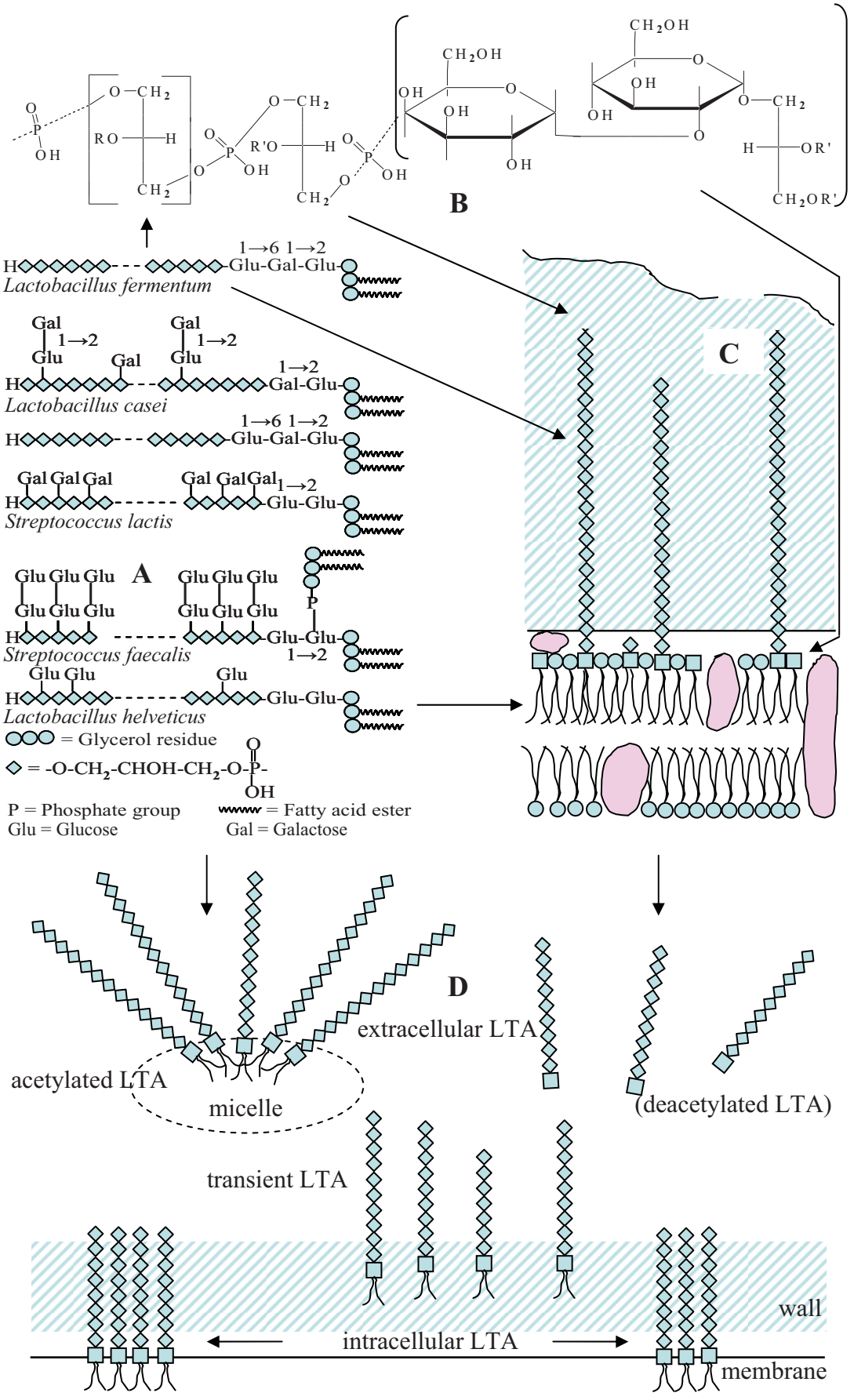
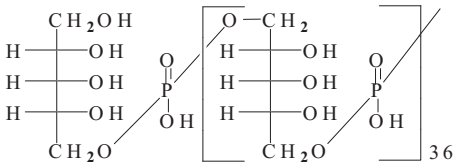


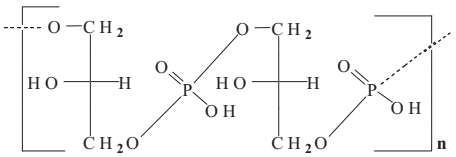
Figure 1.4 Structures (A & B), location (C) and biosynthesis (D) of LTA (Wicken and Knock 1975, Duckworth 1977, Wicken 1980).

A. RIBITOL TEICHOIC ACIDS

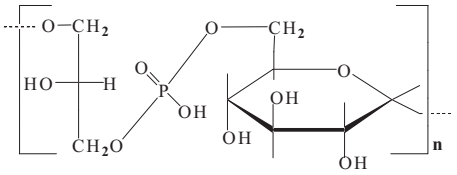


B. GLYCEROL TEICHOIC ACIDS

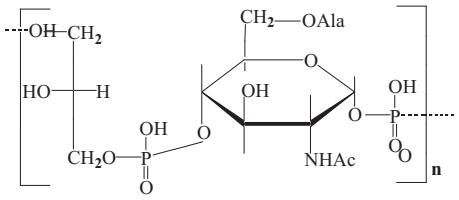
B.1. Polyol phosphate chains



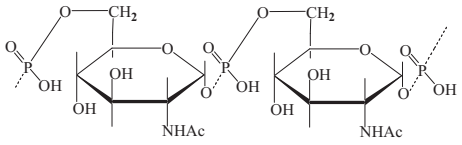
B.2. Polymers in which glycosidically linked sugars are part of the chain



B.3. Glycerol polymers containing sugar 1-phosphate residues



C. POLYMERS OF SUGAR 1-PHOSPHATE RESIDUES (PHOSPHORYLATED POLYSACCHARIDES)



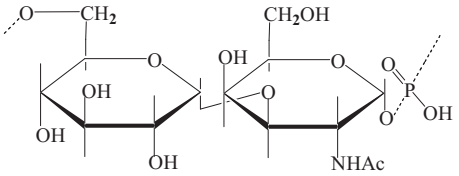


Figure 1.5 Structures of various wall teichoic acids (Duckworth 1977).

monosaccharide analysis of extracellular polysaccharides, and leads to biased results.

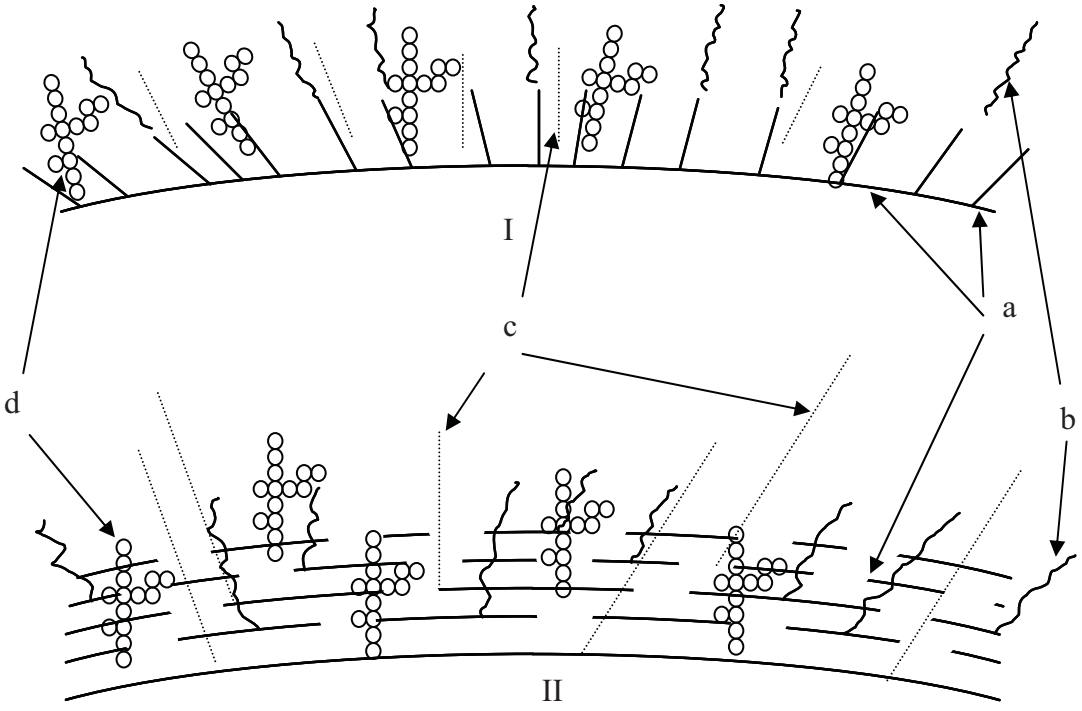


Figure 1.6 Extracellular polymers extended from cell surface (Duckworth 1977).

I and II are perpendicular and parallel arrangement of the glycan chains in bacterial cell walls. a, b, c and d are glycan, wall teichoic acids, membrane teichoic acids (LTA) and polysaccharides (EPS), respectively. For EPS producing bacteria, the extracellular polymer network will be very complex, EPS may associate with a, b or c. Other components (polymer-associated protein, metal and other secreted material) are not presented.

LTA also form closely associated complexes with both protein and polysaccharides. Proteins or contaminating nucleic acids can be easily removed using proteinases or nucleases, but it is difficult to separate the polysaccharides although various techniques such as organic solvent extraction or chromatography

(simple gel permeation, ion exchange, lectin- or hydrophobic affinity) have been applied (Wicken 1980).

LTA are considered more relevant to antigenic determinants (serology) or bacterial attachment to surfaces, and have been reported in various environments. These polymers have been used for classification in *Lactobacillus fermenti* and *Streptococcus faecalis*, based on the reactivity with specific antiserum to the polyglycerophosphate backbone of LTA (Wicken *et al.* 1963; Knox *et al.* 1970, Wicken and Knock 1970, 1971, 1975; Wicken 1980), and is commonly used to detect the presence of LTA produced by a microorganism. *B. fibrisolvens* have been reported to contain both wall and membrane teichoic acids (Sharpe *et al* . 1975, Markham *et al.* 1975, Hewett *et al*. 1976). However, the biological role of these polymers in *B. fibrisolvens* has been neglected. The most recent analysis of extracellular polymers in *B. fibrisolvens* stressed the importance of EPS but avoided the possibility that the examined strains may also produce teichoic acids, which are very difficult to separate during purification.

In attempting to classify *B. fibrisolvens* based on serological activity (reacting toward antiserum of phospho-glycerophosphate determinants), Sharpe *et al.* (1975) found that 33 out of 52 strains tested gave unexpectedly strong positive results. Further analysis showed that the LTA isolated from these organisms contained phosphorous, glycerol, hexose and ester-linked amino acids. Hewett *et al.* (1976) found that 3 strains (No's 37, IL6-3I and 49) produced LTA and 2 strains (D1 and 7A) did not. Two types of LTA were identified in the 3 positive strains, acetylated LTA which consisted of a conventional 1,3-phosphodiester–

linked chain of glycerol phosphate joined covalently to a glycolipid, and a non-micellar deacetylated LTA with lower molecular weight which had similar chemical constituents but lacked fatty acid esters. The presence of 2 forms of LTA (in bacterial extracts and in the external medium) have been reported for other bacteria (Markham *et al*. 1975, Wicken and Knox 1975, Joseph and Shockman 1975).

1.2.9 Extracellular polysaccharides (EPS)

Bacterial EPS are either hetero- or homopolysaccharides, generally composed of repeating structures, and are mostly anionic or negatively charged. However, neutral homopolysaccharides are also commonly found (Sutherland 1985). Heterogeneity in molecular size is found even in homopolysaccarides produced by either Gram positive or Gram negative bacteria (levan/homopolymer of fructose, glucans, dextran or bacterial cellulose). The molecular size of *Klebsiella aerogenes* EPS ranges between 10^5 - 10^6 daltons while the smallest EPS (glucans of *A*. *tumefaciens* and *Rhizobium* sp.) has a molecular size of 10^3 daltons (Sutherland 1982). The non-carbohydrate substituents, monosaccharide components, and linkage types are thought to be factors affecting structural diversity amongst bacterial EPS (Kenne and Linberg 1983), which varies with either the growth phase or the growth conditions used, and are frequently present in non-stoichiometric amounts (Sutherland 1982, Sherwood *et al*. 1984).

The ability of bacteria to produce EPS has been identified in a variety of ecological niches, and is considered to be a direct and logical response to selective pressure in the natural environment of the microorganism (Dudman 1977). Thus

the functions ascribed to EPS are often of a protective nature (Whitfield 1988). It has been reported that EPS plays protective roles against desiccation and predation by protozoans, and has significant effects on diffusion (both into and out of the cell) properties through the presence of a gelled polysaccharide layer around the cell (Dudman 1977). It also is reported to act as an antibacterial agent (Costerton *et al*. 1987). Furthermore, the penetration of both useful and toxic metal ions may be affected or controlled by anionic EPS binding or interactions (Dudman 1977). This has considerable practical importance in the corrosion of metallic surfaces (Costerton *et al*. 1987) or in the use of EPS as a metal chelating agent (Stack 1988).

The roles for EPS in cellular recognition have been observed in several situations such as the specificity of certain *Rhizobium* sp. for some leguminous plant groups with which they form symbiotic relationships. Plant lectins (polysaccharide binding proteins) may direct the establishment of the symbiotic association and its specificity for a particular *Rhizobium* and cell surface polysaccharides may determine which species can form such associations (Dudman 1977, Whitfield 1988). Even subtle changes in the non-carbohydrate substituents of EPS during the growth cycle (Sherwood *et al*. 1984), or mutant strains (Gardiol *et al*. 1987) can be recognized by lectins such as trifolin.

The roles of EPS in microbial adherence are seen in a variety of environments where survival depends on attachment to surfaces (Griffin *et al*. 1995). In some systems other cellular factors (eg. pili) may also be involved in the initial adhesive interaction. However, in many systems (*Hyphomonas*, *Pseudomonas*

solanacearum, *Vibrio* cholerae, *Haemophilus* influenzae, and others), EPS alone appears to be the primary adhesin and therefore EPS$^-$ variants do not attach to surfaces (Brooker *et al*. 1993, Quintero and Weiner 1995).

Most strains of *B. fibrisolvens* are reported to produce heteropolysaccharides having pseudoplastic (thickening) properties (Cheng *et al*. 1977, Stack 1988, Ha *et al*. 1991). The yield ranges from 2-4 mg/L to over 500 mg/L, but even for a given strain the yields are often variable (Stack *et al*. 1988a). This production can be influenced by nitrogen nutrition (Wachenheim and Patterson 1990), and further manipulation of cultural conditions may lead to enhancement for large scale production (Wachenheim and Patterson 1988, Ha *et al*. 1991). Several unusual sugars are found as common constituents, such as L-altrose (Stack 1987, Stack *et al*. 1988a), 4-O-(1-carboxyethyl)-D-galactose (Stack *et al*.1988b) and 4-O-(1-carboxyethyl)-L-rhamnose (Stack and Weisleder 1990). EPS remains relatively stable even after cell lysis and death, indicating that it has no function as a storage polysaccharide. Furthermore, L-monosaccharide incorporation into the EPS may have a protective role such as preventing recognition and subsequent hydrolysis by glycanase and glycosidase enzymes present in the GI tract (Stack and Ericson 1988). Further physical and chemical characterization has revealed that EPS is associated with replaceable mono- and divalent metal ions with different affinities, which may lead to its use as a commercial metal chelating agent (Ha *et al*. 1991). EPS also contains one or more acidic sugar components such as lactyl-galactose or 4-O-(-carboxyethyl)-D-galactose which may present as carboxylate groups capable of being disproportionately neutralized by basic metal ions, or as metal-carboxylate conjugates, or as free carboxylic acids (Ha *et al*.

1991). Disproportionality and viscosities are pH dependent. Therefore the degree of neutralization may reflect the viscosity differences among EPSs and their rheological properties may be controllable by pH adjustment (Ha *et al*. 1991).

EPS production, as well as the rheological, physical and chemical properties of a variety of *B. fibrisolvens* strains have been reported. However, it is still unclear how these bacteria adhere to surfaces, whether or not EPS is involved in the attachment, and what gene(s) are responsible for the biosynthesis of its EPS or attachment proteins. This was mainly due to difficulties in developing suitable vectors and a transformation system for this species. However, recent research has successfully developed cloning vectors and gene transfer systems capable of replication and expression in this species (Berger *et al*. 1989, 1990; Lin *et al*. 1990; Hazlewood *et al*.1990a,b; Rumbak *et al*. 1991; Ware *et al*. 1992; Whitehead 1992, Hefford *et al*. 1993; Brooker and Lum 1993; Brooker and Miller 1995). One such system (Beard *et al*. 1995) will be used in the current research presented in this thesis.

1.2.10 Extracellular polymer biosynthesis

Although EP compositions of various *B. fibrisolvens* strains (either as EPS or teichoic acids) have been reported, mechanisms of biosynthesis remain unknown. This section describes EP biosynthesis in various microorganisms for comparison.

1.2.10.1 Biosynthesis of teichoic acids

1.2.10.1.1 Biosynthesis of wall teichoic acids

In the synthesis of poly-ribitol phosphate in *S. aureus* or *Lactobacillus plantarum*, the ribitol phosphate units were transferred from CDP-ribitol to LTA onto which the ribitol phosphate chain was assembled, and the assembled poly-ribitol phosphate was then transferred from the glycerol phosphate chain of the acceptor to the glycan chains of peptidoglycan (Fiedler and Glaser 1974, Duckworth 1977). Some of the glycerol phosphate residues might be transferred to the glycan together with the poly-ribitol phosphate, since glycerol phosphate chains were present between the glycan and the ribitol teichoic acid. Since the precursor of this linkage unit (tri-glycerol phosphate) was CDP glycerol, it is also possible that the glycerol phosphate residues were added from CDP-glycerol, during total acceptor biosynthesis (Braca and Glaser 1972, Hancock and Baddiley 1972, Fiedler and Glasser 1974, Duckworth 1977). Similarly in the synthesis of poly-glycerol phosphate in *Bacillus licheniformis* and *B. subtilis*, except UDP-glycerol was used as substrate instead of UDP-ribose (Glaser and Burger 1964, Mauck and Glaser 1972, Duckworth 1977).

Sugar residue addition to the wall-glycerol or –ribitol teichoic acids has been known for sometime, *B. subtilis* enzyme can also transfer glucose residues from UDP-glucose to the free hydroxyl groups of a polymer to form α-D-glucopyranosyl-substituted-glycerol polymer (Glaser and Burger 1964, Duckworth 1977). Enzyme systems of various *S. aureus* strains were also able to incorporate N-acetylglucosamine from UDP-acetylglucosamine into poly-ribitol phosphate, and CDP-ribitol may serve as the acceptor, since the addition of CDP-

ribitol results in a much greater rate of N-acetylglucosamine incorporation (Mauck and Glaser 1972, Glaser and Burger 1964, Ishimoto and Strominger 1966, Duckworth 1977).

In the biosynthesis of poly-glucosyl glycerol phosphate in *Bacillus licheniformis*, glucose from UDP-glucose was transferred to a phospholipid (Hancock and Baddiley 1972), forming a glucose-phosphate-lipid complex. Glycerol phosphate from CDP-glycerol was then incorporated to form a glycerol-phosphate-glucose-phosphate-lipid, and the glycerol phosphate unit was then added to this growing polymer chain (Hancock and Baddiley 1972, Duckworth 1977). Similarly, in biosynthesis of *Staphylococci lactis* cell wall teichoic acid, N-acetylglucosamine l-phosphate residues from UDP-N-acetylglucosamine is transferred to a phospholipid (Hussey and Baddiley, 1972). The repeating unit of the polymer was assembled by transferring a glycerol phosphate residue from CDP-glycerol to a 4-hydroxyl group of N-acetylglucosamine. This repeating unit was then transferred to a growing teichoic acid chain, and the lipid phosphate was released. The lipids may participate as those in the biosynthesis of peptidoglycan, but the nature of the lipid moiety has not been directly established (Archibald 1974, Duckworth 1977). Phosphorylated polysaccharide in *S. lactis* is also synthesized by incorporating N-acetylglucosamine 1-phosphate (from UDP-N-acetylglucosa-mine) to a phospholipid intermediate and so onto the growing polysaccharide chain (Brooks and Baddiley 1969). The non-reducing end of the phosphorylated polysaccharide is extended in the same direction as in conventional teichoic acids (Brooks and Baddiley 1969, Duckworth 1977).

The synthesis of teichoic acid and peptidoglycan may be related (Mauck and Glaser 1972). In *S. lactis*, wall teichoic acid is linked to glycan chains, which are being synthesised at the same time as the teichoic acid is being produced. The peptidoglycan, to which teichoic acid was already linked, may serve as the export unit. However, these polymers may also be linked to pre-existing cell wall structures or linked to a cell wall disaccharide that is then attached to pre-existing cell wall material (Mauck and Glaser 1972, Duckworth 1977).

1.2.10.1.2 Biosynthesis of lipoteichoic acids

The wall teichoic acids (WTA) are synthesized at the membrane and therefore need a lipid anchor for their solubilisation, i.e. the intermediate is either an isoprenoid phosphate or a LTA carrier (Duckworth 1977). In contrast, the LTA has a lipid residue as part of their structure and therefore do not necessarily need a lipid residue for their solubilisation. In this case, the lipid end of the LTA should be synthesized first to provide an anchor point for the growing molecule within the membrane (Duckworth 1977). Therefore, the biosynthesis of the LTA and the WTA may be through completely different mechanisms. Unlike wall teichoic acids, LTA could not be biosynthesized from CDP-glycerol (Glaser and Lindsay 1974). Van Golde *et* al. (1973) observed that for phosphatidyl glycerol and cardiolipin of *E. coli*, both phosphate and glycerol from this lipid were incorporated into a group of related polysaccharides. These polymers possessed a polyglucose backbone substituted with equal amounts of glycerol and phosphate, and closely resembled the teichoic acids of Gram positive organisms. In LTA biosynthesis of *S. aureus* (Glaser and Lindsay 1974) and *S. sanguis* (Emdur and Chiu 1974), glycerol was transferred from the chloroform/methanol extractable

phosphatidyl glycerol to a water-soluble polymer, which was a lipoteichoic acid-like compound that may participate as the acceptor in WTA biosynthesis. LTA may be required as the precursors of the bridging regions between peptidoglycan and other wall polymers. However, the extent to which LTA normally occurs (approx. 2-3 % of the cell weight) and the fact that some WTA-lacking bacteria still have LTA, make it unlikely that this is their only function, but it is not clear whether all LTA fractions can act as acceptor or whether carrier molecules are different in location or structural respects (Duckworth 1977). Various functions of LTA have been reported, such as controlling the availability of cations at the membrane; protecting cell-autolysis; maintaining the repulsive charges on the surface of unicellular organisms; serological activity; or acting as a binding ligand in cell attachment to surfaces (Knox and Wicken, 1973, Wicken and Knox 1975, Duckworth 1977, Ofek and Beachey 1980, Sutherland 1985, Fletcher 1991, Griffin *et al*. 1995, Kranenburg *et al*. 1997).

1.2.10.2 Extracellular polysaccharides biosynthesis

Based on the site and the nature of the precursors, EPS biosynthesis is classified into two categories, synthesis outside the cell or at the cell membrane. However some exceptions (other systems) have also been reported (Whitfield 1988). Synthesis outside the cell, such as levans or dextrans of several Gram positive cocci are catalysed by a series of extracellular (glycosyl transferase) enzymes, which require sucrose or an alternative oligosaccharide as an activated precursor (Sutherland 1982, Whitfield 1988, Kranenburg *et al*. 1997). This process has been found to be complicated, as polymers are variable in branching degree and contain both $\alpha 1 \rightarrow 3$ and $\alpha 1 \rightarrow 6$ linkages (Whitfield 1988).

The essential process of synthesis at the cell membrane in Gram negative or Gram positive bacteria is shown in Figure 1.7 (Troy *et al*. 1979, Sutherland 1985, Whitfield 1988, Kranenburg *et al*. 1997). During precursor synthesis, nucleotide diphosphate or monophosphate sugars serve as activated glycosyl donors, and synthesis is catalysed by cytoplasmic or loosely-associated-cytoplasmic-membrane enzymes, allowing spatial organization with glycosyl transferase enzymes (Sutherland and Norval 1970).

Figure 1.7 Possible mechanisms of EPS biosynthesis and assembly (Whitfield 1988).

To assemble the EPS repeating units, sugars are sequentially transferred to lipid-linked intermediates. Some of the polymerisation may take place at the lipid intermediate level, but the precise role of lipid, in many cases, is unknown (Sutherland 1985, Whitfield 1988, Griffin *et al*. 1995, Kranenburg *et al*. 1997).

It has been suggested that the lipid is involved in facilitating the accuracy and ordered formation of the repeating unit structure, solubilization of hydrophilic oligosaccharides in a hydrophobic membrane domain, transport across the membrane, regulation of priorities in polysaccharide synthesis on the basis of lipid availability, or a combination of these events (Troy 1979, Sutherland 1982, Whitfield 1988). The lipid involved is undecaprenol phosphate lipid which is identical to the lipid carrier in peptidoglycan and lipopolysaccharide biosynthesis in a variety of bacteria (Troy *et al*. 1970, Troy 1979, Sutherland 1982, Whitfield 1988, Kranenburg *et al*. 1997).

During assembly and/or elongation of polysaccharide, nascent oligosaccharides are transferred directly from lipid intermediates (Troy and McCloskey 1979) to endogenous acceptors consisting of growing EPS chains in the cell wall (Smith *et al*. 1961, Troy 1979). The role played by active protein synthesis in the assembly of new acceptors has not been resolved. However, protein-synthesis inhibition also inhibits (both *in vitro* and *in vivo*) the initiation of synthesis of new acceptors, but not the elongation of pre-existing acceptors, suggesting that initiation and elongation reactions may be discrete processes (Whitfield *et al*. 1984a,b; Whitfield and Troy 1984; Whitfield 1988; Kranenburg *et al*. 1997). In the addition of phospholipids that anchor some EPS to the cell surface, is still not known whether the polysaccharide is assembled on the anchor molecule or whether this component is added after complete polymerisation (Troy 1979, Sutherland 1982, Whitfield 1988).

Although in the early stages, biosynthetic and assembly reactions may be similar in both Gram negative and Gram positive bacteria, the transfer of oligosaccharides to endogenous acceptors in Gram negative bacteria may be more complex, constraints imposed by cell wall structure may create biosynthetic problems; and extra steps such as periplasmic assembly and subsequent translocation may be required (Figure 1.7) (Whitfield 1988). Since polysaccharides accumulate in the periplasm of certain insertion mutants (Roberts *et al*. 1986, Boulnois *et al*. 1987, Silver *et al*. 1987).

EPS biosynthesis may be regulated at the level of the lipid intermediate (Sutherland 1982) or precursor availability by either feedback inhibition (Kornfield and Ginsburg 1966) or degradation (Vimr and Troy 1985). Although for the most part, this is still unclear (Whitfield 1988). Other regulatory elements acting on EPS biosynthesis have also been defined, such as Ca^{2+}- (Ross *et al*. 1987), self-proteolysis- (Torres-Cabassa and Gottesman 1987, Whitfield 1988), homologous recombinations- (Deretic *et al*. 1987, Hoiseth *et al*. 1986, Whitfield 1988), or temperature-sensitive-control (Ohman and Chakrabarty 1980, Goldberg and Ohman 1984).

Other systems of EPS biosynthesis, such as without involving lipid intermediates (Zaar 1979, Aloni 1983, Lin *et al*. 1985, Whitfield 1988) or involving extracellular modification (Pindar and Bucke 1975, Skjak-Braek and Larsen 1985), have also been reported.

1.2.11 Extracellular polymer-biosynthetic genes

The genes encoding EP biosynthesis may be encoded by plasmid, chromosome, or both. In *Lactococcus lactis*, EP biosynthesis is controlled by a plasmid-dependent gene cluster (Kranenburg *et* al. 1997), and in *E. coli* is clustered within the chromosome (Roberts *et al*. 1986, Boulnois *et al*. 1987), while in *R. melioti* is divided between the chromosome and plasmid (Leigh *et al*. 1985). The common features of EP biosynthetic genes are regulator, polymerase (chain length determination and biosynthesis of the oligosaccharide repeating units), export, and glycosyl transferase proteins (Kranenburg *et al*. 1997). The assembly of the repeating unit is usually catalysed by specific glycosyl transferase, and in many cases, is essential since a single gene disruption results in an EP-deficient mutant (Brown *et al*. 1992, Morona *et al*. 1994, Kranenburg *et al*. 1997).

EP biosynthetic genes may be arranged in a single or multiple clusters (Kranenburg *et al*. 1997). An example of a plasmid-dependent EP-gene cluster is that of *Lactococcus lactis*, a 12 kb region containing 14 genes, which is transcribed as a single polycistronic mRNA (Kranenburg *et al*. *1997*). Similar EP-gene clusters have also been reported from *Salmonella* (Jiang *et al*. 1991), *Shigella flexneri* (Morona *et al*. 1994), *Streptococci* (Rubens *et al*. 1993, Kolkman *et al*. 1996), and *Rhizobium meliloti* (Stingele *et al*. 1996). For further discussion on EP biosynthethic genes cluster can be found in Kranenburg *et al*. (1997).

In some cases, EP biosynthetic genes may also be arranged in multiple clusters, such as in *X. xampestris* (Harding *et al*. 1987, Thorne *et al*. 1987) and *Klebsiella* K20 (Whitfield 1988). The biosynthesis of *S. typhimurium* EP is also known to

involve either plasmid or chromosomal DNA (Markovittz 1977, Whitfield 1988), while alginate biosynthesis system in P. *aeruginosa* is controlled by at least two regions in the chromosomal DNA (Fyfe and Govan 1980).

1.2.12 Genetic manipulation in *Butyrivibrio fibrisolvens*

Increased productivity in the livestock industries could be obtained through improvements in the nutrition and health of ruminant animals. The benefits to animal production that might result from genetic manipulation of rumen bacteria have drawn considerable attention (Teather 1985, Forsberg *et al*. 1986, Gregg *et al*. 1987, Orpin *et al*. 1988, Russell and Wilson 1988, Brooker and Miller 1995). However, progress has been somewhat slow because of a lack of the basic tools, such as stable vectors and efficient DNA delivery systems. Amongst rumen bacterial species, *B. fibrisolvens* (Ware *et al*. 1992, Whitehead 1992, Beard *et al*. 1995) and *Prevotella ruminicola* (Klieve *et al*. 1994) are the most frequently considered as recipients of new genetic material. Although the development of vectors for *P. ruminicola* is showing great promise, progress has been much slower for *B. fibrisolvens* because there is a shortage of genetic information available on this species (Beard *et al*. 1995).

Various genes of *B. fibrisolvens* have been successfully cloned in *Escherichia coli*, including those genes encoding xylosidase (Sewell *et al*. 1989), β-glucanase (Romaniec *et al*. 1987a; Mann 1986; Berger *et al*. 1989; Berger *et al*. 1990; Hazlewood *et al*. 1990a,b), xylanase (Mannarelli *et al*. 1990a), xylosidase and arabinosidase (Utt *et al*. 1991), α-amylase (Rumbak *et al*. 1991), branching enzymes (Rumbak *et al*. 1991) and cinnamoyl ester hydrolase (Dalrymple *et al*.

1996). However, there has been only limited success reported in the transfer of DNA into *B. fibrisolvens*. Examples are the transfer of the broad-host range plasmids RP4 from *E. coli* (by conjugation, imparting ampicillin resistance to the recipient strain) (Teather 1985) and pRK248 (by PEG treatment of spheroplasts) (Hazlewood and Teather 1988). However these studies were not pursued because of plasmid instability. Electroporation of *B. fibrisolvens* with recombinant plasmids and measurements of plasmid stability have demonstrated plasmid maintainance over a number of generations (Ware *et al* 1992), but the lack of a suitable selectable marker in the plasmid (a cryptic plasmid pBf1 from *B. fibrisolvens* AR10) has made it difficult to employ it as a practical DNA transfer vector. Transformation of *B. fibrisolvens* by conjugation using the *Enterococcus faecalis* plasmid pAMβ1 has been demonstrated by Hespell and Whitehead (1991), but the large size of pAMβ1 (26.5 kb) makes it difficult to manipulate as a transformation vector. Although the feasibility of the system has been successfully demonstrated by electroporating an *E. coli/B. subtilis* shuttle vector into *B. fibrisolvens* (Whitehead 1992), the transformation efficiency was low (25 transformants/μg DNA), and the use of this vector to transfer additional DNA has yet to be reported. However, since most *B. fibrisolvens* strains produce extracellular polysaccharide with various degrees of viscosity (Stack 1988, Ha *et al*. 1991), it has been suggested that the higher viscosity exopolysaccharides produced by some strains would interfere with their ability to be transformed using electroporation. Therefore, electroporation protocols developed for one strain may not necessarily be appropriate for other strains of *B. fibrisolvens* (Clark *et al*. 1994).

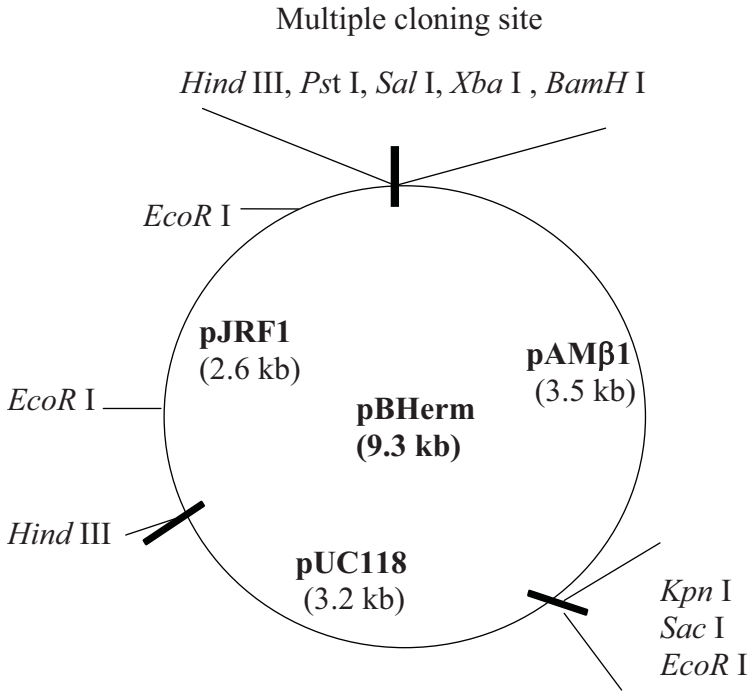


Figure 1.8 Map of pBHerm plasmid (Beard *et al.* 1995).

To minimize problems of instability that are often associated with plasmids derived from other species, effort is aimed at using a well-characterized plasmid that is native to *B. fibrisolvens*. The cloning and complete sequencing of a small native plasmid from strain OB156 (Hefford *et al*. 1993) provided the information needed to construct a shuttle vector without disturbing genetic elements of the plasmid that might be important for replication and stability. Recently, Beard *et al*. (1995) constructed an *Escherichia coli/B. fibrisolvens* shuttle vector pBHerm of 9.3 kb (Figure 1.8) capable of replication and selection in both *E. coli* and *B. fibrisolvens*. The replication functions and ampicillin resistance in *E. coli* were derived from plasmid pUC118. The well-characterized native plasmid pJRF1 from strain OB156 (Hefford *et al*. 1993) and a 3.5 kb fragment of plasmid pAMβ1

containing erythromycin resistance (Whitehead and Hespell 1991) controlled the replication functions and erythromycin resistance in *B. fibrisolvens*, respectively.

The features of pBHerm seem to provide a promising method as a tool for the study of the molecular genetics of this species. The transformation efficiency of *B. fibrisolvens* cells using pBHerm was 10^4/μg DNA (when pBHerm was isolated from *B. fibrisolvens*) and 10^2/μg DNA (when pBHerm was isolated from *E. coli*) (Beard *et al*. 1995). However, although pBHerm has been shown to be a practical tool for making genetic alterations to the host organism (Gregg *et al*. 1994), and the size of pBHerm is relatively small (9.3 kb) compared with other plasmids described for genetic studies on *B. fibrisolvens*, it is still relatively large compared to more commonly used *E. coli* vectors such as pUC18, and therefore may result in lower transformation efficieny.

1.2.13 Aims

The aims of the present study are:

- To study the growth behaviour of S and L variants and the stability of their phenotype, and to investigate the differences in their extracellular protein and genetic profiles (chromosomal DNA, plasmid content and phylogeny). The effect of carbon sources on protein profiles and the presence of molecule(s) that may mediate attachment of the S variant or inhibit attachment of the L variant will also be explored.
- To study and compare the effects of attachment (S and L variants) on nutrient utilization and enzymatic activities toward carbon sources commonly found in plant polysaccharides.

- To study the differences in morphology of S and L variants at various stages of growth as well as any correlation between morphology and carbon source used for growth.
- To compare the level of EP production between S and L variants, as well as the composition (monosaccharide and fatty acid content) of the EP produced by the two variants. The effect of various carbon sources on the level of EP production and composition will also be elucidated.
- To conduct a preliminary study aimed at cloning *B. fibrisolvens* gene(s) involved in attachment.

CHAPTER 2

MATERIALS AND METHODS

All molecular biological techniques were as described in either Current Protocols in Molecular Biology (Ausubel *et al* 1989) or Molecular Cloning: A Laboratory Manual (Sambrook *et al* 1989), unless otherwise indicated. The recipes for solutions, buffers and medium used are included in Appendix C.

2.1 General materials

2.1.1 Strains

The following bacterial strains were used in this project:

- *Butyrivibrio fibrisolvens* E14 variants sticky (S) and loose (L); and *Butyrivibrio fibrisolvens* H17c were obtained from Dr. J. D. Brooker's Lab., Department of Animal Science, University of Adelaide.
- *Escherichia coli* K-12 ED8299 (ampicillin sensitive) lacZΔM15 was obtained from Dr. J. Rood, Monash University, Melbourne, Australia
- *E. coli* K-12 DB11 (erythromycin sensitive) was obtained from Dr. B. White, University of Illinois, USA

2.1.2 Plasmids

Plasmid pUC19 used in this project was obtained from Dr. J. D. Brooker's laboratory, while plasmid pBHerm was obtained from Dr. R. M. Teather, Centre

for Food and Animal Research, Agricultural and Agri-food, Canada, Ottawa, Ontario.

2.1.3 Chemicals

Casamino acids and Brain Heart Infusion (BHI) were purchased from Difco Laboratories, Detroit, USA. Tryptone and agar were prepared from Oxoid Ltd., Basingstoke, Hampshire, England. Resazurin, acetic acid, propionic acid, butyric acid, isobutyric acid, *n*-valeric acid, isovaleric acid and DL-α-methylbutyric acid were obtained from Aldrich Chemical Company, Milwaukee, USA. Vitamins (biotin, riboflavin, folic acid, lipoic acid, nicotinamide, thiamin hydrochloride, pyridoxamine and calcium pantothenate), hemin (bovine) and L-cysteine were obtained from Sigma, Chemical Company St. Louis, USA.

Sodium dodecyl sulphate (SDS), tris (hydroxymethyl) aminoethane, acrylamide and N,N'-methylenebisacrylamide were obtained from Sigma Chemical, Company, St. Louis, USA. Bromophenol blue was from Ajax Chemical, Sydney, Australia. Coomassie Brilliant Blue G-250 and N,N,N',N'-tetrametylethylenediamine (TEMED) were obtained from Bio-Rad Laboratories, Richmond, CA., USA.

Paraformaldehyde was purchased from Sigma Chemical Company, USA. Glutaraldehyde, paraformaldehyde, osmium tetroxide and Spurr's resin (obtained as kit) were purchased from Probing Structure, Queensland, Australia.

Standard monosaccharides for GC analysis were obtained from Matreya, Inc., California, USA, catalog no. 1124 (rhamnose, fucose, ribose and arabinose) and no. 1125 (mannose, galactose, glucose and inositol). The internal standard inositol was obtained from Sigma. The internal standard (C19) for fatty acid analyses was kindly provided by Dr. Brian Siebert, Animal Science Department, University of Adelaide.

All other chemicals, of the highest purity available, were obtained from the following sources: Ajax Chemicals, Sydney, Australia; B.D.H. Chemicals Australia Ltd., Boronia and Kilsyth, Australia.

2.2 Methods

2.2.1 Working cultures

Stocks of *B. fibrisolvens* variants S and L were stored at -80^{o}C in 20 % glycerol and experiments were always inoculated from glycerol stocks.

2.2.2 Culture preparations

Routine culture preparations (Figure 2.1) were always used for all experiments involving S and L cultures, unless otherwise indicated. Starting culture inoculation in liquid medium was 1 % v/v, unless specified.

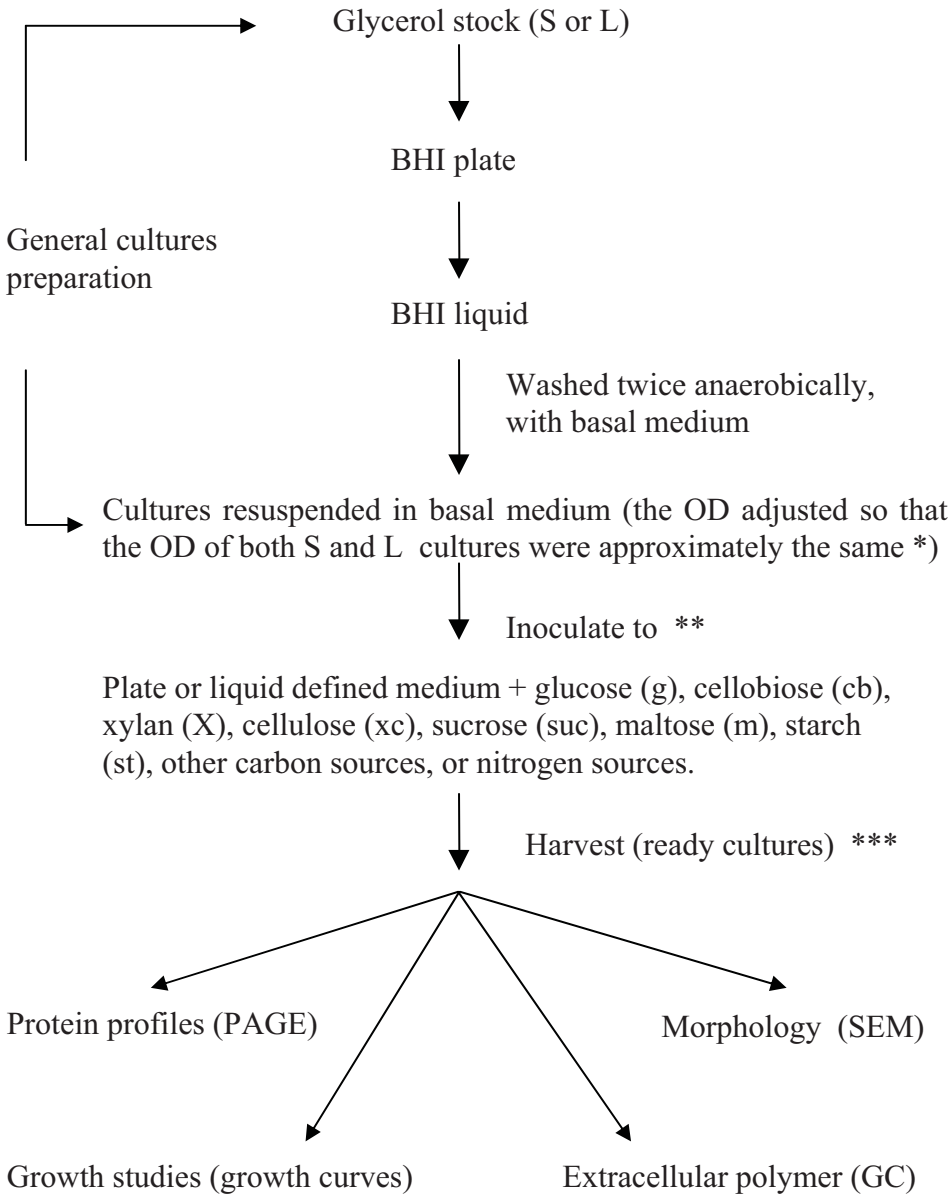


Figure 2.1 General procedures for culture preparations.

* Usually the OD of S was slightly higher than L. To obtain the same OD between S and L for the starting cultures, after washing L was resuspended in basal medium to its original volume (10 ml), while S was resuspended in (OD S/OD L x 10) ml medium.

** The starting cultures were 1 % (v/v) for broth, and ± 5µl of diluted (1000 x) for plates, unless specified.

*** These cultures are ready for sample preparation. Cultures preparation that were used in all in experiments (growth studies, protein profiles, morphological examinations, studies of extracellular polymer production and composition) were obtained though this procedures, unless specified.

2.2.3 Medium

All ingredients and preparations for BHI and defined medium (DF) were done as described by Nili and Brooker (1995), except for the carbon sources. LB medium (broth and agar), was prepared according to Sambrook *et al*. (1989). Addition of antibiotics (ampicillin or erythromycin) for plate or broth aerobic and plate anaerobic selective medium were as described in Sambrook *et al* (1989). For broth anaerobic medium; the antibiotic stock solution was left 4 hours inside the anaerobic chamber for oxygen exchange and filter sterilized inside the anaerobic chamber before use.

2.2.4 Carbon sources

Unless otherwise indicated, all carbon sources used in the medium preparation were 0.5 % w/v (final concentration), and were filter sterilised and added separately, except for xylan, cellulose and starch, which were sterilised together with other medium components. Sg, Scb, Sx, Sxc, Ssuc, Sm, Sst are abbreviations used for S cultures (or samples isolated from these cultures) grown on glucose, cellobiose, xylan, crystalline cellulose, sucrose, maltose and starch, respectively. Similar abbreviations applied to L cultures.

2.2.5 Preparation of anaerobic medium

For broth medium, all components were mixed in a glass Schott bottle, adjusted to the correct pH and boiled in a microwave oven (5 x 20 seconds). The bottle was then capped and placed inside a Coy anaerobic chamber with an internal atmosphere of 95 % v/v CO_2/5 % v/v H_2 and maintained with a palladium catalyst and circulator unit. The medium was left inside the hood for a period of at least 4

hours during which time gas exchange occurred and the medium became completely anaerobic. Aliquots were added to 15 ml Hungate tubes or to another bottle which had been stored anaerobically at least overnight. The tubes or Schott bottles were then capped, removed from the anaerobic chamber and sterilised by autoclaving for 15 minutes at 121^{o}C and 15 psi. For solid (plate) medium, 1.5 % agar (w/v) was added to the mixture at the correct pH, autoclaved as above and poured in plates in a laminar-flow cabinet once the medium had cooled down to approximately 45^{o}C. The agar plates were then transferred to the Coy anaerobic chamber and left overnight to allow the medium to become anaerobic.

2.2.6 Protein determination

Protein concentration was determined using the Bradford method (1976) and BSA as a standard.

2.2.7 Estimation of DNA concentration

DNA concentration and purity were estimated by the absorbance of samples at 260 nm and 280 nm against an reverse osmosis H_2O blank as described in Sambrook *et al.* 1989), using a Shimadzu UV-160A spectrophotometer with 1.0 cm path length and quartz glass cuvettes. One absorbance of samples at 260 nm = 50 µg/mL. A pure sample of DNA has an OD 260:280 ratio of 1.8.

2.2.8 Phenol-chloroform extraction

A half volume of TE buffer (25 mM Tris HCl, 10 mM EDTA, pH 7.8) saturated phenol (Sambrook *et al.* 1989) was added to a DNA sample dissolved in TE or

water, followed by addition of a half volume of chloroform. After thorough mixing and centrifugation (10,000 *xg*, 5min), the upper aqueous layer was transferred to another tube. The extraction of the aqueous layer was repeated until the interface was clear and it was then extracted twice with an equal volume of chloroform.

2.2.9 Ethanol precipitation

Two and a half volumes of chilled absolute ethanol and 0.1 volumes of 3 M sodium acetate pH 4.8 were added to the nucleic acid solution, and, after gently mixing by inverting the tube several times, the solution was placed at –20^{o}C for at least 2 hours. The nucleic acid was recovered by centrifugation at 10,000-15,000 *xg* for 10 minutes at 4^{o}C. After removing the supernatant, the nucleic acid pellet was washed twice with 70 % ethanol, and dried in a Speedivac for 1-5 minutes.

2.2.10 Plasmid isolation from *E. coli*

Plasmid (pBHerm) isolation from *E. coli* was carried out using the alkaline lysis method as described in Sambrook *et al.* (1989) as modified by Beard et al. (1995). 10 ml of *E. coli* (containing pBHerm and derivatives) were centrifuged at 8,000 *xg*, at 4^{o}C, for 10 minutes. After discarding the supernatant, the cell pellet was washed in 2 x 5 ml of STE (10mM Tris.HCl, 20 % w/v sucrose, 1mM EDTA, pH 8.0) buffer, with centrifugation in between as above. The cell pellet was resuspended in 500 µl of solution I (STE buffer containing 5 mg/ml of lysozyme), transferred to Eppendorf tubes (100 µl each) and incubated at 37^{o}C for 10 minutes. 200 µl of solution II (containing 0.2 M NaOH freshly diluted from

a 10 M stock and 1 % SDS) was added to each sample. The sample was mixed at RT for approximately 5-10 minutes. 150 µl of ice-cold solution III (3 M potassium acetate, 11.5 % v/v glacial acetic acid) were added to each sample and the mixture was held on ice for 15 minutes. It was then centrifuged at 10.000 *xg*, at 4^{o}C for 15 minutes and the supernatant was transferred to another eppendorf tube, phenol-chloroform extracted (section 2.2.8), ethanol precipitated (section 2.2.9), and dried under vacuum for 2 minutes. Plasmid DNA was dissolved in 500 µl of TE buffer pH 7.4, and any RNA was degraded using 1µl of 20 µg/ml DNAase-free RNAase (Boehringer Mannheim) incubated at 37^{o}C for 8 minutes. The sample was then phenol extracted once, ethanol precipitated, and dried under vacuum for 2 minutes. The dried sample was kept at 4^{o}C until used. Usually from 10 ml of culture, the sample was dissolved in 25 µl water, the concentration (A260 nm) and the purity (A260 nm/A280 nm) was determined by spectroscopy as described in section 2.2.7, and the DNA was analysed by agarose gel electrophoresis (section 2.2.13).

2.2.11 Plasmid isolation from *B. fibrisolvens*

Plasmid isolation from *B. fibrisolvens* was carried out as in section 2.2.10, except cells were washed 3 times with CE buffer (50 mM Na_2CO_3, 25 mM EDTA, pH 10) to remove the extracellular polymer before washing with STE buffer, and longer incubation time with lysozyme was used (30 minutes). Usually from 10 ml of S or L culture, the plasmid DNA was dissolved in 20 µl of water.

2.2.12 Chromosomal DNA isolation from *B. fibrisolvens*

Chromosomal DNA was prepared by alkaline lysis as described in Ausubel *et al.* (1989). The cells for chromosomal DNA preparation were obtained from an overnight culture of *B. fibrisolvens* (variant S or L) in defined medium containing cellobiose. 10 ml of S or L culture were centrifuged at 8,000 *xg*, at 4^{o}C for 10 minutes. After discarding the supernatant, the cell pellet was washed in 3 x 10 ml of CE (to remove the extracellular polymer), followed by 2 x 5 ml of STE buffer, with centrifugation in between as above. The cell pellet was resuspended in 900 µl of STE buffer containing lysozyme (5mg/ml) and incubated at 37^{o}C for 2 hours. SDS to a final concentration of 2 % w/v and proteinase K (final concentration 300 µg/ml) were added to the cell suspension and it was further incubated at 37^{o}C for 3 hour or until the lysate became clear.

The cell lysate was divided into two (500 µl each), phenol/chloroform extracted (section 2.2.8, usually at least 5 times), and ethanol precipitated (section 2.2.9). The DNA was dissolved in an appropriate volume (500 µl) of TE buffer pH 7.4, and the RNA was degraded using 1µl of 20 µg/ml DNase-free RNase and incubated at 37^{o}C for 8 minutes. The sample was then phenol extracted once, followed by ethanol precipitation, it was then dried under vacuum for 2 minutes. The dried sample was kept at 4^{o}C until further use. Usually from 10 ml of culture, the chromosomal DNA sample was dissolved in 25 µl of water, and the concentration (A260 nm) and the purity (A260 nm/A280 nm) was determined by spectroscopy as described in section 2.2.7 and agarose gel electrophoresis (section 2.2.13)

2.2.13 Agarose gel electrophoresis

DNA samples were mixed with one-sixth volume of 6x gel loading buffer (30 % Glycerol, 0.25 % Xylene cyanol, 0.25 % Bromophenol Blue) and loaded onto a 0.8 % w/v agarose gel. Electrophoresis was at 7.5 v/cm in 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) buffer until the bromophenol blue reached about the end of the gel. After electrophoresis, the gel was stained in 0.5 μg/ml ethidium bromide for 15 minutes, and either viewed on a transilluminator (UV wavelength: 300 nm) and photographed or viewed using a BioRad gel documentation system.

2.2.14 Purification of DNA from agarose gel

The DNA sample excised from an agarose gel, was put into a small plastic bag and snap frozen in liquid nitrogen. The gel was squeezed as it thawed (Wu and Welsh 1995), the expressed solution was then transferred onto a clean tube and was purified by phenol/chloroform extraction followed by ethanol precipitation.

2.2.15 Restriction digestion (Sambrook *et al.* 1989, Ausubel *et al*. 1989)

Approximately 2 μg of DNA sample was restriction digested (Boehringer Manheim) (2 units/μg DNA) in a total volume of 20 μl. The reaction mixture was incubated at the appropriate temperature as described by the manufacture, usually 3 hours for complete digestion of plasmid or 16S rDNA, and overnight for chromosomal DNA. Partial digestion of chromosomal DNA was carried out for 1, 2 and 3 hours). The reaction was stopped by heating (90^{o}C, 30 seconds) followed by phenol-chloroform extraction (section 2.2.8), ethanol precipitation (2.2.9), and drying under vacuum for 2 minutes. The dried sample was kept at 4^{o}C. The

sample was dissolved in water and the concentration was determined spectrophotometrically (section 2.2.7), prior to use.

2.2.16 Southern transfer

Following electrophoresis of restriction enzyme digested DNA, the agarose gel (section 2.2.13) was soaked in 0.25 M HCl solution for 15 minutes to partially depurinate the DNA, then washed with 0.5 M NaCl solution twice for 30 and 5 minutes, respectively. After removing the NaCl solution, the DNA was transferred to Hybond^{TM}-N+ nylon membrane (Amersham) by capillary transfer as described by Southern (1975) for at least 6 hours in 20 x SSC (3 M NaCl, 0.3 M sodium citrate) and finally UV crosslinked to the membrane using a Stratagene UV1800 transilluminator (254 nm) for 2 minutes.

2.2.17 Prehybridisation and hybridisation

The DNA bound membrane (section 2.2.15) was incubated with prehybridization (0.01 % w/v calf thymus DNA, 0.2 % w/v SDS, 10 % w/v skim milk powder, 5 x SSC) solution for at least 6 hours at 65^{o}C in a Hybaid glass hybridisation bottle. The prehybridisation solution was then replaced with hybridisation solution (4 % v/v polyethylene glycol, 1 % w/v skim milk powder, 5 x SSC) containing denatured ^{32}P-dCTP-labeled DNA probe (section 2.2.15) followed by incubation for at least 12 hours at 65^{o}C. The membrane was then washed with gentle agitation in a solution containing 2 x SSC and 0.5 % SDS at 42^{o}C for 30 minutes, 2 x SSC and 0.1 % SDS at 42^{o}C for 15 minutes, and 2 x SSC and 0.5 % SDS at 65^{o}C for 15 minutes. After drying in air, the membrane was covered in Glad Wrap

and placed in an autoradiograph cassette with an intensifying screen for autoradiography (section 2.2.17). Autoradiography is described in section 2.2.19.

2.2.18 Oligo-labelling of a DNA probe

Five µl of primer (Megaprime labelling primer, Amersham) solution was added to 10 µl of (25-100ng) DNA solution to be labelled, and the volume was brought to 37 µl by adding 22 µl of distilled water. The probe was denatured by boiling for 5 minutes and allowed to cool on ice. After a brief centrifugation at room temperature, 10µl of labelling buffer (Megaprime labelling buffer, Amersham), 1µl of α-^{32}P dCTP and 2 µl of enzyme (1unit/µl, Klenow fragment, Amersham) were added. The reagents were mixed gently and incubated at 37^{o}C for 30 minutes. The reaction was stopped by adding 5µl of 0.2M EDTA. The labelled probe was denatured by boiling for 5 minutes and cooled on ice before it was added to the hybridisation solution.

2.2.19 Autoradiography

X-ray film (Fuji medical X-ray film, Fuji photo Co., Ltd., Tokyo, Japan) was placed on top of the membrane and exposed at -70^{o}C for an appropriate period. The exposed X-ray film was developed by subsequently placing it in (a:) developer (Phenisol X-ray developer, Ilford Aust. Pty., Ltd. Victoria) for at least 2.5 minutes, fixer (Hypam X-ray Rapid fixer, Ilford Aust. Pty, Victoria) for approximately 2.5 minutes, and distilled water for approximately 2.5 minutes. The film was then allowed to drip dry in air.

2.2.20 Stripping of membranes

A boiling solution of 0.5 % w/v SDS was poured over the membrane and allowed to cool at room temperature according to the manufacture (Amersham). The above procedure was repeated until the level of radiation fell to zero as determined by hand-held mini-monitor (Neomedix Systems).

2.2.21 Amplification of 16S rDNA

The ingredients of the PCR mixture were: 50 ng of DNA sample; 5 µl of 10 x PCR buffer, 0.4 mM of dNTP; 30 pmols of universal forward primer FD1 (AAGCTTGGATCCGGTTACCTTGTTACGACTT) and reverse primer, Rp2, (GAATTCGTCGACAGAGTTTGATCCTGGCTCAG); 1.5 mM of $MgCl_2$; 2.5 unit of Taq polymerase (Gibco) and water to make to 50 µl. Reactions were overlaid with light mineral oil (Sigma), and were incubated in a DNA Cetus Thermalcycler (Perkin Elmer) under the following conditions: 94^{o}C (4 minutes), 1 cycle; 94^{o}C (5 minutes), 57^{o}C (2 minutes), 72^{o}C (2 minutes), 1 cycle; 94^{o}C (5 minutes), 57^{o}C (2 minutes), 72^{o}C (2 minutes), 30 cycles; 72^{o}C (2 minutes), 1 cycle; and 4^{o}C (held indefinitely). The PCR products were then pipetted away from the mineral oil, placed in clean microcentrifuge tubes, ethanol precipitated and dried. Samples were then stored at -20^{o}C until required. PCR samples after digestion with restriction enzymes, were analysed by polyacrylamide gel electrophoresis and stained using a DNA Silver Staining Kit (Pharmacia Biotech, code no. 17-6000-30) as described by the manufacture or by agarose gel electrophoresis (section 2.2.13 with 1.2 % agarose, 85 volts, for 90 minutes). The

DNA size standard was Amplisize 50-2,000 bp ladder (BioRad, catalog no. 170-8200).

2.2.22 Determination of dry cell weight

A known volume of bacterial cell suspension was centrifuged and the pellet was dried under vacuum at 60^{o}C then weighed. For cells grown in liquid medium with insoluble substrates such as crystalline cellulose, a known volume of suspension (cell and remaining cellulose) was filtered using a preweighed filter paper. It was then dried at 101^{o}C for at least 3 hours to obtain was constant weight. Cells were lyzed by adding 5 ml of formic acid. Five 5 ml of water was then added to wash the remaining formic acid. The sample was then dried as above and weighed to obtain the weight of paper + cellulose. The weight of the remaining cellulose as well as the cell weight could then be calculated. Since the acid treatment may also partially degrade the paper, the cell weight was corrected using a control in which an appropriate amount of cellulose suspension from uninoculated medium was treated similarly to the samples.

2.2.23 Growth characteristics of variants grown on plates

To examine the behaviour (growth ability, stickiness and colony shape and colour) of S & L variants on solid medium, cultures obtained through general culture preparation (section 2.2.2) were streaked onto BHI plates and defined medium containing various carbon sources, such as glucose, cellobiose, xylan (oat spelt, washed oat spelt or beechwood), carboxymethyl cellulose (CMC), crystalline cellulose (size 20, 50 or 100), fibrous cellulose, sucrose, fructose, lactose, galactose, maltose or soluble starch. Cultures were incubated

anaerobically in hood at 39^{o}C overnight (except for cellulose, where the incubation was carried out for at least 36-48 hours), and the colonies were examined.

2.2.24 Variant stability

The stability of the variants was examined by growing the cultures sequentially for up to 10 passages in liquid medium and on plates containing glucose (DFg) and the characteristic behaviour of each variant (stickiness, colony shape and colour in solid medium, and cell clumps in liquid medium) was examined to determine whether any changes had occurred during sequential inoculation. S or L variants were streaked onto plate DFg medium and incubated anaerobically at 39^{0}C for 16-24 hours. Single colonies were transferred from plates into liquid DFg medium and incubated similarly. For the first passage, the S or L liquid grown culture was streaked on plate or inoculated in liquid DFg and incubated as above. For the second passage, the liquid grown culture of the first passage was then inoculated to plate or liquid DFg medium as above. This procedure was repeated until the tenth passage.

2.2.25 Cell to cell communication

The S variant was streaked onto a DFg plate and incubated anaerobically at 39^{0}C for 16-24 hours. A single colony was transferred from plate into liquid DFg medium and incubated similarly, except the length of incubation was carried out until the OD of the culture reach 0.3 (early log phase) and approximately 1.0 (early stationary phase). The liquid grown cultures (OD 0.3 and <1.0) were centrifuged (8,000 *xg*, 15 minutes) and filter sterilized using a 0.21 μm Millipore

filter. A sample of each cell free medium was added to an L culture (<20 %, v/v) at a starting OD of 0.0, <0.3 and <1.0. The remaining cell free medium was added (<20 %, v/v) to the preparation of plate DFg medium used for inoculating the L variant. The behaviour (stickiness as well as colony shape in solid medium, and clumping in liquid medium) of the liquid and plate grown L variants were examined.

2.2.26 Comparison of extracellular protein profiles

The extracellular protein profiles of S and L variants were compared using SDS-PAGE. Samples were prepared from plates (total extracellular) and liquid (mostly cell- and extracellular polymer-associated proteins) grown cultures. Samples were concentrated by freeze-drying or by microcentrifugal concentration. The effects of various carbon sources on the protein profiles were also studied. For total extracellular proteins, samples were prepared from plate grown cultures. S and L cultures obtained through general procedure preparation (section 2.2.2) were inoculated on plates of defined medium containing various carbon sources (glucose, cellobiose, xylan, cellulose, sucrose, maltose or starch), then incubated anaerobically at 39^{o}C for approximately 16 hours. For each carbon source, one plate was used for each variant, except for cellulose where two plates were used for each variant and incubated for two days. The plate grown cultures were harvested with a minimal amount (<10 ml) of 0.05 M phosphate buffer pH 6.8 containing 5 mM DTT and 1 mM PMSF to inhibit proteolytic activity that may degrade the protein samples. The cell suspensions were then transferred to clean tubes, the volume adjusted to 10 ml using the same buffer, and left at 4^{o}C for 2 hours with vortexing every 30 minutes. Samples were then centrifuged at 8,000

xg, 4^{o}C for 15 minutes, and the supernatants were transferred to a clean tube and concentrated by freeze-drying or microcentrifugal concentration. Samples were snap frozen in liquid nitrogen and freeze dried overnight. The dried samples were then dissolved in a minimal amount of water (approximately 1 ml), and the protein concentration was determined using the Bradford method. In the microcentrifugal method, samples were concentrated at 8,000 *xg*, and 4^{o}C for 30 – 60 minutes.

For EP-associated extracellular proteins, samples were prepared from broth grown cultures. Experiments were done as above, except broth was used instead of plate medium. For each carbon source, 100 ml of medium (1ml culture/100 ml medium) was used for each variant, except for cellulose where two x 200 ml of medium was used for each variant and incubated for two days. Cells were recovered by centrifugation (8,000 *xg*, 4^{o}C, 15 minutes), and the cell free medium were removed and concentrated by freeze-drying or microcentrifugal concentration as above.

Denaturing PAGE was performed using vertical gels (BioRad Laboratories Ltd., Watford) in the presence of SDS as described in Ausubel *et al.* (1989), with 4 % w/v stacking and 10 % w/v separating gels. Approximately 30 µg of S and L extracellular protein samples were mixed with one volume of 2x gel-loading buffer, and boiled for 3 minutes. Samples were run on 7.3 cm x 8.0 cm x 0.75 mm gels at 85 V for 30 minutes and 120 V for 1 hour. Gels were stained for one hour in 20 % w/v Coomassie Blue, destained in water-methanol-acetic acid (60:

30: 10) and dried onto Whatman 3 MM filter papers using a gel drier (BioRad Laboratories Ltd., Watford) at 65^{o}C for 20 minutes.

2.2.27 Comparison of plasmid profiles

To compare and study the homology between S and L host plasmids, S & L plasmids were isolated (section 2.2.11) and digested (1 μg) with *Hind* III, *Pst* I, *Sal* I, *BamH* I, *Xba* I or *Pst* I-*Xba* I (section 2.2.15) Samples were then run on an agarose gel (section 2.2.13), transferred to a nylon membrane (section 2.2.16), and hybridised with the smallest or the largest Pst I fragment of S or L plasmid (section 2.2.17, 2.2.18 and 2.2.19), which had been previously labbed as described in section 2.2.18.

2.2.28 Estimation of cellulose degradation in culture

The ability of S and L variants to degrade crystalline cellulose was compared using a gravimetric method. Defined medium containing sigmacel (1 %, w/v) was inoculated with S or L glucose-grown cultures (5 %, v/v starting culture) at OD 1.0. Cultures were then incubated anaerobically at 39^{o}C for 2-3 days. The pellets (cell and the remaining cellulose) obtained after centrifugation (8,000 *xg*, 4^{o}C, 10 minutes) were transferred to a preweighed filter paper (a). Five ml of formic acid was added (x2) to the samples to lyse the cells. Five ml of water was then added (twice) to remove the remaining formic acid. Samples were then dried and weighed (remaining cellulose and paper)(b). The weight of the remaining cellulose (c) was calculated from b-a, and the % hydrolysis was calculated from: c/initial cellulose weight x 100 %. For a control, the same amount of cellulose

suspension from uninoculated medium was treated similarly. The weight of the remaining cellulose obtained above, (c), was corrected for the decrease in the control.

2.2.29 Xylanase plate assay

To compare xylanase activity, S or L variants (section 2.2.2) were streaked onto plates containing xylan as a sole carbon source and incubated anaerobically at 39^{o}C for 2 days. Plates were examined for clearing zones as a result of bacterial xylanase activity.

2.2.30 CMCase plate assay

β-glucan hydrolysis was visualized by staining the CMC plates with 0.1 % (w/v) Congo red solution for 15 minutes, destaining with 1 M NaCl for 15 minutes and stabilizing the visualized zones by flooding the plate with 5 % (v/v) acetic acid (Teather and Wood 1982).

2.2.31 Quantitative CMCase assay

CMCase activity of S and L variants was compared quantitatively. The enzyme was prepared from total extracellular protein harvested from plate grown cultures of S or L variants. Enzyme preparation was as described in section 2.2.26, except the cultures were only grown on plates of defined medium containing glucose, and several plates were used for each variant to obtain an appropriate amount of protein sample. The assay was carried out as described by Wood and Bhat (1988), except that phosphate buffer (Groleau and Forsberg 1981, Min *et al.* 1994) was used instead of PC buffer. A volume (500 μl) of 2 % CMC substrate was

prewarmed to 39^{o}C for 15 minutes, 500µl of diluted enzyme in 50 mM sodium phosphate buffer pH 6.8 was added, and the reaction was incubated at 39^{o}C for 30 minutes. The reaction was stopped by adding 1 ml of the Somogyi copper reagent solution (Wood and Bhat (1988) followed by boiling for 20 minutes. The production of reducing sugar was determined using the Nelson-Somogyi reagent (Ashwell 1957). One ml of the Nelson (arseno-molybdate) reagent was added after cooling and the tubes were left to stand at room temperature for 10 minutes to allow for colour development. The assay volume was diluted to 5ml with milli-Q water followed by centrifugation at 3,000 *xg* for 10 minutes to sediment the unhydrolysed CMC. The absorbance of the supernatant was read at 520 nm using a 1.0 cm quartz cuvette in a Shimadzu Spectrophotometer (Shimadzu UV-160A, Shimadzu Corporation, Kyoto, Japan). The control mixtures contained the same ingredients and were treated similarly as the sample, except the enzyme was boiled for 10 minutes before mixing with substrate. The glucose concentration was obtained by plotting the sample absorbance against a 0-50 µg D-glucose standard curve that was determined separately. The activity was expressed in international units (IU). One unit of enzyme activity represents one µmole of glucose released per minute under the assay conditions. Specific activity was expressed as activity per mg of protein (IU/mg protein).

2.2.32 Xylanase activity assay

Xylanase activity of S and L variants was measured quantitatively, as in section 2.2.31, except xylan and xylose were used as substrate and standard for reducing sugar, respectively.

2.2.33 Growth comparison in casein

To compare the growth of S and L variants using various nitrogen sources, cultures of S or L variants (prepared according to section 2.2.2) were inoculated (1 %, v/v starting culture) in defined medium containing casein (0.5 %, w/v) or a mixture of 0.25 % casein and 0.25 % ammonium chloride, and incubated anaerobically at 39^0C for 16-36 hours. Cell growth was monitored by optical density (OD 600 nm).

2.2.34 Semiquantitative bacterial proteolysis assay

S or L cultures (prepared as described in section 2.2.2) were streaked on plates containing defined medium plus 0.5 % w/v casein or a mixture of 0.25 % w/v casein and 0.25 % w/v ammonium chloride and incubated anaerobically at 39^oC for 1-2 days. Plates were then flooded with 1 M HCl to stop the reaction and to precipitate the remaining casein. Clearing zones as a result of casein degradation were examined.

2.2.35 Quantitative proteolytic assay

Proteolytic activities of S and L variants were analysed. Enzyme extracts were prepared as described in section 2.2.26 (only fraction 2 was used) and the assay was carried out using the method of Cotta and Hespell (1986). A mixture of 0.5 ml of azocasein substrate (Sigma) (0.8 % w/v azocasein in 10 mM sodium phosphate buffer pH 6.8) and 0.5 ml of enzyme solution (fraction 2 obtained from section 2.2.39) was incubated at 39^oC for 3 hours. The reaction was stopped by the addition of 0.5 ml of cold 1.5 M $HClO_4$. The mixture was then incubated on

ice for 30 minutes and centrifuged at 12,000 *xg*, 4°C for 5 minutes. One ml of the supernatant was mixed with 1 ml of 1 M NaOH, left at room temperature for 5 minutes, and then the absorbance at 440 nm was measured. As a control, enzyme was mixed with the $HClO_4$ in advance, before adding the substrate, then incubated and treated as above. 1 OD ≈ 320 μg/ml (Cotta and Hespell 1986). One unit of activity is defined as the amount of casein degraded (μg) per hour under specified condition and the activity was expressed as total activity (unit/ml culture).

2.2.36 Determination of attachment using SEM

To study the possible steps in attachment or extracellular polymer biosynthesis, S or L cultures were examined under scanning electron microscopy (SEM) at various stages of growth. S or L cultures (prepared according to section 2.2.2) were streaked onto plates containing defined medium plus maltose or starch (0.5 %, w/v) and then incubated anaerobically at 39°C for 12, 19, 25, or 28 hours. Cells were harvested using a glass rod, when tiny colonies appeared on the agar plates (approximately early log phase), when colonies were bigger but still strongly attached to the agar plates (approximately log phase), when colonies became removable from the agar plates but still tend to stick together (approximately early stationary phase), and when colonies became separable (approximately stationary or late stationary phase). Cells were then prepared for SEM using the method of Karnovsky (1965). Cells were gently resuspended in fixative solution (0.15 %, v/v ruthenium red in PBS buffer) and left overnight at 4°C (Karnovsky 1965). The suspensions were centrifuged (10,000 *xg*, 4°C, 5 minutes) and the pellets were washed (2 x 30 min each) in PBS buffer containing

4% (w/v) sucrose. The pellets were suspended in 1% (w/v) osmium tetroxide solution in RO water and left at room temperature for 2 h. The cells were further centrifuged and dehydrated through a graded series of ethanol solutions (70, 90, 95, 100%), 2 changes of 30 min in each and an extra change of 1 h in 100% ethanol. The bacteria were spotted onto aluminium SEM stubs, allowed to dry overnight before coating with gold-palladium-carbon, and examined under a Phillips XL30 Scanning Electron Microscope.

2.2.37 Isolation of extracellular polymer using a gradient centrifugation method

Cell-associated EP was isolated using the gradient centrifugation method (Hewet *et al*. 1976). S or L cultures (prepared as described in section 2.2.2) were inoculated (1 %, v/v starting culture) into liquid defined medium containing sucrose (0.5 %, w/v) and incubated anaerobically at 39^{o}C for 16-24 hours. Cells were harvested (from 100 ml cultures, at OD 1.0) by centrifugation (8,000 *xg*, 4^{o}C, 15 minutes). The cell pellets were suspended in 4 ml medium, 1 ml was taken for dry cell weight determination and the remaining suspension was fractionated by gradient centrifugation as described in section 2.2.41. The polymer obtained at the interface was collected and washed twice with water, followed by centrifugation (10,000 *xg*, 10 minutes), and the amount of EP was measured using the phenol-sulphuric method (Dubois 1956, Nili and Brooker 1995). 2.5 ml of concentrated sulfuric acid was added to 500 µl of EP sample, mixed thoroughly and left for 25 minutes at RT. 500 µl of 5 % w/v phenol was added, and the absorbance at 488 nm was measured using spectrophotometer. The sample absorbance was plotted against a glucose standard curve determined separately.

EP production was expressed in mg EP (based on glucose content) per mg dry cell weight.

2.2.38 Measurements of extracellular polymer production

Total (cell-associated and secreted) EP was isolated from plate grown cultures of S or L variants according to the method of Berri and Rollings (1995), except 2 M NaOH was used instead of 1 M. The effect of carbon source on EP production was examined by growing the two variants on nutrient agar plates containing defined medium plus various carbon sources (glucose, cellobiose, xylan, cellulose, sucrose, maltose or starch). S or L cultures (prepared as described in section 2.2.2) were streaked onto these plates and incubated anaerobically at 39^{o}C overnight, except for crystalline cellulose where two plates were prepared and these were incubated for two days. Cultures were then harvested with a minimal amount (< 10 ml) of 2 M NaOH, transferred to clean sterile yellow-capped tubes and adjusted to 10 ml with 2 M NaOH. One ml of each sample was immediately transferred to an Eppendorf tube, and centrifuged at 10,000 *xg*, at 4^{o}C for 10 min. After removing the supernatant, the pellets were dried under vacuum and heated (50^{o}C) for dry cell weight determination. The remaining 9 ml of sample was held at 4^{o}C for one hour with 2-5 minutes vortexing every 15 minutes, centrifuged at 3,000 *xg* and 4^{o}C for one hour, and the supernatant was dialyzed against 2-3 changes of water overnight. All the dialyzed samples were then centrifuged as above and the pellets were freeze-dried and designated as fraction I. The supernatants were adjusted to pH 4.0 with 0.1 M acetic acid to further remove contaminating proteins and centrifuged as above. Both the pellets and the

supernatant were freeze dried and designated as fractions A and N, respectively. The cell weight and EP weight were calculated as follows:

Cell weight /plate = 10 x the cell weight measured from 1 ml sample = a

EP weight/plate = 10/9 x EP weight measured from 9 ml samples = b

EP/cell weight = b/a

For a control, uninoculated plates containing defined medium plus various carbon sources were treated similarly.

2.2.39 Analysis of extracellular polymer by the cell free medium method

To examine secreted EP production, EP was isolated from defined-liquid medium-grown cultures of S or L variants according to Stack, 1987. The effect of carbon source on EP production was elucidated by growing the two variants in defined liquid medium plus various carbon sources (glucose, cellobiose, xylan, cellulose, sucrose, maltose or starch). S or L cultures (prepared as described in section 2.2.2) were inoculated (1 %, v/v starting culture) in the above medium and incubated anaerobically at 39^{o}C overnight (until stationary phase). For each specific carbon source, 500 ml of the above medium was used, except for crystalline cellulose where 1 L of medium was used and it was incubated for 48 hours. Cultures were centrifuged at 8,000 *xg*, 4^{o}C for 20 minutes and the cell pellets were transferred to Eppendorf tubes, dried under vacuum and heated for dry cell weight determination. The cell free media was concentrated to 35 ml using rotavapor, dialyzed against water (3 changes for the first and second days, and 2 changes for the consecutive days) until there was no further decrease in brown color (usually after 5 days). Samples were then centrifuged at 3,000 *xg*, and 4^{o}C for one hour. Pellets were then freeze-dried and designated as fraction I (insoluble material).

The supernatants were divided into three, the first fraction was freeze dried directly and designated as fraction M. The second fraction was phenol extracted, left for two hours at room temperature and vortexed every 30 minutes, then it was centrifuged (3000 *xg*, 4^{o}C) for one hour. The water fraction (upper) was extracted x 2 with phenol as above, then freeze-dried and designated as fraction PD. The third fraction was treated the same way as the second fraction, except it was dialyzed overnight against three changes of water, before being freeze-dried and designated as fraction P. The EP production was calculated as follows:

cell weight/500 ml medium = a

EP/500 ml medium = 3 x EP weight (M fraction) = b

EP/cell weight = b/a

For a control, uninoculated defined liquid medium plus various carbon sources were treated similarly.

2.2.40 Monosaccharide composition of extracellular polymer

To compare monosaccharide composition of EP, various EP fractions obtained from plates (section 2.2.46) and cell free media (section 2.2.47) were degraded and the monosaccharide composition (in the form of alditol acetate) was determined using Gas Chromatography (GC). Samples were prepared according to the method of Albersheim et al. (1967), which was modified by Stack (1987). Five mg of EP samples containing 0.5 mg internal standard inositol (Sigma, St. Louise, USA) were dissolved in 0.5 ml of water containing 76.5 µl of trifluoroacetate anhydrous (Sigma, St. Louise, USA), and the mixtures were hydrolyzed at 100^{o}C for 1 hour. Samples were then evaporated to dryness at 50^{o}C

under a stream of nitrogen gas. Reduction was done by adding 0.25 ml of 0.25 M sodium borohydride in 1 M ammonia to the dried samples, mixing the samples, and incubating them at 40^{o}C for 90 minutes. Glacial acetic acid (250 µl) was added to decompose the excess borohydride, and the samples were then evaporated to dryness as above. Acetylation was carried out by dissolving the dried material in 0.25 ml acetic anhydride, mixing and incubating at 121^{o}C for 3 hours. Five hundred µl of water were added to decompose the remaining acetic anhydride. The prepared alditol acetates were then dissolved in 0.5 ml of methylene chloride, and 0.1 µl injections were made into a Hewlett Packard 5890 gas chromatograph equipped with a 0.25 µm x 30 m DB-225 fused silica capillary column (J&W Scientific, Rancho, Cordova, CA, USA). Due to the limited amount of fraction I, and samples obtained from cellulose grown cultures, the procedures were scaled down to 1 mg of EP sample containing 0.1 mg of standard inositol. All reactions were carried out with one fifth amount of the above procedures and at the end were extracted and dissolved in 50 µl of methylene chloride. Hence, the concentration remained the same (1 µg of EP containing 10 % standard inositol per 0.1 µl of methylene chloride) and injections were done using 0.1 µl of the methylene chloride fraction. Trials were carried out using N and M fractions to check this scaled down procedure.

Injections were run isothermally at 210^{o}C, split 15:1, attenuator 1, and detected by flame ionization. A Hewlett-Packard 3392A recording integrator plotted the results and calculated peak areas. Most injections were done using 0.1 µl samples. Higher amounts of samples were also carried out, however although the

chromatogram peaks were higher, the relative proportions remained the same. All chromatograms presented in this thesis were obtained from 0.1 µl injections.

The relative proportion of each peak was expressed as mmol per mg sample and was calculated as follow:

$$\frac{\%\ \text{peak area}}{\%\ \text{internal standard inositol}} \times \text{mmol inositol} \times 1/5\ \text{mg}$$

2.2.41 Fatty acid composition of extracellular polymer

To compare the fatty acid composition, various EP fractions obtained from plates (section 2.2.43) and cell free media (section 2.2.44) were methylated and the fatty acid composition was determined using GC. Samples were prepared as described in Horwitz *et al.* (1980).

Five mg of EP sample was dissolved in a mixture of 1 ml water and 5 ml methanol, and was extracted with 4 ml chloroform. After thorough mixing for approximately 5 minutes, the sample was centrifuged at 1,000-3,000 *xg* for 5 to 10 minutes and the chloroform layer at the bottom was transferred to another clean tube. The remaining water-methanol fraction was re-extracted with chloroform as above, and the chloroform fraction obtained was pooled with the previous one. The sample was then dried under a stream of nitrogen and was methylated by adding 1.5 ml of acidified water-free methanol, mixing and incubating at 100^{o}C for 1 hour. The methylated sample was cooled at room temperature, then extracted with a mixture of 3 ml water and 5 ml of petroleum ether (boiling point 40^{o}-60^{o}C), mixed (vortex) for 5 minutes and then centrifuged at 1,000-3,000 *xg* for 5

minutes. The petroleum ether fraction (upper layer) was transferred to a clean tube, while the water-methanol (bottom layer) was re-extracted with 5 ml of petroleum ether as above, and the petroleum ether obtained was combined with the previous one. The petroleum ether fraction was then dried under a stream of nitrogen, and dissolved in a mixture of 90 µl isooctane and 10 µl methylated internal C19 standard (stock, 1 mg/ml, w/v) (Matreya, Inc., CA, USA). The concentration of each sample (per 100 µl) was 5 mg EP containing 10 µg internal standard. Normally 10 % internal standard is used for GC analysis. However due to the low proportion of fatty acid in EP samples, much less internal standard was required (10 µg/5mg EP sample or approximately 0.2 %). Therefore the internal standard was methylated separately and added to the sample at the end of methylation process. Injection was done as in section 2.2.48, except the attenuator was set at 0. The relative proportion of each peak was expressed as µmol per mg sample and was calculated as follows:

$$\frac{\%\ \text{peak area}}{\%\ \text{internal standard C19}}\ \text{x µmol C19 x 1/5 mg}$$

2.2.42 Dephosphorylation of linearized plasmid DNA

Approximately 2 µg (1 pmole) of linearized plasmid DNA was dephosphorylated using bovine alkaline phosphatase (Boehringer Manheim) (1 unit/pmole DNA) in a total volume of 10 µl. The reaction mixture was incubated at 37^{o}C for 30 minutes. The reaction was stopped by heating (90^{o}C, 30 seconds) followed by phenol-chloroform extraction (section 2.2.8), ethanol precipitation (2.2.9), and drying under vacuum for 2 minutes. The dried sample was kept in 4^{o}C. The

sample was dissolved in water and the concentration was determined spectrophotometrically (section 2.2.7), prior to use.

2.2.43 Ligation

Dephosphorylated linear plasmid DNA (1 μg) was ligated with restriction enzyme-digested chromosomal DNA (3 μg) using bacteriophage T4 DNA ligase (Boehringer Manheim) (1unit) in a total volume of 10 μl. The reaction mixture was incubated at 16^{o}C for 8 hours. The mixture was then phenol-chloroform extracted (section 2.2.8), ethanol precipitated (2.2.9), and dried under vacuum for 2 minutes. The dried sample was kept in 4^{o}C. The sample was dissolved in water and the concentration was determined spectrophotometrically (section 2.2.7), prior to use.

2.2.44 Preparation of competent *E. coli* cells for electroporation

E. coli ED8299 (glycerol stock) was streaked onto a plate containing LB medium and incubated at 37^{o}C overnight. A single colony was transferred into 5 ml of LB liquid medium and was incubated at 37^{o}C with moderate shaking until OD (600 nm) 0.7 was reached. The culture was then diluted with 500 ml of liquid LB medium and incubated similarly until OD (600 nm) 0.5-0.7 was reached. It was then centrifuged at 8,000 *xg*, and 4^{o}C for 10 minutes. After discarding the supernatant, the cell pellet was chilled on ice for 10 minutes, suspended in 50 ml of ice-cold 10 % glycerol, and centrifuged as above. The procedure was repeated using 5ml and 1 ml of 10 % glycerol. The cell pellet was suspended in 1 ml of ice cold 10 % glycerol and was aliquoted into ice-cold Eppendorf tubes (100 μl) and stored at –80^{o}C until required.

2.2.45 Preparation of competent *B. fibrisolvens* cells for electroporation

S or L cells (glycerol stock) were streaked on defined solid medium containing cellobiose and were incubated anaerobically at 39^{o}C overnight. A single colony was transferred into liquid defined medium containing cellobiose and incubated anaerobically at 39^{o}C until OD (600nm) = 1-1.3 was reached. 30 µl of the culture was then inoculated into 30 ml of liquid defined medium containing cellobiose and incubated as previously. 20 ml of prewarmed (39^{o}C) liquid defined medium containing cellobiose was added to the culture when the culture reached OD (600 nm) = 1.0 or other ODs as described in results, and was further incubated for one hour. The culture was then transferred into an ice-cold 50 ml anaerobic, sterile centrifuge tube with air tight cap and chilled thoroughly in ice water for 15 minutes. The sample was centrifuged at 8,000 *xg*, and 4^{o}C for 15 minutes. The subsequent steps were carried out anaerobically at a temperature < 4^{o}C. After centrifugation, the supernatant was discarded and the remaining supernatant was removed. The cell pellet was rinsed with 5 ml of ice-cold electroporation buffer (EB) (dithiothreitol 0.1 mM, resazurin 0.1 % w/v, sorbitol 5.25 % w/v) without disturbing the pellet and the EB was removed. The cell pellet was then suspended in 10 ml of ice-cold EB, mixed and another 30 ml of ice-cold EB was added to the cell suspension. It was then centrifuged (8,000 *xg*, and 4^{o}C for 15 minutes) and the buffer was removed as above. Cells were suspended in 100 µl of ice-cold EB, mixed, transferred into ice-cold Eppendorf tubes (42 µl each) and stored at –80^{o}C until further used. For large scale preparation (up to 500 ml of culture), the reagent volumes used were multiplied 10 times.

2.2.46 Electroporation of *E. coli*

Competent cells were defrosted slowly on ice, 0.5 μg (unless indicated) of plasmid DNA or ligation mixture was added, mixed, transferred into an ice-cold electroporation cuvette, incubated on ice for 5 minutes and electroporated at 2.5 kV and 200 or 400 Ω. The time constant was recorded, and the transformation mixture was incubated on ice for 30 seconds. 1 ml of LB medium was added and the cells were incubated at 37^{o}C for 1 hour. The sample was diluted 1000 x with LB medium (no dilution was used for pBHerm) and was streaked (100 μl/plate) on LB plates containing 50μg/ml or 20 μg/ml ampicillin for pUC19 or pBHerm transformants, respectively. Plates were incubated at 37^{o}C overnight, the colonies were counted and the insert-containing transformants (white colonies) were picked and restreaked on selective medium for further purification. A single colony of purified transformant was inoculated in liquid medium, and glycerol stocks were prepared.

2.2.47 Electroporation of *B. fibrisolvens*

Electroporation was carried out as in section 2.2.46, except *B. fibrisolvens* (S or L variant) were used as the competent cells and the conditions of electroporation (voltage, resistance, DNA concentration and cell competent preparations) were varied, as indicated in the results. The electroporation and incubation (39^{o}C) were carried out anaerobically. Growth of transformants was in defined medium (Nili and Brooker 1995) containing sucrose with and without 10 μg/ml of erythromycin.

CHAPTER 3

PRELIMINARY COMPARISON OF THE VARIANTS

3.1 Introduction

Previous researchers have described several strains of *Butyrivibrio fibrisolvens* that attach to surfaces (Dehority and Grubb 1977, 1981; Rassmusen *et al.* 1989). These organisms readily attach to cellulose fibres (Rasmussen *et al.* 1989), to feed and other particles in the rumen (Dinsdale *et al.* 1978, Dehority and Grubb 1981), and to sheep rumen epithelia (Mead and Jones 1981). However, attachment characteristics of *B. fibrisolvens* strain E14 have not been reported. The existence of sticky (S) and loose (L) variants of E14 (Nili 1996) provides an opportunity to study the mechanism of attachment of *B. fibrisolvens* to surfaces, especially to plant fibre.

In this chapter I describe the growth characteristics of the two variants in solid and liquid medium containing various soluble and insoluble carbon sources, as well as their phenotypic stability. The existence of molecule(s) that may mediate or inhibit cell attachment was also studied. The variants were also compared at the protein (extracellular) and genetic (plasmid, chromosomal and 16S rDNA) levels.

3.2 Results

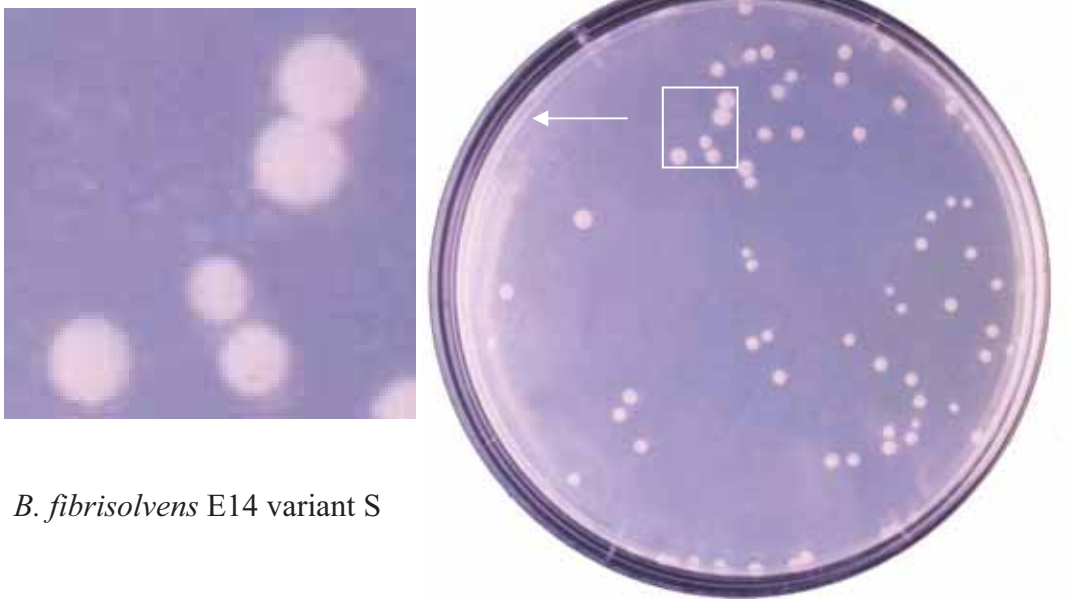
3.2.1 Growth characteristics of variants on plate and liquid media

To study the growth characteristics of S and L variants on various carbon sources, the two variants were grown on solid or liquid defined medium containing various soluble (glucose, cellobiose, sucrose, maltose, starch) or insoluble (xylan, cellulose) plant sugars. On an agar plates, at early log phase, S cells were attached firmly to the agar surface and could not be removed without scraping the agar (Figure 3.1). After longer periods (16 hours) corresponding to stationary phase, the cells became removable, but the cells within the colonies still tended to stick together. After 20 hours on agar, the S cells no longer stuck together and were easy to separate. In contrast L cells could easily be removed from the agar plate and separated at all stages of growth.

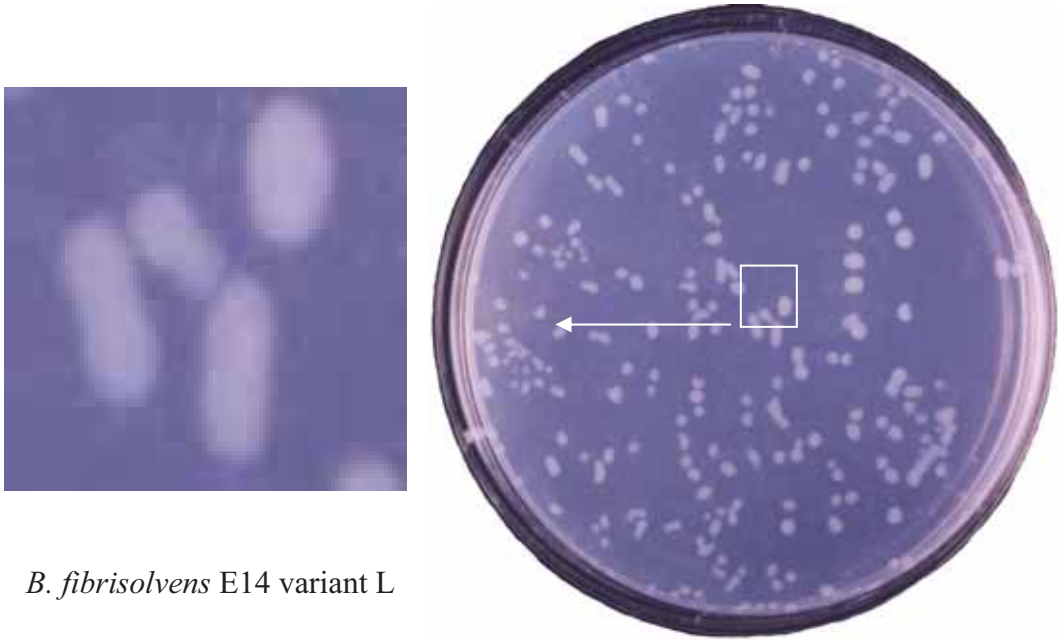
The S colonies were shinier, round and more yellow compared to L colonies (Figure 3.1). In addition, the S colonies were spherical in shape, compared with L colonies, in which the top surface was flattened. When late phase S cells were removed from the plate, the agar remained undisturbed (Figure 3.2).

The results presented in Figure 3.1 and 3.2 were observed on all carbon sources, except cellulose.

In liquid defined medium (Figure 3.3) containing soluble carbon sources (glucose, cellobiose, sucrose, maltose or soluble starch), the S cells tended to clump during the early stage of growth, and be dispersed at a later stage. The L cells grew throughout of the medium at all stages of growth.



B. fibrisolvens E14 variant S

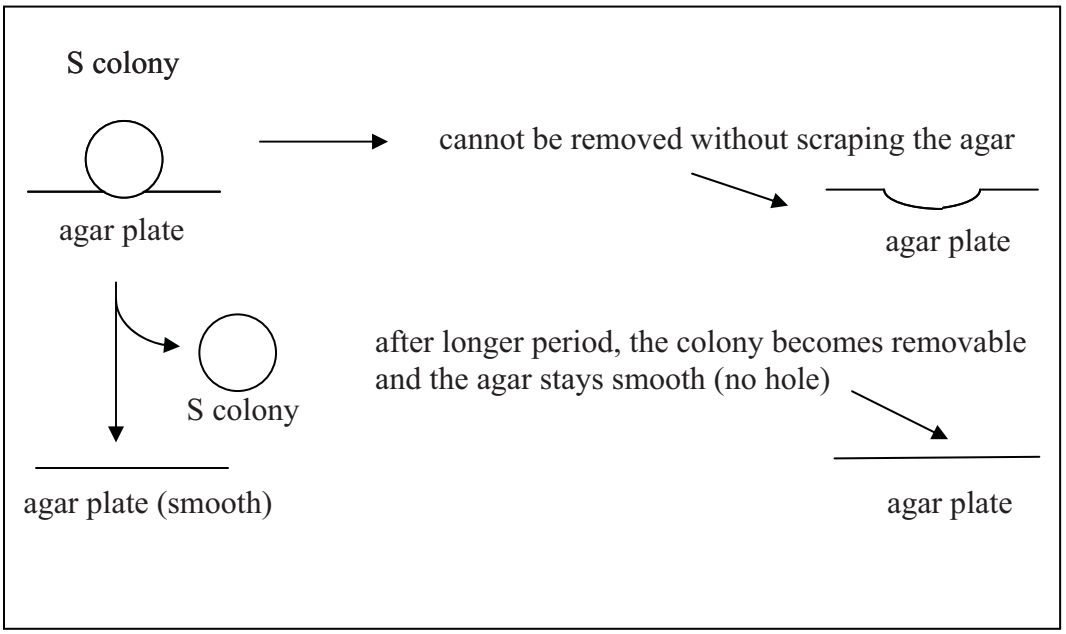


B. fibrisolvens E14 variant L

Figure 3.1 Colonies of S and L variants on agar plate.

B. fibrisolvens E14, variant S or L, was streaked onto solid defined medium (Nili and Brooker 1995) containing glucose, and were incubated anaerobically at 39^{o}C for 16 hours. Similar results were obtained when cellobiose, xylan, sucrose, maltose or starch were used as carbon sources, instead of glucose.

B. fibrisolvens variant S



B. fibrisolvens variant L

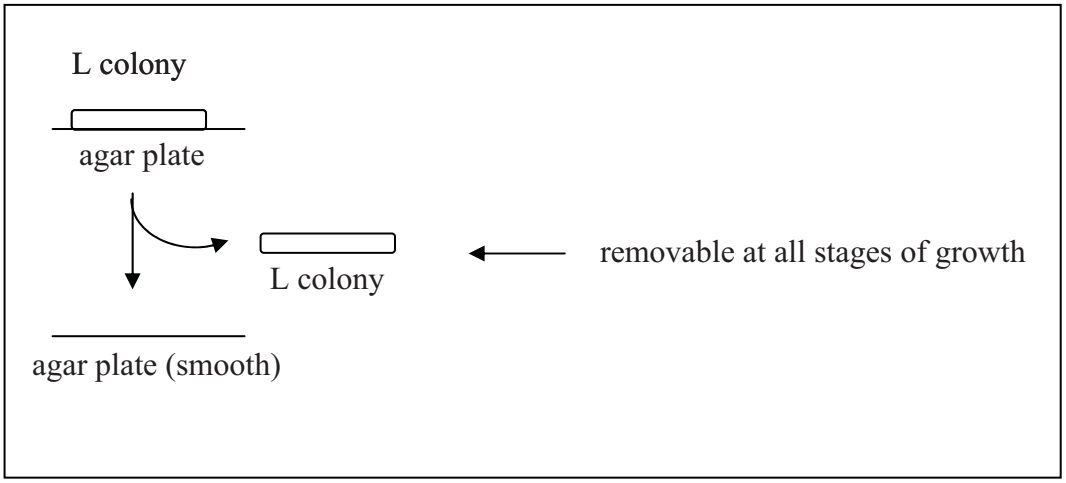


Figure 3.2 Colony shapes of *B. fibrisolvens* E14 variant S or L.

S = *B. fibrisolvens* E14 variant S
L = *B. fibrisolvens* E14 variant L

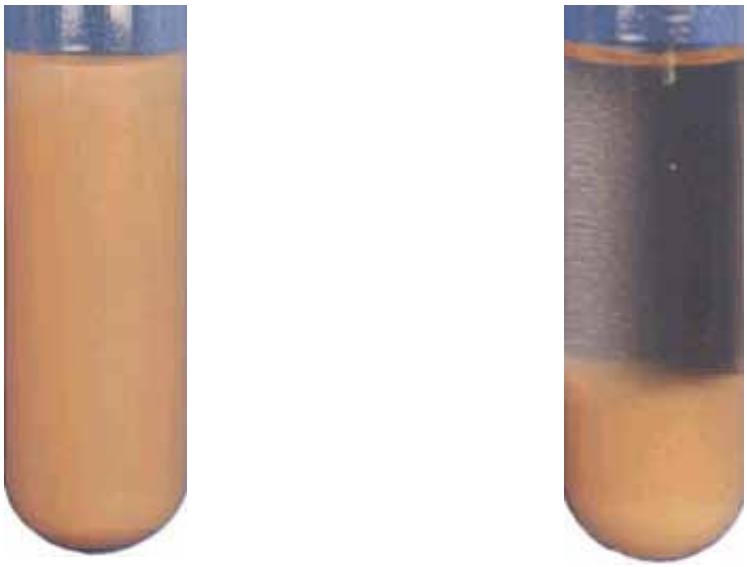
S L

Figure 3.3 Broth culture of *B. fibrisolvens* E14 variants S or L.

S = broth culture of *B. fibrisolvens* E14 variant S
L = broth culture of *B. fibrisolvens* E14 variant L

S or L were each grown in liquid defined medium (Nili and Brooker 1995) containing glucose, and were incubated anaerobically at 39^{o}C for 16 hours. The S cells tended to clump at the bottom of the tube, while the L cells were dispersed throughout the medium. A similar result was obtained when cellobiose, sucrose, maltose or starch were used as carbon sources, instead of glucose.

The differences between S and L variants in liquid medium were not clear when insoluble carbon sources such as xylan (Figure 3.4) or crystalline cellulose (Figure 3.5) were used, due to interference from the insoluble substrates. On xylan, both S and L behaved similarly. Similar results were obtained when the xylan was washed with water three times to remove the soluble fraction prior to incorporation into the medium.



M C

Figure 3.4 Broth culture of *B. fibrisolvens* variant S or L cells in xylan.

M = defined medium containing xylan (before inoculation)
C = defined medium containing xylan, after inoculation with *B. fibrisolvens* E14 variant S or L

Defined medium containing xylan was innoculated with S or L variants and it was incubated anaerobically at 39^{o}C for 2 days.

In crystalline cellulose (Figure 3.5), both S and L cells grew poorly. Similar results were obtained when larger inocula were used (2-5 %) or when the cells (S and L) were adapted to growth in liquid media containing 0.5 % w/v crystalline cellulose and decreasing glucose, cellobiose or both, as outlined in Figure 3.5. On solid medium containing crystalline cellulose, only tiny colonies were observed after three days of incubation, and it was difficult to differentiate between S and L variants. Both variants behaved similarly on plates or in liquid medium.

Glucose and cellobiose concentration for culture adaptation

Glucose	Cellobiose	Mixture of Glucose and cellobiose
0.5 %	0.5 %	0.25 % glucose and 0.25 % cellobiose
0.20 %	0.20 %	0.10 % glucose and 0.10 % cellobiose
0.10 %	0.10 %	0.005 % glucose and 0.005 % cellobiose
0.005 %	0.005 %	

↓

Inoculate (2-5 %, v/v) defined medium containing crystalline cellulose

↓

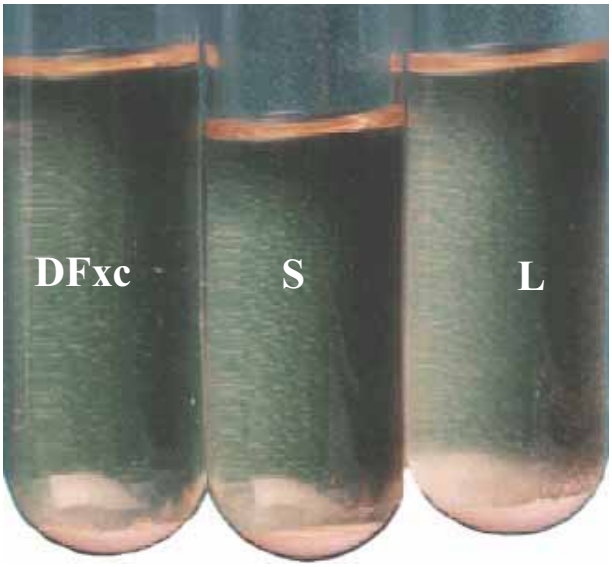


Figure 3.5 Growth of S and L variants in crystalline cellulose.

DFxc = defined medium (Nili and Brooker 1995) containing crystalline cellulose
S = culture of *B. fibrisolvens* variant S in DFxc
L = culture of *B. fibrisolvens* variant L in DFxc

B. fibrisolvens variants S or L were adapted in DFxc containing decreasing glucose, cellobiose or both as described in the method and text. The incubation was carried out at 39^{o}C for 2-3 days.

The ability of S and L variants to grow on solid and in liquid medium containing various carbon sources (Table 3.1) was similar. Both variants were able to grow in all carbon sources tested, except on crystalline cellulose.

Table 3.1 Growth ability of S and L variants using various carbon sources.

Carbon sources		Number of colonies (cells, *) or OD 600 nm (#)	
		S	L
Brain Heart Infusion	#	1.439 ± 0.027	1.281 ± 0.024
Glucose	#	0.632 ± 0.011	0.615 ± 0.008
Cellobiose	#	0.538 ± 0.008	0.519 ± 0.007
Sucrose	#	0.260 ± 0.005	0.114 ± 0.004
Fructose	#	0.025 ± 0.004	0.022 ± 0.005
Lactose	#	0.016 ± 0.003	0.014 ± 0.004
Galactose	#	0.049 ± 0.005	0.047 ± 0.006
Maltose	#	0.624 ± 0.010	0.576 ± 0.011
Soluble starch	#	0.129 ± 0.009	0.116 ± 0.010
Oat spelt xylan	*	$(201 \pm 23)\times10^4$	$(195 \pm 18)\times10^4$
Berchwood xylan	*	$(179 \pm 20)\times10^4$	$(153 \pm 16)\times10^4$
Washed (oat spelt) xylan	*	$(184 \pm 17)\times10^4$	$(172 \pm 21)\times10^4$
Carboxylmethyl cellulose	*	$(158 \pm 12)\times10^2$	$(140 \pm 15)\times10^2$
Cellulose size 20	*	121 ± 12	103 ± 11
Cellulose size 50	*	94 ± 10	83 ± 9
Cellulose size 100	*	81 ± 7	71 ± 10
Cellulose fibrous	*	67 ± 8	52 ± 5

S = *B. fibrisolvens* E14 variant S
L = *B. fibrisolvens* E14 variant L

B. fibrisolvens variants S or L were grown on solid (*) or in liquid (#) defined medium containing various carbon sources, and were incubated anaerobically at 39°C for 16 hours. Cell growth was determined by OD 600 nm (for liquid medium, #) or colony counts (for plates, *). Values represent the mean plus standard error of two independent experiments.

3.2.2 Stability of variants

The phenotypic stability of S and L variants was studied by serial passage on solid or liquid medium, as described in the methods and outlined in Figure 3.6. No changes were observed in the behaviour of S and L cells after ten serial passages on solid or liquid medium. The S cells remained sticky on solid medium and clumpy during early growth in liquid medium. L cells remained loose on solid medium and grew dispersed throughout the liquid medium at all stages of growth. The shape of S and L colonies remained spherical and flat, respectively. S colonies remained yellow compared to the white colour of L cell colonies.

3.2.3 Cell to cell communication

Cell to cell communication experiments were carried out to explore the possibility that S cells produced molecule(s) that may mediate cell attachment to surfaces or that L cells produced molecule(s) that inhibited cell attachment. The S cell-free medium (isolated at approximately OD 600 nm 0.3 or 1.0) was added to L cultures (at approximately inoculation, midlog or late log phase), as described in the methods and outlined in Figure 3.7.

No effect was observed when S cell-free medium was added (20 %, v/v) to L cell cultures or when L cell-free medium was added to S cell cultures. However, it was found that the S cell-free medium was much more difficult to pass through a membrane filter (0.22 μm), and the filter had to be changed for every 1 ml of sample. L cell-free medium passed through the membrane filter easily and the filter did not need to be changed.

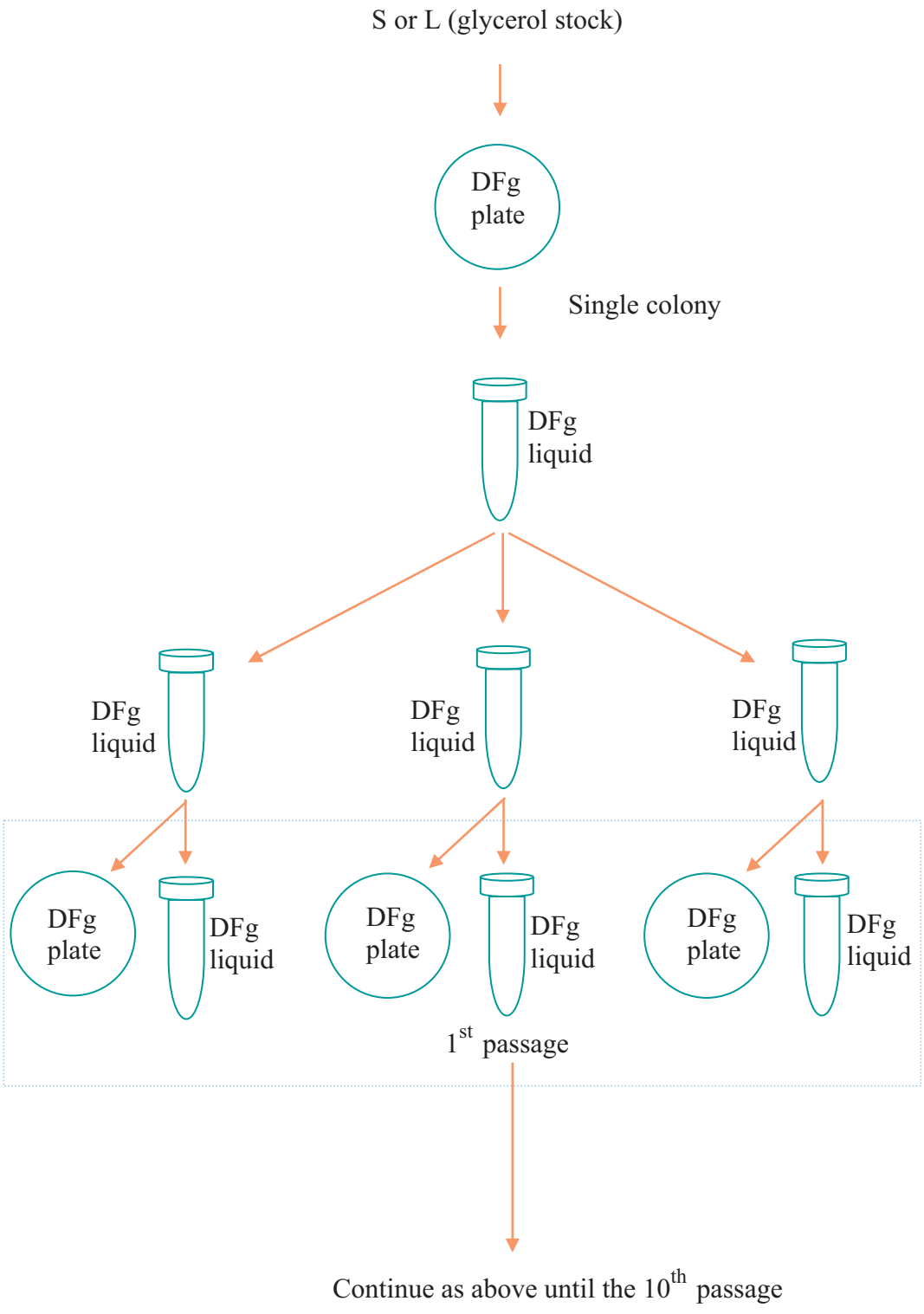


Figure 3.6 Diagrammatic representation of stability experiment.

Dfg was defined medium (Nili and Brooker 1995) containing glucose. Cultures were incubated anaerobically at 39°C for 16-24 hours.

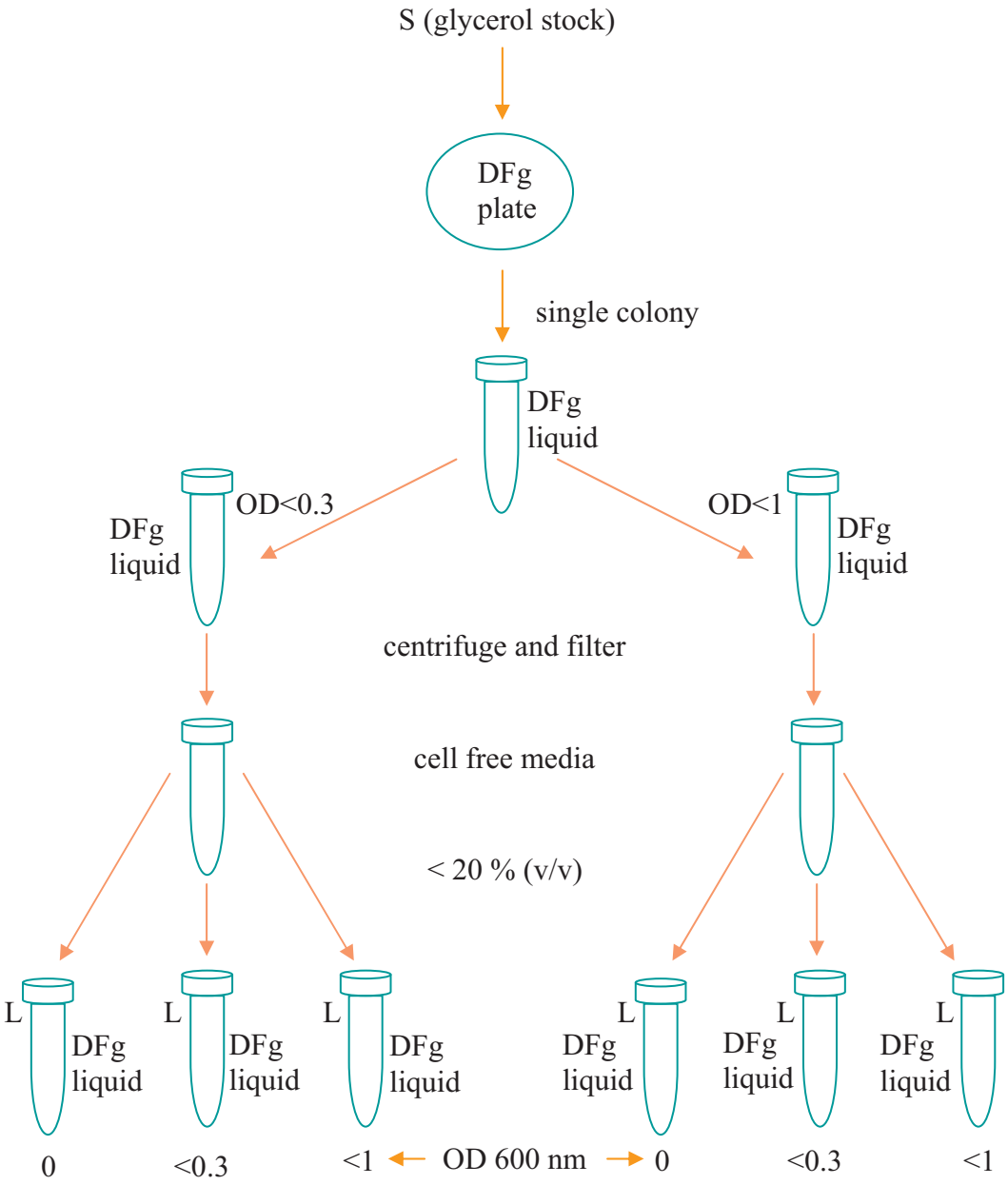


Figure 3.7 Diagrammatic representation of cell to cell communication analysis using L cultures.

Dfg, defined medium (Nili and Brooker 1995) containing glucose. All incubations were carried out anaerobically at 39^{o}C for 16-24 hours. Cell free media was added at the time of culture inoculation. The same experiment was carried out using S cultures (L-cell free media added to S cultures).

3.2.4 Comparison of extracellular protein profiles

(Nili and Brooker (1995) showed that there was no significance difference between the cytoplasmic protein profile of S and L variants. Therefore, in this study, the extracellular protein profiles of S and L variants were compared. Samples were prepared from liquid or solid medium grown cultures, and were concentrated using freeze-drying or microcentrifugal concentration. The effect of carbon source on the protein profiles was also studied using samples prepared from cultures grown on various carbon sources.

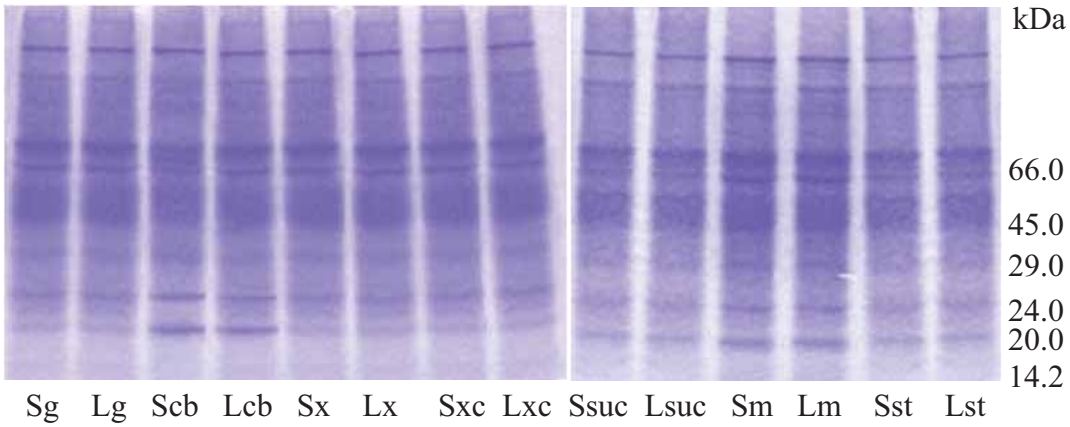


Figure 3.8 Extracellular protein profiles of *B. fibrisolvens* variants S and L.

Sg, Scb, Sx, Sxc, Ssuc, Sm and Sst were samples isolated from *B. fibrisolvens* variant S grown in defined solid medium (Nili and Brooker 1995) containing glucose, cellobiose, xylan, crystalline cellulose, sucrose, maltose or starch, respectively. Lg, Lcb, Lx, Lxc, Lsuc, Lm, Lst were samples isolated from *B. fibrisolvens* variant L similarly. Solid medium grown cultures were harvested and extracted with 10 ml of 0.05 M phosphate buffer pH 6.8 containing 5 mM DTT and 1 mM PMSF, as described in the Methods. These were then concentrated by the freeze drying, and approximately 30 μg was fractionated using SDS PAGE and visualized by Coomassie Blue staining methods.

No significance differences were found between S and L extracellular protein profiles using samples that were isolated from plates (Figure 3.8). Similar results were obtained when samples were isolated from liquid medium-grown cultures. In addition, no significant differences were observed between samples that were concentrated by microcentrifugal concentration or freeze-drying. There was no significant effect of carbon source on the protein profiles.

3.2.5 Comparison of chromosomal DNA profiles

The chromosomal DNA restriction patterns of S and L variants were compared. There were no significant differences between the uncut, *Sal* I- and *Pst* I-cut chromosomal DNA profiles of S and L variants (Figure 3.9). It was observed that besides chromosomal DNA, both S and L variants contained an endogenous plasmid.

3.2.6 Comparison of plasmid profiles

It has been reported (Leigh *et al*. 1985, Whitfield 1988) for some plasmid-bearing bacteria that together with chromosomal DNA, plasmid DNA may be involved in the production of extracellular polymer that may mediate attachment to surfaces. Since both S and L variants contained an endogenous plasmid, I examined these for evidence of any difference between S and L variants. The plasmids from S and L variants were compared. There were no significant difference between the uncut, *Hind* III-, *Pst* I-, *Sal* I-, *Bam*H I-, *Xba* I- and *Pst* I-*Xba* I- cut endogenous plasmid profiles of S and L variants (Figure 3.10). Southern blot analysis using the largest and the smallest *Pst* I fragments from S or L plasmids as probes (Figure 3.11) gave positive results for both variants (Figure 3.10).

kb kb kb

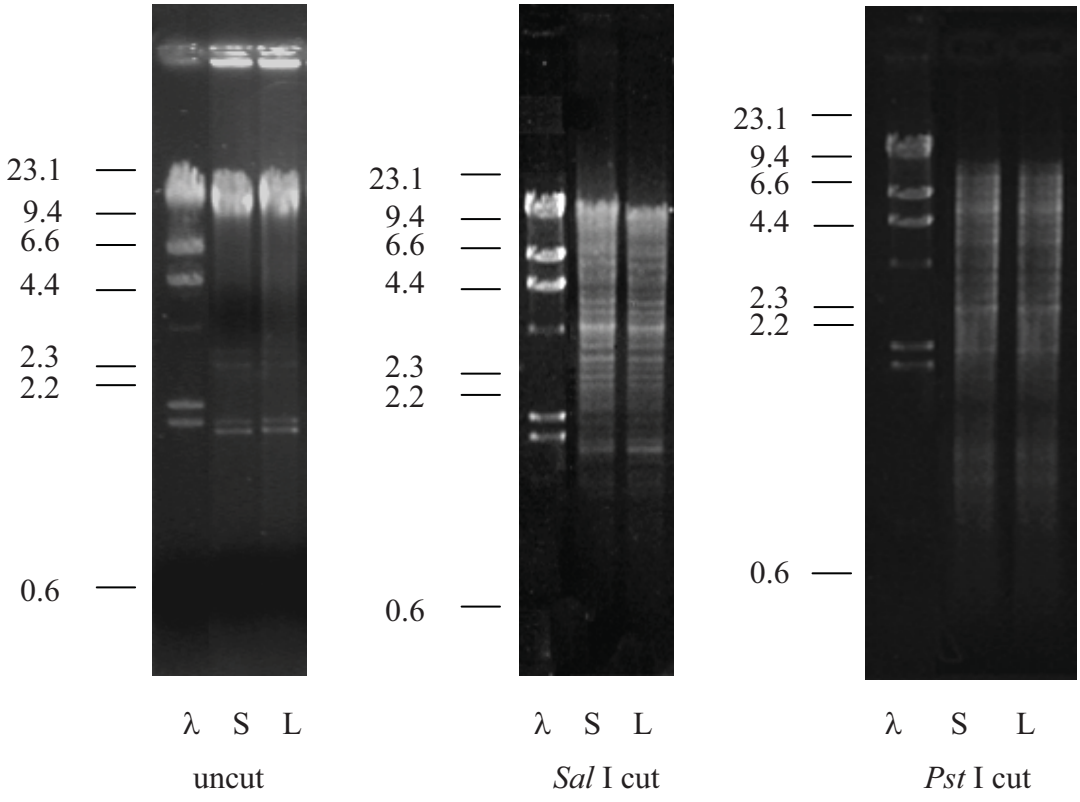


Figure 3.9 Chromosomal DNA profiles of *B. fibrisolvens* E14 variants S and L.

S = uncut, *Sal* I- or Pst I-cut chromosomal DNA of *B. fibrisolvens* variant S
L = uncut, *Sal* I- or Pst I-cut chromosomal DNA of *B. fibrisolvens* variant L
λ = *Hind* III-cut λ DNA

The chromosomal DNA of *B. fibrisolvens* E14 variant S or L was isolated and the uncut, *Sal* I-, *Pst* I-cut profiles were compared by agarose (0.8 %, w/v) gel electrophoresis and staining with ethidium bromide (0.5 μg/ml), as described in the Methods.

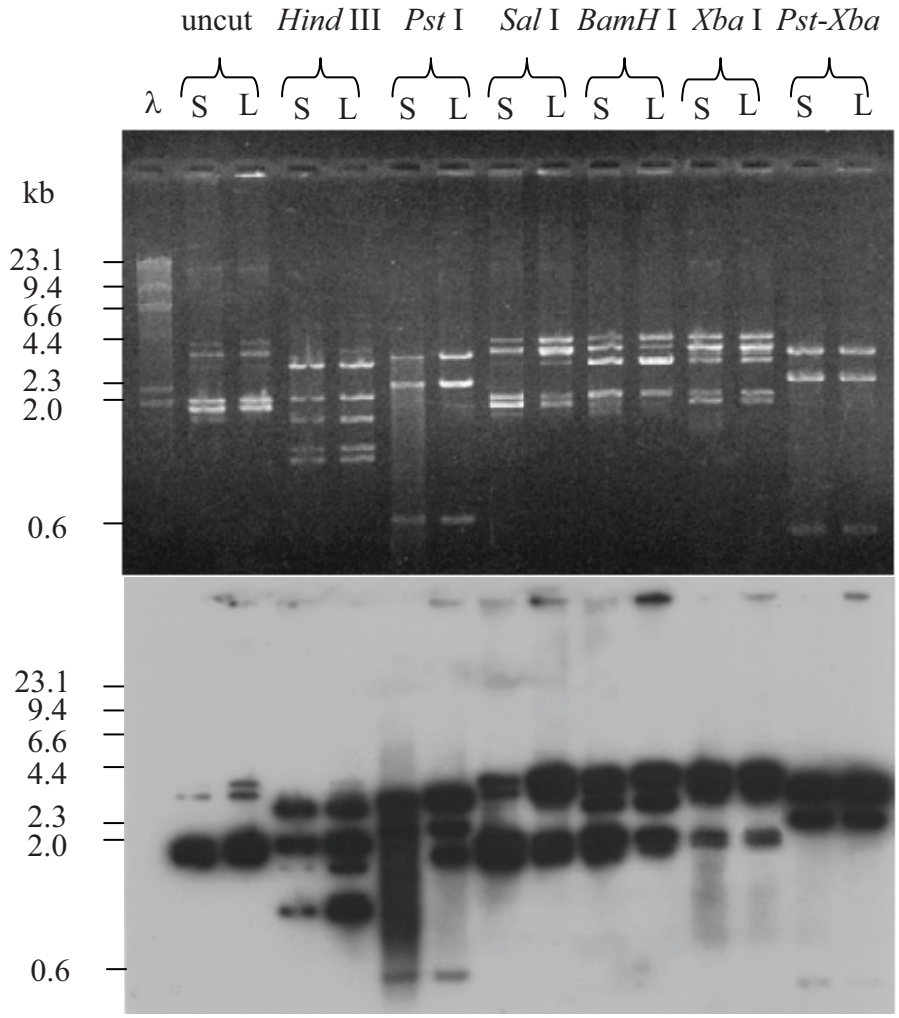


Figure 3.10 Plasmid profiles of S and L variants.

S = uncut, *Hind* III-, *Pst* I-, *BamH* I-, *Xba* I- or *Pst* I-*Xba* I-cut endogenous plasmid of *B. fibrisolvens* variant S
L = uncut, *Hind* III-, *Pst* I-, *BamH* I-, *Xba* I- or *Pst* I-*Xba* I-cut endogenous plasmid of *B. fibrisolvens* variant L
λ = *Hind* III-cut λ DNA

The endogenous plasmid of *B. fibrisolvens* E14 variant S or L was isolated and the uncut, *Hind* III-, *Pst* I-, *BamH* I-, *Xba* I- and *Pst* I-*Xba* I-cut profiles were compared by agarose gel electrophoresis and staining with ethidium bromide (0.5 μg/ml). The DNA was transferred onto Hybond^{TM}-N+ nylon membrane (Amersham) by capillary transfer (Southern 1975) and hybridised against the largest or the smallest *Pst* I fragment of endogenous plasmid isolated from *B. fibrisolvens* E14 variant S or L (Figure 3.11), as described in the Methods

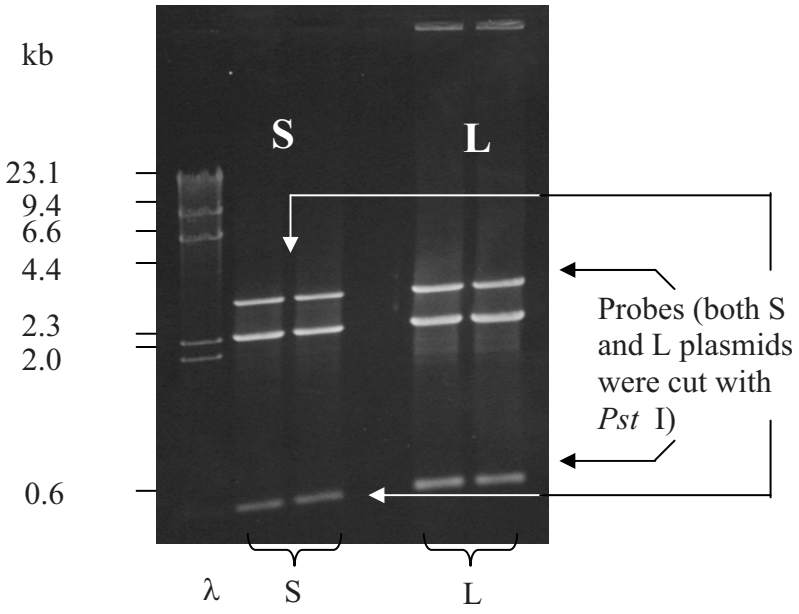


Figure 3.11 Probes used for Southern analysis.

S = *Pst* I-cut plasmid DNA of *B. fibrisolvens* variant S
L = *Pst I*-cut plasmid DNA of *B. fibrisolvens* variant S
λ = *Hind* III-cut λ.DNA

Endogenous plasmid of *B. fibrisolvens* variant S or L was isolated, restriction digested with *Pst* I, fractionated by agarose gel electrophoresis, and stained using ethidium bromide (0.5 μg/ml). The largest and the smallest fragments of S and L plasmids were isolated, further purified by ethanol precipitation and used as probes for Southern Blot analysis (Figure 3.10) as described in the Methods.

3.2.7 Comparison of 16S rDNA profiles

The chromosomal DNA of S and L variants was isolated, the 16Sr-DNA was PCR-amplified as described in the methods, and the 16S rDNA restriction profiles of both variants were compared. There were no significant difference between the *Hae* III-, *Alu* I- and *Taq* I- 16S rDNA restriction profile of S and L variants

(Figure 3.12). The 16S rDNA profiles of *B. fibrisolvens* strain H17c was included as control.

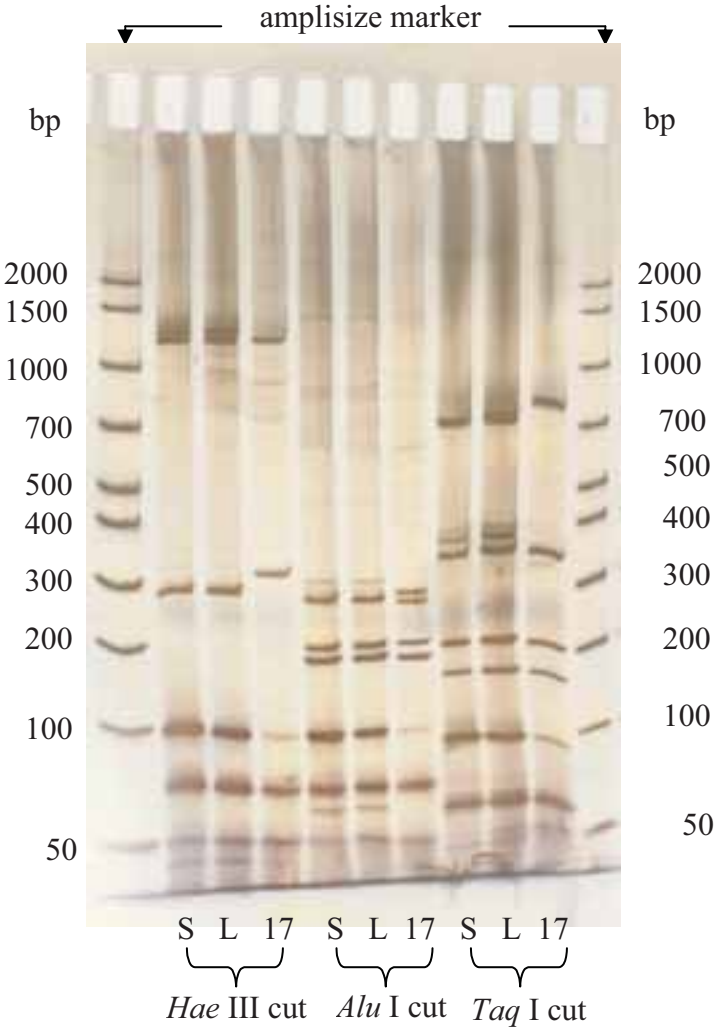


Figure 3.12 Phylogenetic analysis of S and L variants.

S = uncut, *Hae* III-, *Alu* I-cut or *Taq* I-cut 16S rDNA of *B. fibrisolvens* variant S
L = uncut, *Hae* III-, *Alu* I-cut or *Taq* I-cut 16S rDNA of *B. fibrisolvens* variant L
17 = uncut, *Hae* III-, *Alu* I-cut or *Taq* I-cut 16S rDNA of *B. fibrisolvens* H17c
λ = *Hind* III-cut λ DNA

The 16S rDNA of *B. fibrisolvens* variant S or L was PCR-amplified as described, and the uncut, *Hae* III-, *Alu* I- and *Taq*-I cut profiles were compared using PAGE and silver staining as described in the Methods.

3.3 Discussion

The growth characteristics of the L variant was like most other strains *of B. fibrisolvens*, with the cells being non-sticky, dispersed throughout the medium, and easily removed from an agar plate. In contrast, the S variant, clumped together in liquid media, attached to agar surfaces and the cells tended to stick together. After longer periods (approximately early stationary phase), the S variant behaved more like the L variant; being more dispersed in liquid medium, not attached to the agar surface and the cells became separable. A similar behaviour has been reported for *B. fibrisolvens* OB156 (Beard et al 1995), which was clumpy during early growth and was dispersed throughout the liquid medium after longer incubation. This may reflect the natural role of the S variant to attach to surfaces within the rumen and may indicate different nutrient preferences *in vivo*. Detachment at late log/early stationary phase may be a mechanism to maintain its adherent population in logarithmic phase. Since without this detachment mechanism, the epithelial cell surfaces may be covered by old or dead cells, and may inhibit the cell attachment of the next generation to the epithelial cell surfaces. For the detachment mechanism to occur, cells must be unattached or removable from the surface, i.e. the S cells at the end of their growth phase. Attachment blocking by polymer adsorption onto substrata have been described previously. Examples include attachment inhibition of *Pseudomonas* to polystyrene by albumin, gelatin, fibrinogen or pepsin (Fletcher 1977, 1980a); or inhibition of attachment of *Streptococci* to glass by whole saliva or serum (Ørstavik 1977, Fletcher 1980b). A decrease in attachment with increased culture age has also been reported for marine *Pseudomonas*, where bacteria from log phase cultures had the greatest tendency to attach to surfaces, and there was a

progressive decline in numbers of bacteria which become attached after the onset of stationary phase and cell death (Fletcher 1977, 1980a). It was suggested that such a decline was due to a progressive change in quality or quantity of cell surface polymer.

Bauchop *et al.* (1975) demonstrated the existence of bacterial populations associated with epithelial cell surfaces, and the most densely packed bacteria covered the epithelium of the roof of the dorsal rumen. These were primarily rod-shape cells. Dehority and Grubb (1977, 1981) found that those were primarily *Butyrivibrio* and *Prevotella.* Studies on the adherence of several *B. fibrisolvens* strains (A38, 49, 12, 49, X6C61) to cellulose have also been reported (Rassmusen *et al.* 1989). These studies suggest that *B. fibrisovens* may preferentially attach to surfaces. However, the results from stability experiments showed that the two variants had stable phenotypes and were not easily convertible. Possibly more than 10 passages (representing more than 30 generations) are required or a different and richer medium may be required to negate the need for attachment since soluble nutrient will be in excess. In addition, for a simple mutation, one might expect a detectable frequency of reversion. This was not observed in these experiments, raising the possibility that if a change in gene expression was the cause, this genotype was now firmly fixed in the chromosome.

Both S and L variants grew well in soluble and insoluble sugars commonly found in plant fibre. However, like most other *B. fibrisolvens* strains, both variants were weakly cellulolytic, especially against crystalline cellulose (sigmacel size 20, 50 and 100, as well as various fibrous celluloses). Although strain E14 has been

reported as being cellulolytic (Orpin *et al.* 1985), the substrate used was CMC instead of crystalline cellulose. Since cellulose is one of the most important plant carbohydrates, we also tried to include it as carbon source for the subsequent experiments. For some carbon sources, there were indications that the S variant grew slightly faster than L, and this was examined separately in growth studies (Chapter 4).

Cell to cell communication experiments were carried out to examine whether S or L cells produced molecule(s) that mediate or inhibit attachment to surfaces. Examples are attachment protein (cipA) in *Clostridium* (Gelhaye *et al*. 1993, Fujino *et al*. 1993, Gerngross *et al*. 1993); or an attachment-inhibitor protein (Concanavalin A) in *Pseudomonas*, *Streptococci*, or *Flexibacter* (Fletcher 1977, 1980a, Marshall and Cruickshank 1973), although the molecule(s) are not necessarily proteins. Results suggested that no specific molecule produced by the S or L variant mediates or inhibits bacterial attachment to surfaces. However, it was found that a higher molecular weight molecule was secreted by the S variant into the cell free medium. This molecule was retained in the membrane filter, and was suspected to be extracellular polymer (EP). The S variant may synthesize higher molecular weight EP than that of the L variant, and the shorter EP in L variant may be due to the lack of certain component(s) or an incomplete elongation process in its EP biosynthesis. This was examined separately in Chapter 6 (characterization of EP).

Comparison of protein profiles were carried out to examine whether S produced attachment specific protein(s) but L cells did not. Such protein(s) may be involved

in attachment to surfaces or EP biosynthesis. There were no significant difference in the extracellular protein profiles of S and L variants. This supports the previous results for cytoplasmic protein profiles (Nili 1996), and the view that they are of the same species. However, it is also possible that a mutation has occured within the structural, not in the regulatory sequence of the gene(s). Therefore, protein(s) would still be expressed but without proper functions; PAGE would not resolve such differences. DNA-DNA hybridisation may resolve such differences, for example, using S DNA as probe, S and L chromosomal DNA can be differentiated by adjusting the stringency during the hybridization. However, this would not be possible if whole chromosomal DNA was used as probe, a few differences in base-pairing would not be significant compared to the whole chromosomal base-pairing, and is unlikely to be detectable by adjusting the hybridization stringency. Small DNA fragments are required as probes, and they must contain sequences with the mutation site. At this stage, we do not have such a probe. Randomly amplified PCR is an alternate method that could be used, but primer containing mutation sequence is required. Therefore, these two methods were not used for further S and L DNA analysis.

The procedure for protein sample preparations did not involve cell lysis; therefore the proteins obtained were predominantly extracellular, and can be categorized as follows:

1. released proteins (proteins that are released to the medium and are not attached to the cell surface or extracellular polymer)
2. cell-associated proteins (proteins that are attached to the cell surface at all times)

3. EP-associated proteins (proteins that are attached to the extracellular polymer at all times)

The extracellular protein samples that were prepared from liquid medium-grown cultures would be predominantly cell- (2) and EP-associated (3) proteins, since released (1) proteins would have been in the cell free medium, which was discarded. Proteins prepared from solid plate cultures were total (1, 2 and 3) extracellular proteins. Both samples (plates and liquid medium) contained cell-associated (2) proteins. Therefore only released (1) and EP-associated (3) proteins may be compared between these samples. If the major extracellular proteins were being released (1), differences should be observed between samples that were prepared from plate and liquid medium (lack of protein 1). The protein profiles of both preparations showed no significant difference, suggesting that most of the proteins were EP associated or that secreted proteins were trapped in the EP network. Similar observations (the existence of EP-associated proteins) have been reported for *B. fibrisolvens* 49 (Cotta and Hespell 1986), where several faint protein bands were observed when EP was fractionated by PAGE, and one of these was identified as a proteolytic enzyme. This was carried out by testing the EP (identified as extracellular polysaccharide/EPS) fraction for proteolytic activity. Problems in obtaining clear gel resolution were encountered due to protein association with EPS. It was also suggested that this association may affect protein migration through the gel, and the actual protein size may be smaller than that indicated. Extracellular protein complexes (EP, cellulosome or fimbriae) have also been reported for attachment proteins in *Clostridium* (Gelhaye *et al*. 1993, Fujino *et al*. 1993, Gerngross *et al*. 1993), *Azoprillum brasilense* (Dufrêne *et al.* 1996), *E. coli (*Salit and Gotschlich 1977a,b), *Gonococci* (Pearce

and Buchanan 1980, Watt and Ward 1980), or *Mycoplasma* (Collier 1980, Ofek and Beachey 1980); or attachment-inhibitor proteins in *Pseudomonas* (Fletcher 1977, 1980a), *Streptococci* (Østarvik 1977) *Flexibacter* (Marshall and Cruickshank 1973), and *Ruminococcus* (Miron *et al.* 2001).

Cellulase has been suspected to be involved in the attachment of *R. albus* to cellulose, and has been found to be associated with capsular and cell wall fractions (Miron *et al.* 2001), similar to those in *Fibrobacter succinogenes* (McGavin *et al.* 1990) and *F. intestinalis* (Miron and Forsberg 1998, 1999). Although it has been reported to be associated with a cellulosome-like complex (Wood *et al.* 1982), Miron *et al.* (2001) found that most of the bacterial cellulase activity was not integrated into cellulosome-like complexes. The enzyme may have been dissociated during isolation of subcellular fractions (Miron et al. 2001). However, noncellulosomal glycanases, such as celA and celB of *R. albus* SY3, endoglucanases I, II, III and IV of *R. albus* F-40, have also been reported (Poole *et al.* 1990; Karita *et al.* 1997; Nagamine *et al.* 1997; White *et al.* 1997; Ohara *et al.* 2000). The status of glycanases in *R. albus* remain to be proven.

There was no significant effect of carbon source (glucose, cellobiose, xylan, crystalline cellulose, sucrose, maltose or starch) on the protein profiles of both variants, and protein profiles were identical. For an insoluble substrate such as cellulose, it was not practical to use liquid medium grown cells, due to interference of the remaining cellulose. In addition, a much larger volume of phosphate buffer was required to harvest the cells, and longer periods of time to concentrate the protein samples. Both concentrating methods (freeze drying or

microcentrifugal concentration) had disadvantages. For freeze-drying, the dried samples also contained buffer salts, and this affected the minimal volume of water required to redissolve the samples. On the other hand, EP often blocked the pores when microcentrifugal concentration was used for concentrating the protein samples.

The *Sal* I and *Pst* I restriction patterns of S and L total chromosomal DNA were similar, suggesting that they are the same species. However, one could be a mutant of the other, since unless the restriction enzyme cut exactly at the mutation site, no pattern change would be observed. Small deletions or insertions would also be unresolved because agarose gel electrophoresis would not resolve differences of a few bases. If differences had been found, they would provide a shortcut to identifying gene(s) involved. However, to detect small differences, restriction with many enzymes would have to be undertaken, with a low probability of success. Therefore, this experiment was more to confirm similarities rather than differences and to verify previous data that showed no difference in *EcoR* I and *Hae* III digestion patterns of S and L DNA profiles (Nili 1996).

Plasmid-dependent adherence or plasmid-dependent EPS biosynthesis has been reported (Ofek and Beachey 1980, Leigh *et* al. 1985, Whitfield 1988, Kranenburg *et al*. 1991). Since both S and L variants contain a plasmid, restriction patterns were examined, to explore the possibility that the differences between S and L variants may be observed in the plasmids. This approach should be more specific and clearer than that of total chromosomal DNA. Results showed no differences in

Hind III, *Pst* I, *Sal* I, *BamH* I, *Xba* I and *Pst* I-*Xba* I restriction profiles. Southern analysis also indicated that the two plasmids were identical. However, the limitations of agarose gel electrophoresis and restriction digestion analysis, are as described for chromosomal DNA. Indigenous plasmids have been isolated from various *B. fibrisolvens* strains (Teather 1982, Mann 1986, Gennaro *et* al. 1987, Ware *et al.* 1992, Hefford *et* al. 1993, Beard *et al*. 1995, Kobayashi *et al*. 1995). Most of these plasmids (pOM1, pRJF1, pRJF2) were very similar in size (approximately 2.8 kb) to those plasmids isolated from S and L cells. This may be a plasmid common to many *B. fibrisolvens* strains, although this was not tested by DNA-DNA hybridisation. Only pOM1 exhibited a distinctly different restriction pattern and replication mechanism (via rolling circle) (Hefford *et al.* 1993). However, the complete nucleotide sequences, as well as the replication mechanisms of the S and L plasmids have not been analysed. Among the plasmid-bearing strains of *B. fibrisolvens*, there are no reports of their attachment properties, and only OB156 exhibited similarities with the S variant. OB156 also displayed a tendency to aggregate during the logarithmic phase, and be dispersed during stationary phase (Beard *et al*. 1995).

A phylogenetic comparison was also undertaken, in which the restriction map for 16S rDNA was analysed. The fact that no difference was observed between S and L 16S rDNA maps supports the hypothesis that they have identical origins. Slight differences were observed when S and L were compared with another strain, *B. fibrisolvens* H17c, and confirms a previous observation of strain differences in 16S rDNA reported by Forster *et al.* 1996. The data therefore support the identification of S and L cells as belonging to *B. fibrisolvens* and both belonging

to the same strain. *B. fibrisolvens* has been classified phylogenetically, and results showed that plasmid bearing strains (OB156, OB157, OB192, OB189 and 49) appeared to be different from non-bearing strains of *B. fibrisolvens* (Forster *et al*. 1996).

In conclusion, the phenotype is stable, there is no indication that the variants produce molecule(s) that inhibit or mediate attachment, and a larger amount of EP is produced by the S variant compared with the L variant. The effects of carbon source on growth and protein profiles were not significant. There was no difference in S and L extracellular protein, chromosomal DNA, plasmid and 16S rDNA profiles. These analyses strongly suggest that both variants are the same strain of *B. fibrisolvens*.

CHAPTER 4

NUTRIENT UTILIZATION

4.1 Introduction

Within the rumen, substrates are often complex and resistant to digestion. They may consist of layers of cellulose, lignin and hemicellulose in plant cell walls or protein coats surrounding starch grains. A number of enzymes must act in combination in order to digest these substrates, and attachment may therefore be especially important (Morris and Cole 1987). Direct examination and *in vitro* studies of ruminal microorganisms have shown that many bacteria must attach to cellulose substrates in order to promote digestion (Kudo *et al*. 1987). An adherence defective mutant of *F. succinogenes* that retained a full complement of cellulase enzymes was found to be unable to digest cellulose (Gong and Forsberg 1989). Within the rumen, starch-digesting bacteria adhere to granules of starch (Costerton *et al.* 1978), and it is known that certain bacteria adhere preferentially to particular types of plant cell walls (Latham *et al.* 1978). These reports demonstrate the importance of bacterial attachment to substrates and are likely to be a significant factor controlling survival of microbial communities, especially within competitive environments such as the rumen.

In some cases, attachment is not necessarily related to substrate digestion. Less refractory types of plant cell wall can be degraded by soluble enzymes without the

need for adhesion (Akin *et al.* 1974, Akin 1980), and studies on several strains of *B. fibrisolvens* have shown that adherence was not always followed by cellulolysis (Rassmussen *et al.* 1989). For some non-ruminal bacteria, cellulose degradation also did not depend on cell-fibre contact (Kauri and Kushner 1985).

Although bacterial surfaces may bind proteins or glycoproteins (Gibbon and Quershi 1978, Ofek and Beachey 1980), there is no conclusive evidence that proteins or peptides in the epithelial cell membranes of various animal tissues are involved in bacterial recognition (Etzler and Branslator 1974, Ofek and Beachey 1980). Sugar moieties are the only residues identified as bacterial receptors on epithelial cells (Ofek *et al.* 1977, Ofek and Beachey 1980). Examples are mannose for certain *E. coli* strains (Ofek *et al.* 1977, Ofek and Beachly 1978), fucose for *Vibrio cholera* (Jones 1980), sialic acid for *Mycoplasma sp.* (Collier 1980), β-galactosyl residues for *E. coli* K-88 (Ofek and Beachey 1980), and N-acetyl-D-galactosamine for *Leptotricia buccalis* (Kondo *et al.* 1976). This may also reflect different bacterial populations in different regions within the rumen or other sites in the gastrointestinal tract (Etzler and Branslator 1974). Therefore, when bacteria attach to plant fibre, it is likely that they attach via sugar moieties on the fibre surface. In the present chapter, various carbohydrates commonly found in plant polysaccharides were used to study the effect of attachment on carbon utilization, as well as the effect of ligands on attachment processes.

Previous studies on S and L variants showed no significant difference in their growth on soluble maltose (Nili 1996). In the present chapter, experiments were carried out to determine whether attachment of S variants has any significant

effect on the utilization of various carbon and nitrogen sources. Soluble (starch) and insoluble (xylan and cellulose) complex sugars, as well as simple (glucose, cellobiose, sucrose and maltose) sugars commonly found in plant polysaccharides were used as carbon sources, while casein was used as the nitrogen source.

4.2 Results

4.2.1 Growth in soluble carbon sources

The growth rates of *B. fibrisolvens* E14 sticky (S) and loose (L) on various soluble carbon sources (glucose, cellobiose, sucrose, maltose or starch) were compared. Mid log BHI grown cells were washed anaerobically then inoculated into defined medium containing various soluble carbon sources. Cell growth was monitored by optical density (OD 600 nm).

Of the 2 variants tested here, the S variant grew slightly faster (Figure 4.1 - 4.5), however the differences were not significant. These results were obtained for cultures that were initially grown in BHI medium before inoculating into defined medium containing various carbon sources. Similar results were obtained when the cultures were initially grown in glucose or cellobiose (data not shown), except for a slightly shorter lag phase and higher final OD 600 nm.

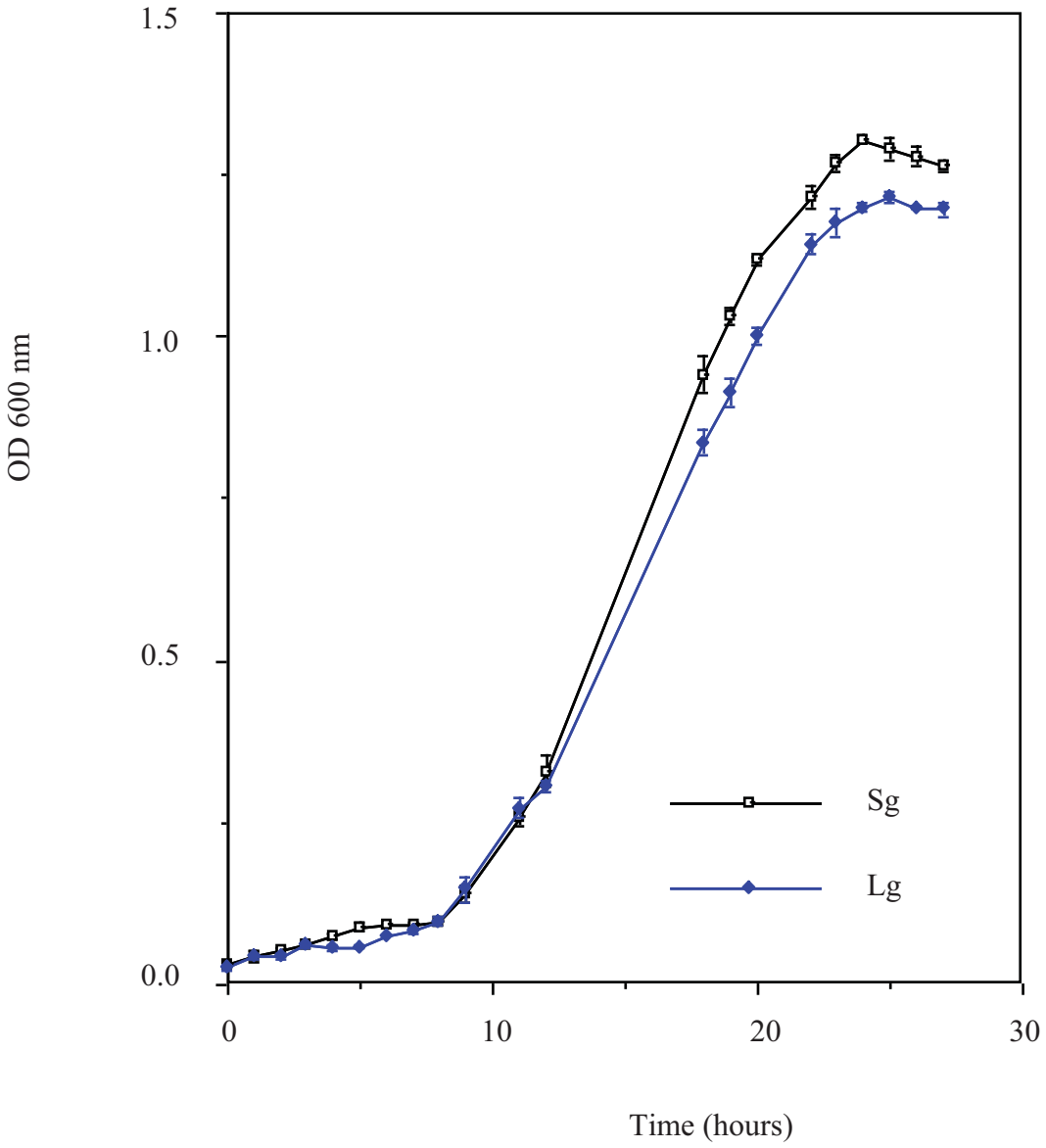


Figure 4.1 Growth of *B. fibrisolvens* E14 variants sticky and loose in glucose as a sole carbon source.

Defined medium (Nili and Brooker 1995) containing 0.5 % (w/v) glucose was inoculated with *B. fibrisolvens* E14 S (Sg) or L (Lg) that had been grown in BHI medium, and was incubated anaerobically at 39^{o}C. Cell growth was monitored by measuring the optical density (OD 600 nm). Values represent the mean and standard error of two independent triplicate experiments.

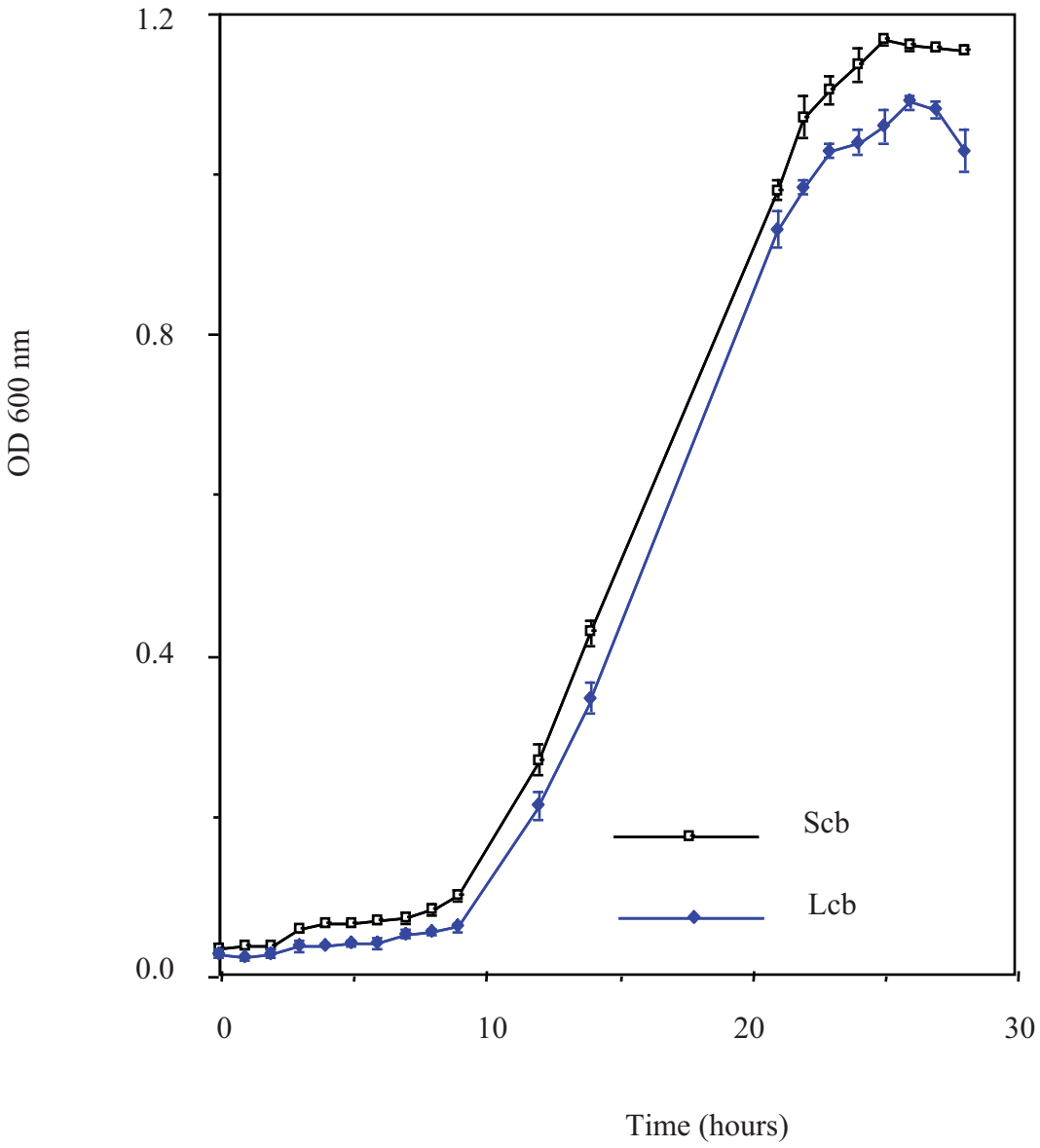


Figure 4.2 Growth of *B. fibrisolvens* E14 variants S and L in cellobiose as a sole carbon source.

Defined medium (Nili and Brooker 1995) containing 0.5 % (w/v) cellobiose was inoculated with *B. fibrisolvens* E14 S (Scb) or L (Lcb) that had been grown in BHI medium, and was incubated anaerobically at 39^{o}C. Cell growth was monitored by measuring the optical density (OD 600 nm). Values represent the mean and standard error of two independent triplicate experiments.

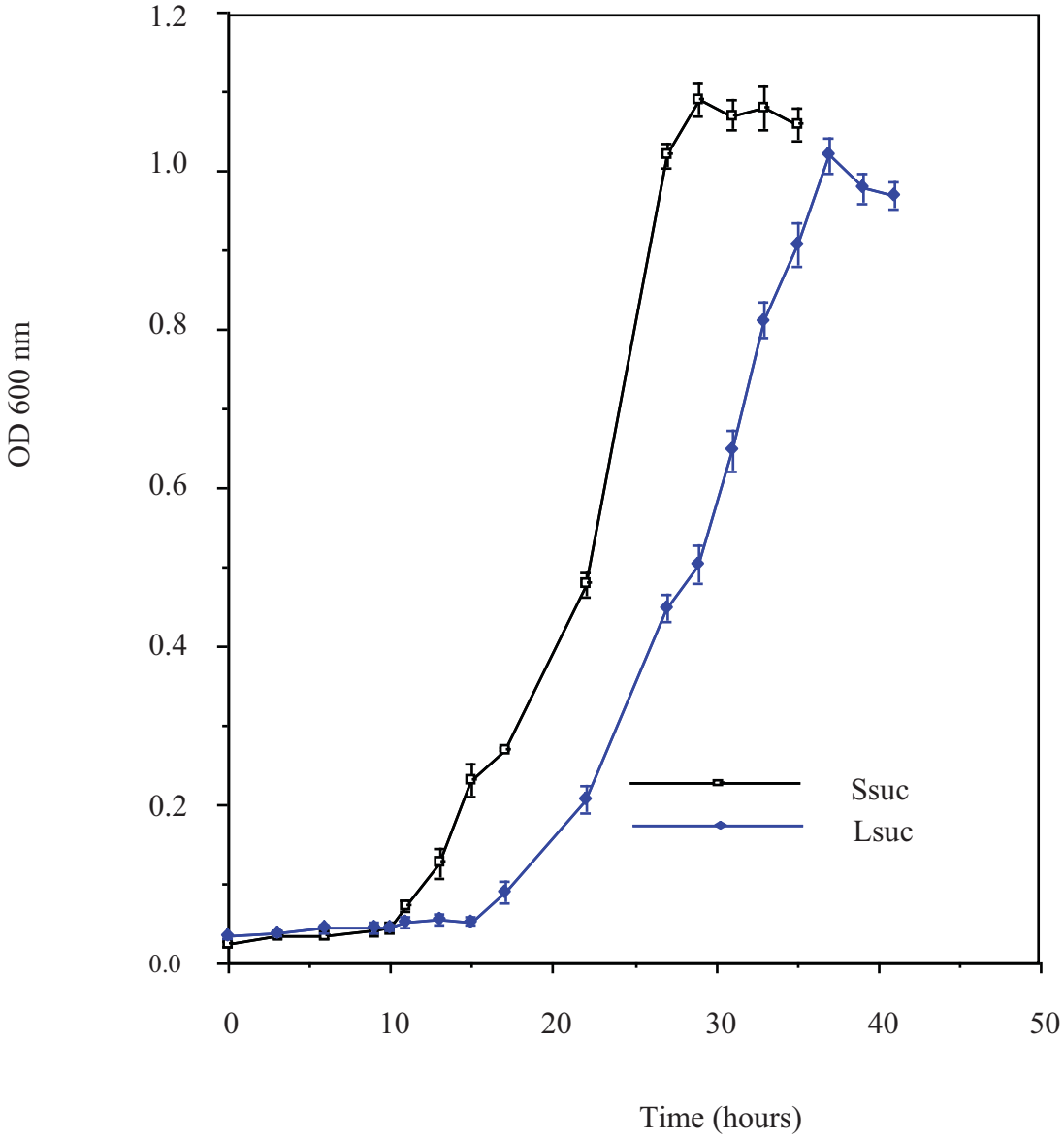


Figure 4.3 Growth of *B. fibrisolvens* E14 variants in sucrose as a sole carbon source.

Defined medium (Nili and Brooker 1995) containing 0.5 % (w/v) sucrose was inoculated with *B. fibrisolvens* E14 S (Ssuc) or L (Lsuc) that had been grown in BHI medium, and was incubated anaerobically at 39^{o}C. Cell growth was monitored by measuring the optical density (OD 600 nm). Values represent the mean and standard error of two independent triplicate experiments.

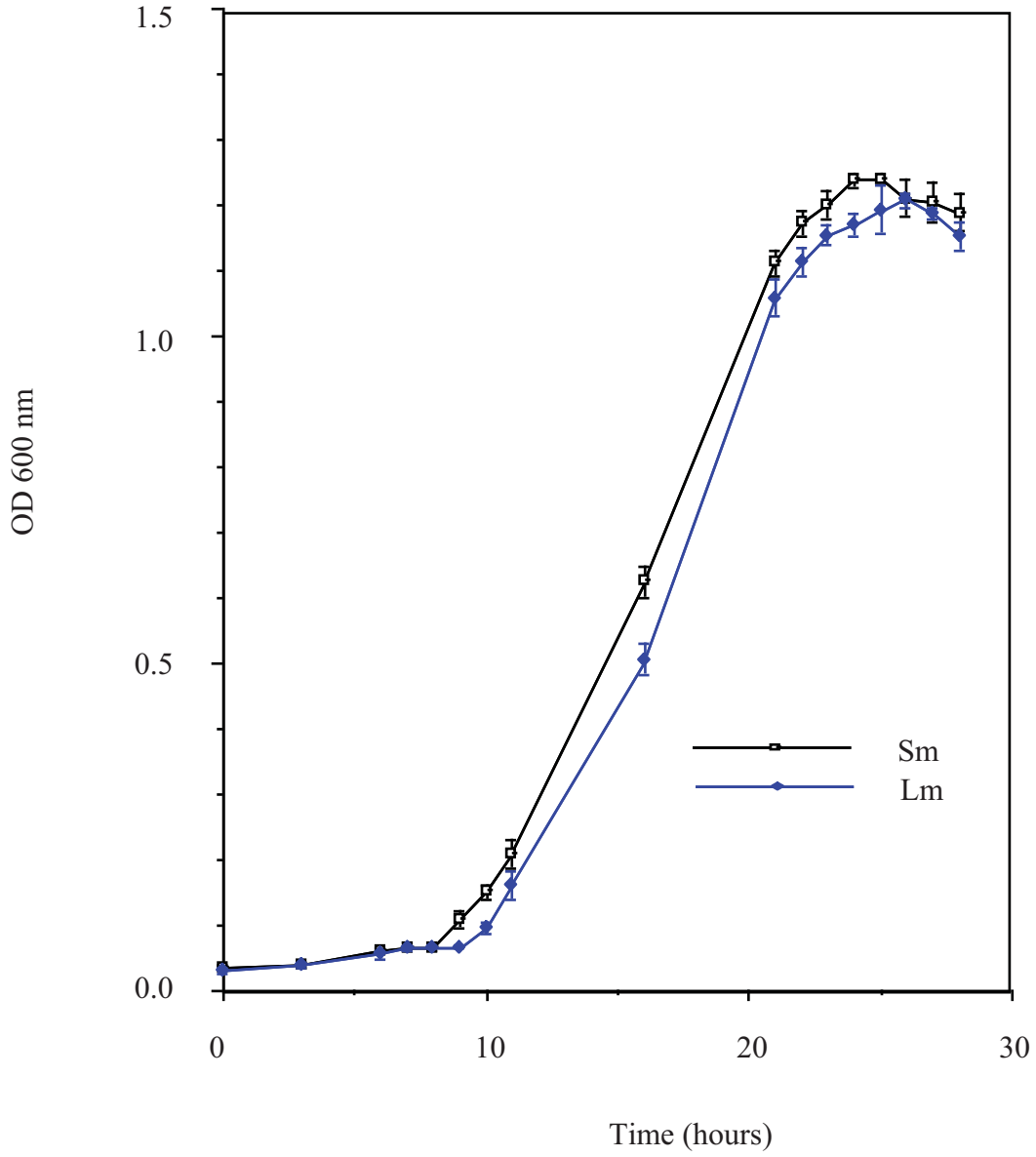


Figure 4.4 Growth of *B. fibrisolvens* E14 variants in maltose as a sole carbon source.

Defined medium (Nili and Brooker 1995) containing 0.5 % (w/v) maltose was inoculated with *B. fibrisolvens* E14 S (Sm) or L (Lm) that had been grown in BHI medium, and was incubated anaerobically at 39^{o}C. Cell growth was monitored by measuring the optical density (OD 600 nm). Values represent the mean and standard error of two independent triplicate experiments.

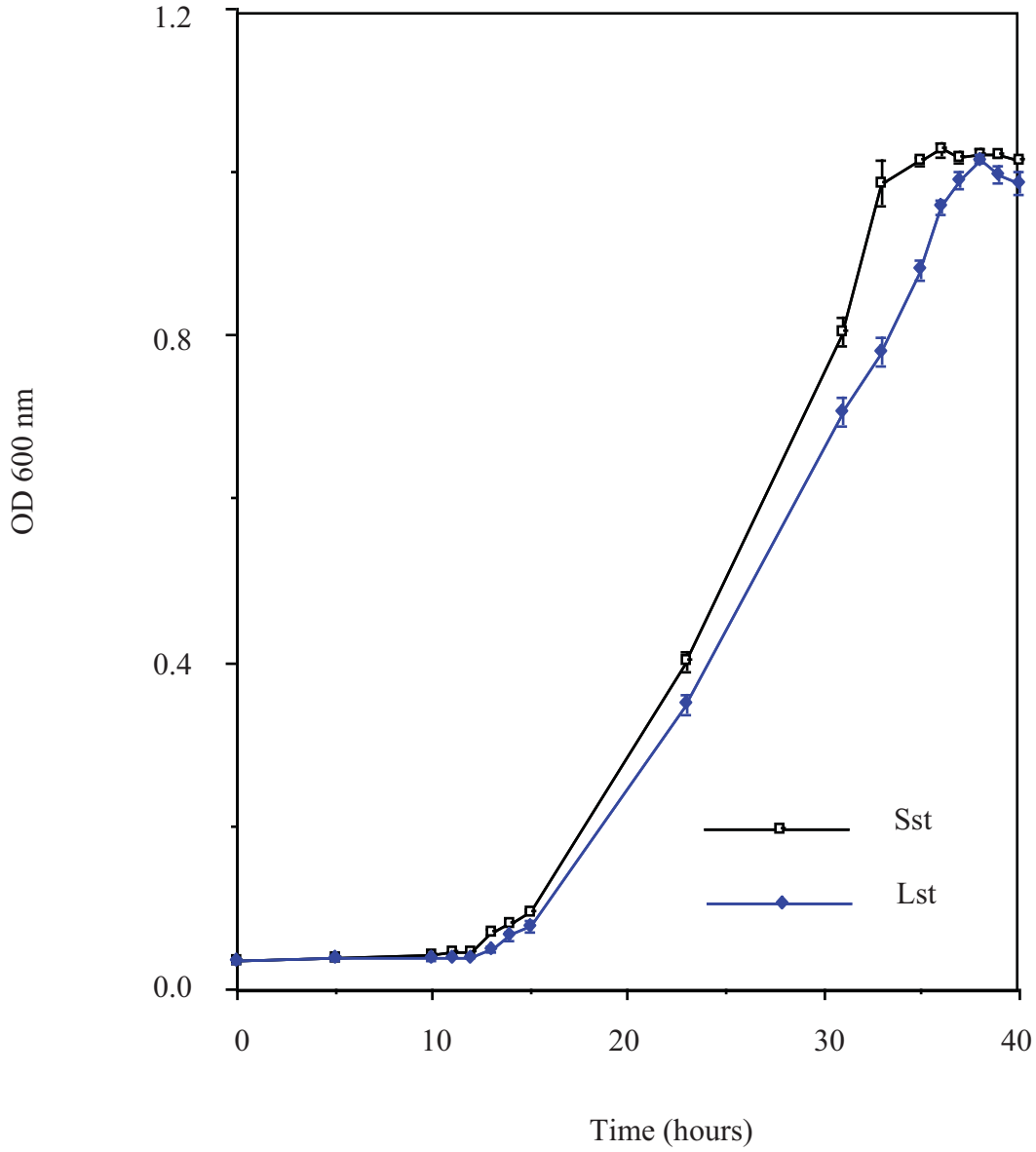


Figure 4.5 Growth of *B. fibrisolvens* E14 variants in starch as a sole carbon source.

Defined medium (Nili and Brooker 1995) containing 0.5 % (w/v) starch was inoculated with *B. fibrisolvens* E14 S (Sst) or L (Lst) that had been grown in BHI medium, and was incubated anaerobically at 39^{o}C. Cell growth was monitored by measuring the optical density (OD 600 nm). Values represent the mean and standard error of two independent triplicate experiments.

4.2.2 Growth in insoluble carbon sources

The growth rates of *B. fibrisolvens* E14 S and L on various insoluble carbon sources (xylan or cellulose) were compared. Mid log BHI grown cells were washed anaerobically then inoculated into defined media containing insoluble carbon sources (xylan or cellulose). Cell growth was monitored by total protein, since it was not possible to monitor cell growth using optical density, due to interference by the insoluble substrate.

The total protein method was standardised before use. S cells were grown in defined media containing glucose. Cell growth was monitored by OD 600 nm and total protein was estimated as described in the methods. The results (Figure 4.6) showed a good correlation between cell growth, protein content, and time.

The S cells grew slightly better in xylan (Figure 4.7) compared to the L cells. No significant difference was observed when washed xylan was used as a substrate (data not shown). It was difficult to compare data for growth on crystalline cellulose (Figure 4.8), because the variations were relatively high, possibly due to the slow cell growth. The experiment was carried out using cultures that were initially grown in BHI medium. Attempts to use cultures initially grown in glucose or cellobiose as outlined in Figure 3.5 were not undertaken, since preliminary observations (Chapter 3) did not show any significant effect on cell growth of both S and L variants.

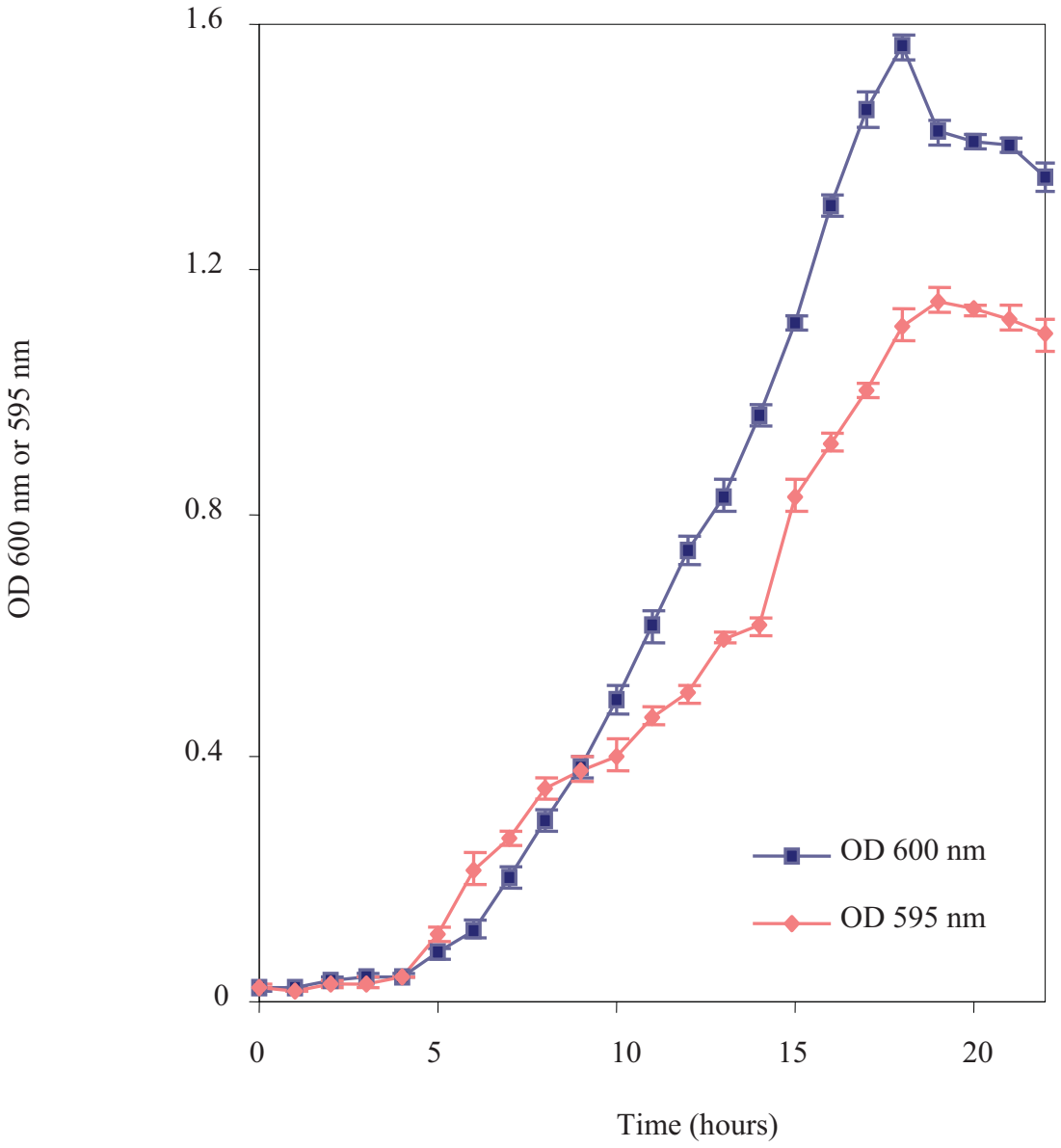


Figure 4.6 Growth of *B. fibrisolvens* E14 S as monitored by optical density and total protein estimation.

Defined medium (Nili and Brooker 1995) containing 0.5 % (w/v) glucose was inoculated with *B. fibrisolvens* E14 S that had been grown in BHI medium, and was incubated anaerobically at 39^{o}C. Cell growth was monitored by measuring the optical density (OD 600 nm) and total bacterial protein estimation (OD 595 nm). Values represent the mean and standard error of two independent triplicate experiments. The total protein was expressed as OD 595 nm instead of protein concentration.

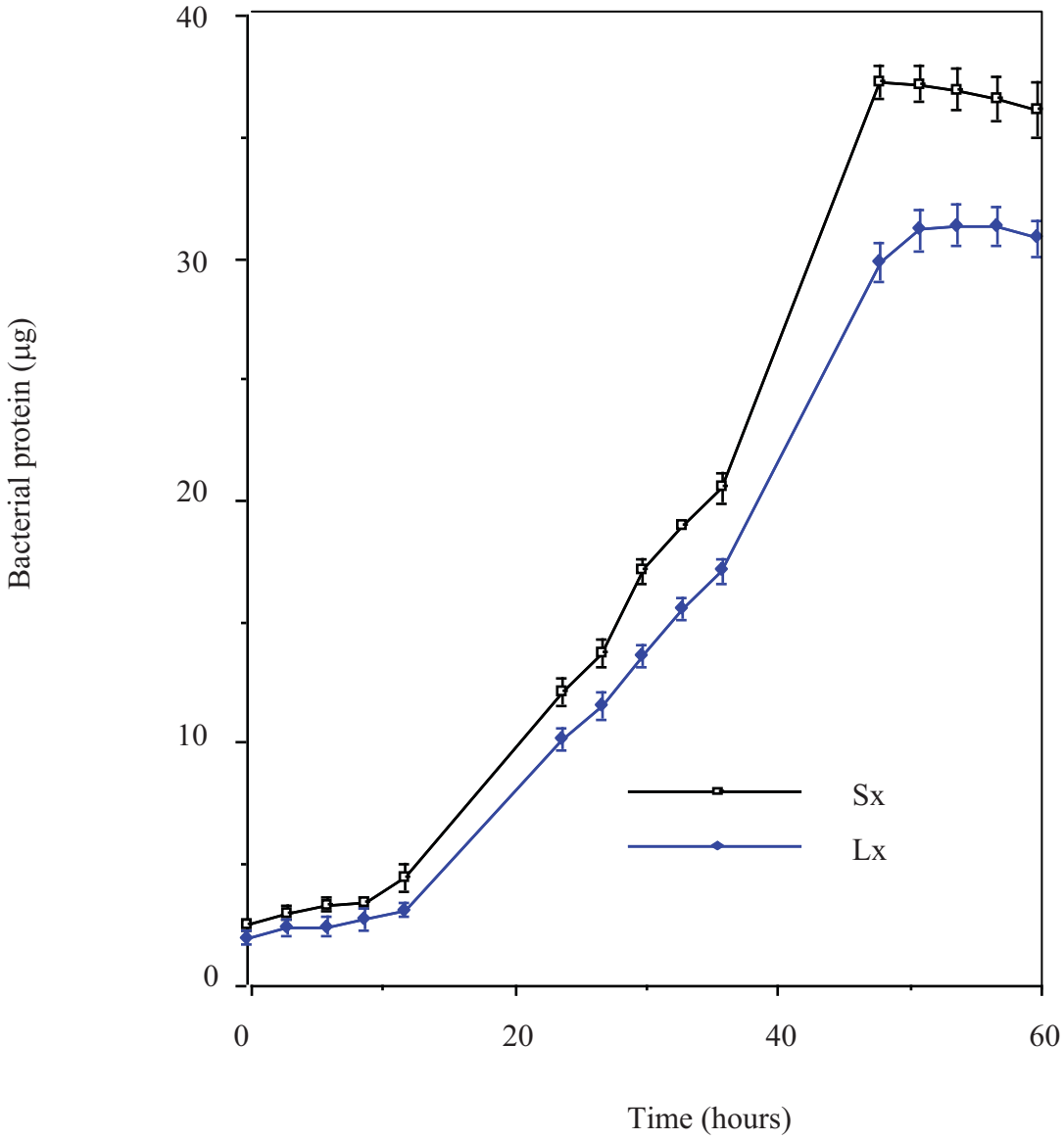


Figure 4.7 Growth of *B. fibrisolvens* E14 variants S and L in defined medium with xylan as a sole carbon source.

Defined medium (Nili and Brooker 1995) containing 0.5 % (w/v) xylan was inoculated with *B. fibrisolvens* E14 S (Sx) or L (Lx) that had been grown in BHI medium, and was incubated anaerobically at 39^{o}C. Cell growth was monitored by total bacterial protein. Values represent the mean plus standard errors of two independent experiments carried out in triplicate.

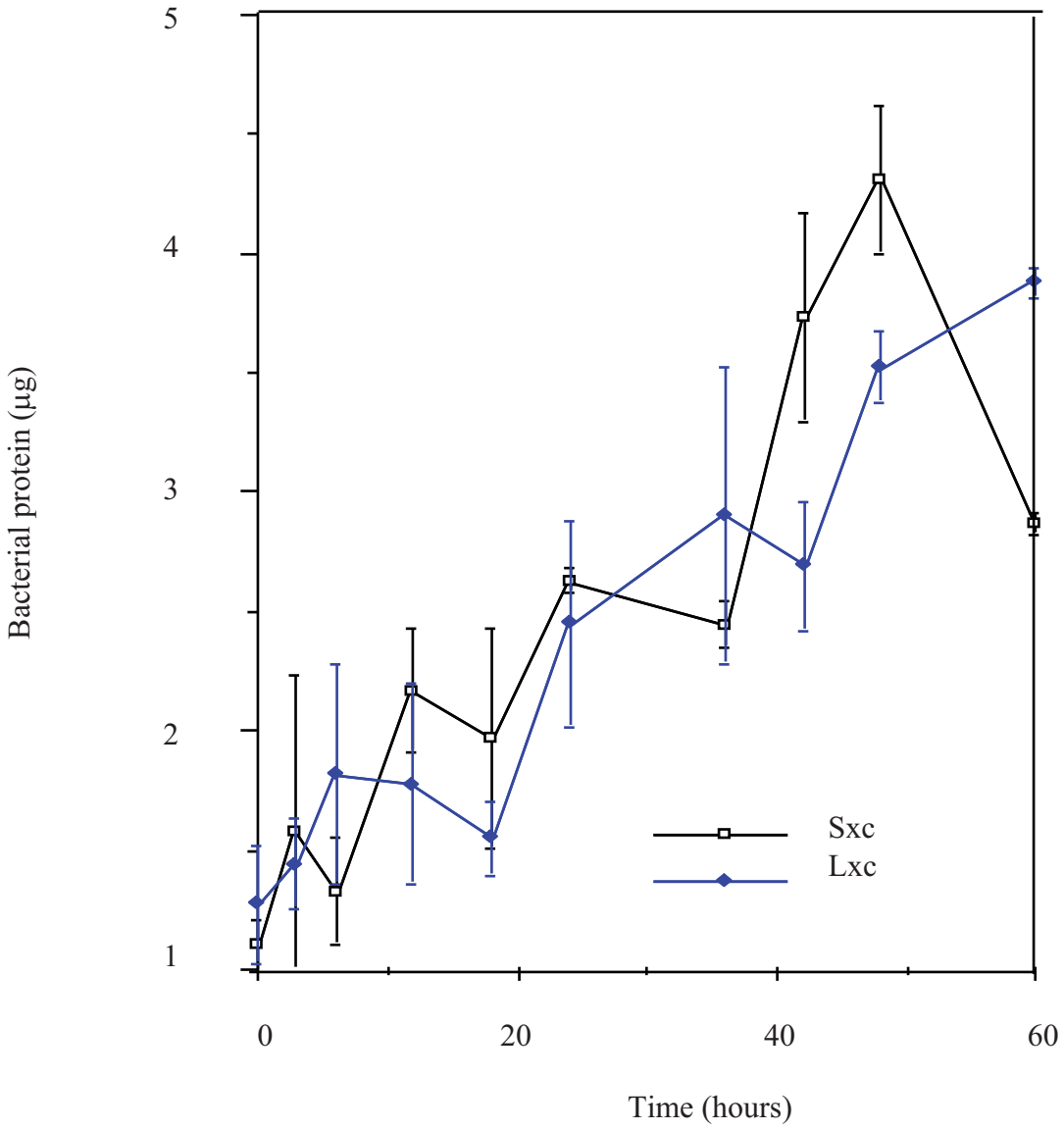
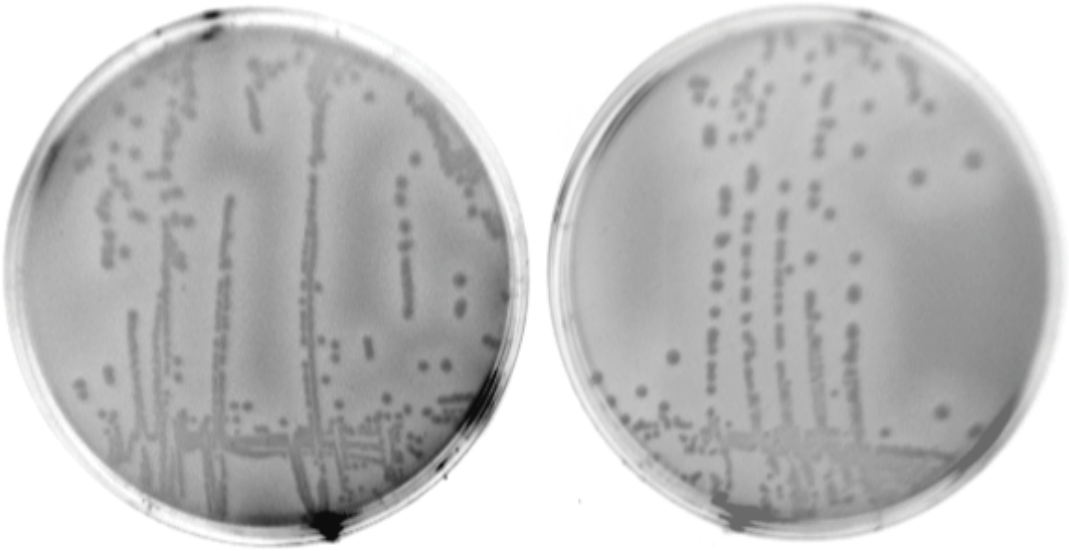


Figure 4.8 Growth of *B. fibrisolvens* E14 variants in defined medium with cellulose as a sole carbon source.

Defined medium (Nili and Brooker 1995) containing 0.5 % (w/v) cellulose was inoculated with *B. fibrisolvens* E14 S (Sxc) or L (Lxc) that had been grown in BHI medium, and was incubated anaerobically at 39^{o}C. Cell growth was monitored by total bacterial protein. Values represent the mean and standard errors of two independent experiments carried out in triplicate.

The activity of S and L variants toward crystalline cellulose was compared quantitatively using the dry weight method (Kellogg and Wood 1988). Cells were grown in defined medium containing a known weight of cellulose, and the remaining cellulose after incubation was measured. Cellulolytic activity was expressed as % hydrolysis, defined as the ratio of the remaining cellulose to the initial cellulose concentration. The results were 4.4 ± 0.2 % and 3.5 ± 0.3 % hydrolysis for S and L cells, respectively.

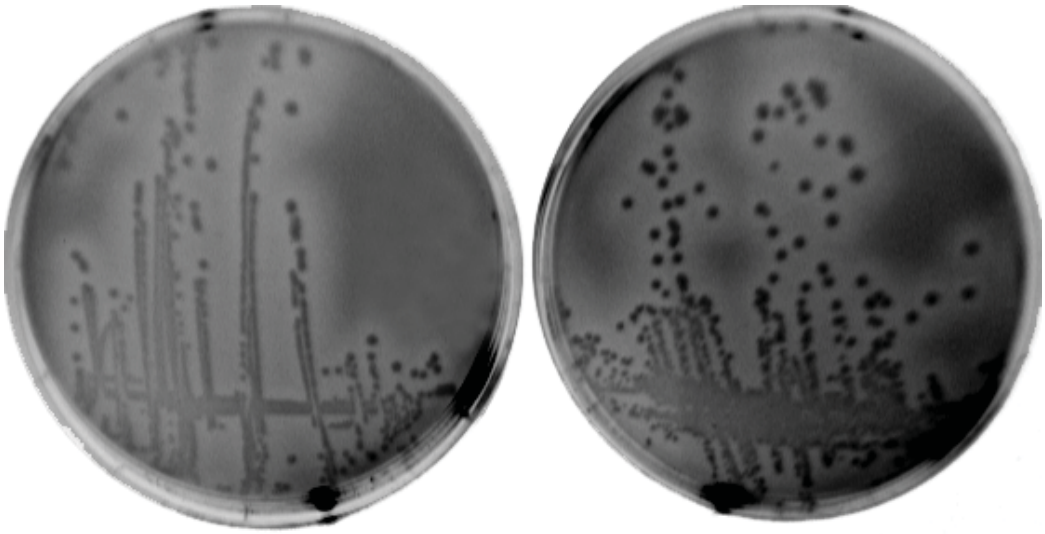
The xylanase and CMCase activities of S and L variants were compared non-quantitatively on agar plates. Cells were streaked onto xylanase or CMC plates and treated as described in the methods. Therewas no significant difference in xylanase (Figure 4.9) or CMCase (Figure 4.10) activities of S and L variants. CMC was used since clear zones were too small to quantify when crystalline cellulose was used as a substrate. Cell growth was very poor, even though incubation was carried out for up to 3 days.



S L

Figure 4.9 Xylanase plate assay of S and L variants.

B. fibrisolvens E14 variants S and L were streaked onto defined medium (Nili and Brooker 1995) containing 0.5 % (w/v) xylan and were incubated anaerobically at 39^{o}C for 36 hours.



S L

Figure 4.10 CMCase plate assay of S and L variants.

B. fibrisolvens E14 S and L were streaked onto defined medium (Nili and Brooker 1995) containing 0.5 % (w/v) CMC and were incubated anaerobically at 39^{o}C for 36 hours. Plates were stained with 0.1 % (w/v) Congo red solution, destained with 1 M NaCl and stabilized with 5 % (v/v) acetic acid.

The enzymatic activity of S and L variants toward xylanase and CMC were compared quantitatively. Total extracellular enzyme was estimated by measuring the reducing sugar released after incubation. The results are shown in Table 4.1.

Table 4.1 Xylanase and CMCase activities of *B. fibrisolvens* E14 S and L

Variant	Specific activity (mU/mg protein)	
	Xylanase	CMCase
S	24.0 ± 0.8	5.1 ± 0.4
L	20.9 ± 1.2	3.4 ± 0.2

Values represent two independent triplicate assays. Activities were expressed in international units (IU), which was defined as the amount of reducing sugar released (μmol glucose or xylose/minute) as determined using the Nelson-Somogy reagent (Ashwell 1957, Wood and Bhat 1988). The specific activity was expressed as IU per mg protein. The assay was performed as described by Groleau and Forsberg (1981).

4.2.3 Comparison of nitrogen utilisation between *B. fibrisolvens* S and L

Since there was no significant effect of attachment on carbon utilisation, an attempt was made to study the effect of attachment on nitrogen utilization. The growth rate and proteolytic activity of S and L variants were compared. For preliminary studies, the two variants were compared semiquantitatively on plates using casein as substrate. The results showed no significant difference (Figure 4.11). However, ammonium chloride was also added, since both variants grew poorly in defined medium with only casein as a nitrogen source. This result may therefore not reflect the full capacity of the two variants to degrade casein. The growth rates of S and L variants were therefore compared in defined medium

containing casein and a mixture of casein and ammonium chloride. There was some difference in growth rates in casein alone (Figure 4.12), but not when casein and ammonium chloride were used together as substrates (Figure 4.13).

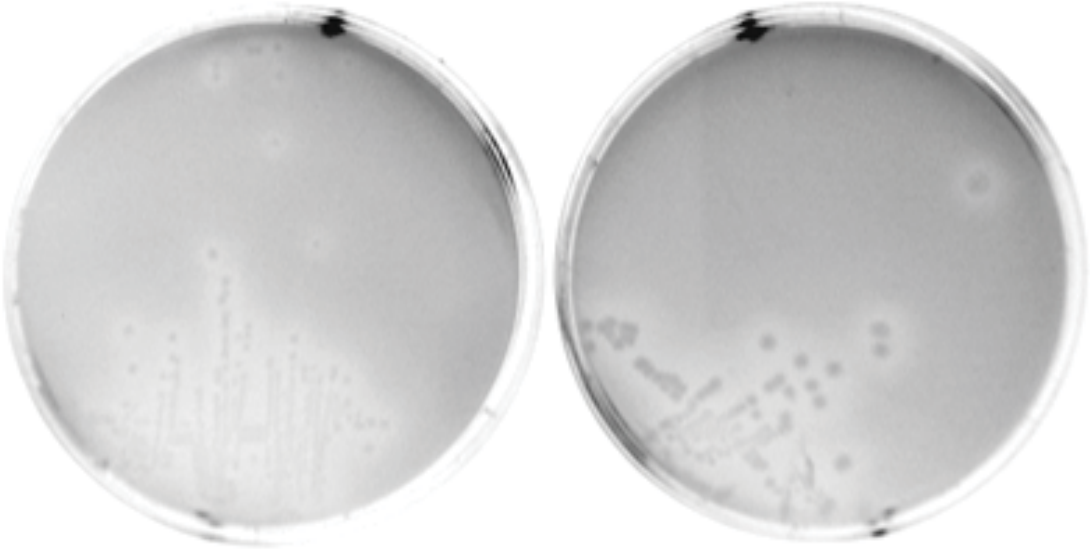


Figure 4.11 Proteolytic plate assay of S and L variants.

B. fibrisolvens E14 S and L were streaked onto defined medium (Nili and Brooker 1995) containing 0.25 % (w/v) casein plus 0.25 % (w/v) ammonium chloride and were incubated anaerobically at 39^{o}C for 36 hours. Plates were then flooded with 1 M HCl to precipitate the remaining casein.

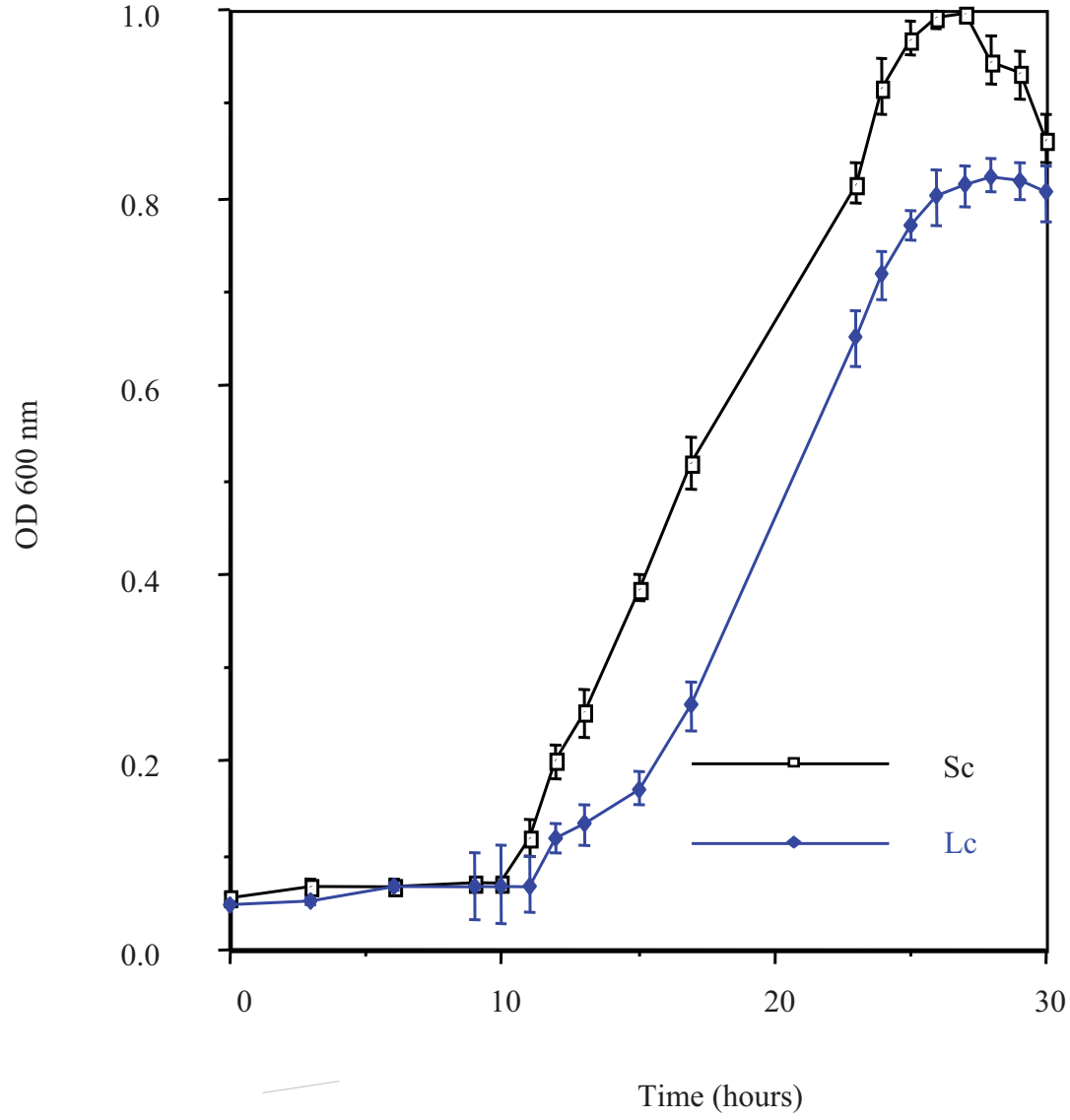


Figure 4.12 Growth of *B. fibrisolvens* E14 S and L in casein as a sole nitrogen source.

Defined medium (Nili and Brooker 1995) containing 0.5 (w/v) casein was inoculated with *B. fibrisolvens* E14 S (Sc) or L (Lc) that had been grown in BHI medium, and was incubated anaerobically at 39^{o}C. Cell growth was monitored by optical density (OD 600 nm). Values represent the mean and standard error of two independent triplicate experiments.

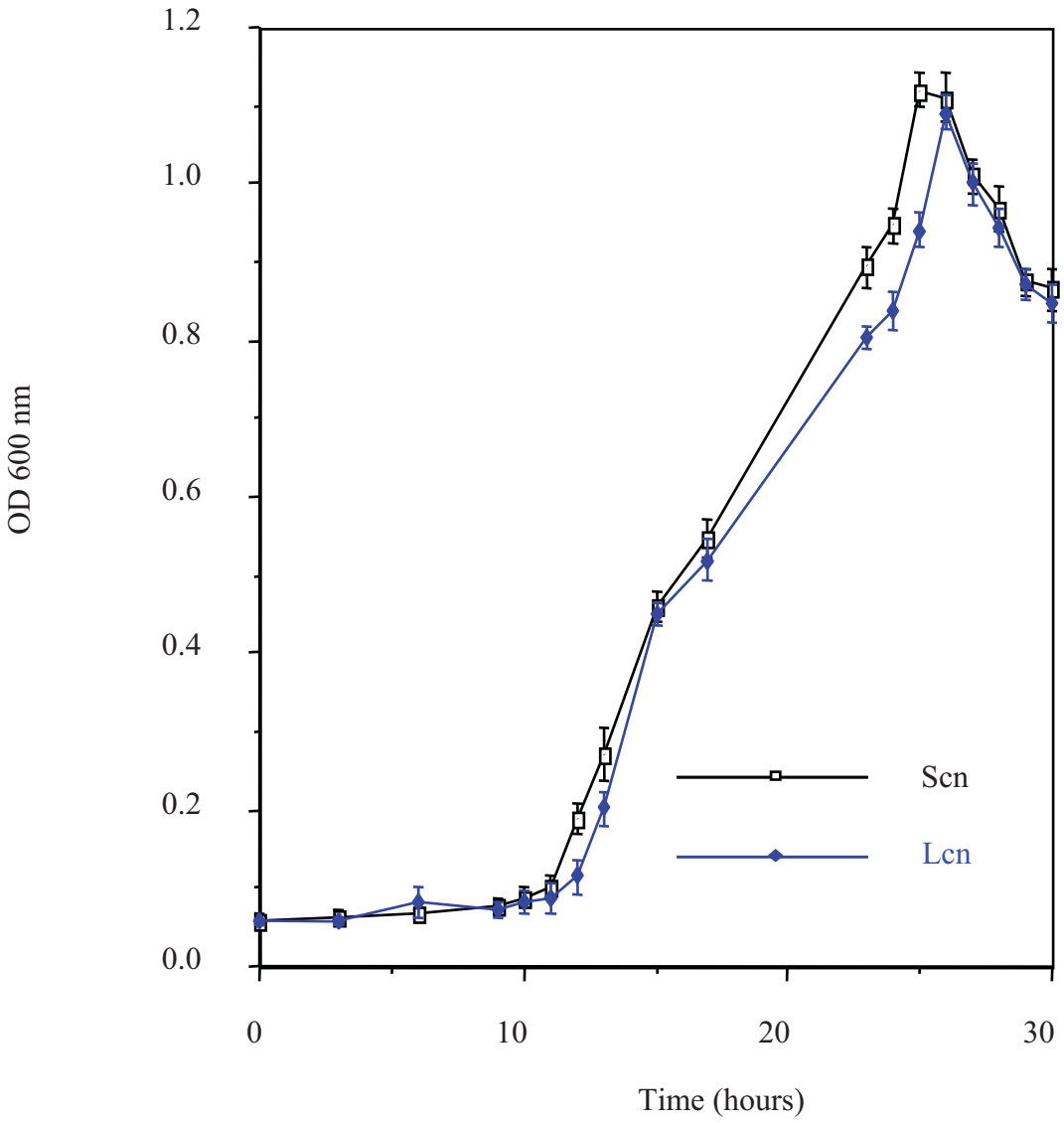


Figure 4.13 Growth of *B. fibrisolvens* E14 S and L in casein and ammonium chloride as nitrogen sources.

Defined medium containing 0.25 % (w/v) casein and 0.25 % (w/v) ammonium chloride was inoculated with *B. fibrisolvens* E14 S (Scn) or L (Lcn) that had been grown in BHI medium, and was incubated anaerobically at 39^{o}C. Cell growth was monitored by optical density (OD 600 nm). Values represent the mean and standard error of two independent triplicate experiments.

A further comparison of proteolytic activity in S and L variants was carried out enzymatically, rather than by cell growth. The proteolytic activity of *B. fibrisolvens* has been reported as extracellular and associated with high molecular weight extracellular polymer (Cotta and Hespell 1986). For that reason, the enzyme was prepared using gradient centrifugation (Figure 4.14), to separate secreted, cell and polymer associated proteins. However, the plate assay (Figure 4.15) showed no significant difference between S and L variants. Most activity seemed to be associated with fraction 2, which was the polymer associated protein.

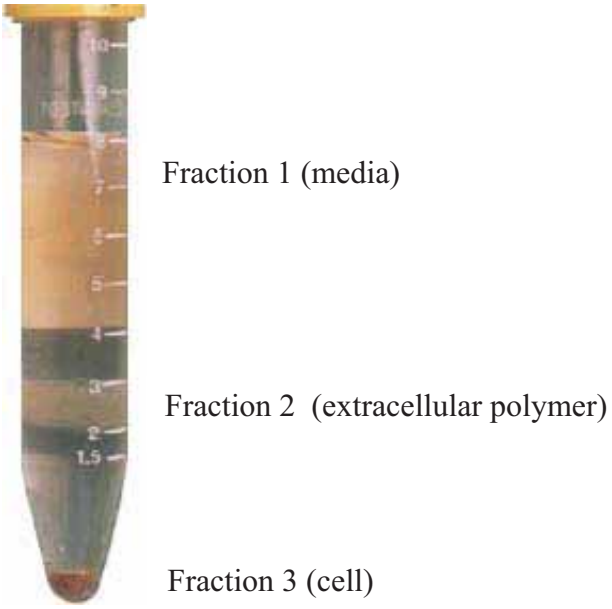


Figure 4.14 Isolation of extracellular proteins by gradient centrifugation.

The cell suspension was applied to the top of a glycerol gradient (30 % - 75 %) and centrifuged at 6000 rpm, 4^{o}C for 30 minutes. Fractions 1, 2 and 3 were collected as secreted, polymer and cell associated proteins, respectively.

Figure 4.15 Proteolytic plate assay.

S1, S2, S3, L1, L2 and L3 were fraction 1, 2 and 3 obtained by gradient centrifugation (Figure 4.14) of S or L samples. M was control buffer. Samples were loaded into the wells and incubated at 39^{o}C for 3 hours. The plate was then flooded with 1 M HCl to stop the reaction and to precipitate the remaining casein.

Proteolytic activity was also measured using azocasein as a substrate as described by Cotta and Hespell (1986). Only fraction 2 was used as a sample. The results were 26.1 ± 2.5 and 24.3 ± 1.9 unit/ml culture for S and L variants, respectively. These values represent the mean of independent triplicate assays.

4.3 Discussion

Throughout the experiments in this chapter, the S cells tended to grow better than L cells. Similarly, for enzymatic activities toward xylan, cellulose and casein. However the differences were not statistically significant.

Because of the tendency for surfaces to adsorb dissolved solutes, it has been proposed that in various environments, nutrients will be adsorbed and concentrated at surfaces. The ability to attach to surfaces should be an advantage for surface-associated cells, and substrate utilization should be enhanced (Fletcher 1991) since this would provide a constantly renewed supply of nutrients (Costerton and Irvin 1981, Leigh and Coplin 1992). Therefore, the S variant should show better growth than the L variant. For soluble and simple substrates (glucose, cellobiose, sucrose or maltose) this may not necessarily be true, since attachment is not really required for these highly soluble substrates. Unlike macromolecules, low-molecular-weight materials are usually transported directly into the cell by diffusion or via membrane permeases, binding proteins or group translocation systems (Fletcher 1991). In addition, the diffusion effect of nutrients within the liquid media may reduce the advantage of attachment. Liquid media with simple soluble nutrients may be considered as an ideal solution. Low-molecular weight solutes in the medium are always in equilibrium, with both adsorption and desorption continuously occurring. Each time a substrate is taken up by the cell, a diffusion gradient would be established, which would continuously deliver fresh substrate to the cell. Therefore, direct substrate transport and diffusion effects may reduce the advantage of attachment in S toward soluble and simple substrates, resulting in slightly different growth, compared to L cells.

The diffusion effect would be less if the rate of diffusion is lower than the rate of nutrient utilization. This may occur at a very low concentration of substrate (Marshall *et al.* 1971, Fletcher 1991). It has been demonstrated using computer-

enhanced microscopy that for surface-attached bacteria, the growth rate increased with laminar flow velocity at a low concentration (0.01 %) of glucose, but no relationship was observed at high glucose concentrations (0.1 %) (Fletcher 1991). The diffusion gradient may be considered as the laminar flow. It has also been reported for marine pseudomonads that attachment was favoured at a carbon source (glucose) concentration of 7 mg/l. Attachment was reduced at higher levels of 14 and 21 mg/l, and completely inhibited at 30 and 70 mg/l (Marshall *et al.* 1971, Fletcher 1980b). The results presented in this thesis show that S cells tended to grow slightly faster than L cells. However, the difference was not statistically significant. The concentration of carbon source that was used in the experiment may be too high. As low as 0.01 % substrate concentration may be required to see a significant difference between the growth of S and L cells (Marshall *et al.* 1971, Fletcher 1991, Leigh and Coplin 1992). However, at this low concentration, measurement of cell growth by OD or total protein could not be used, due to the low cell growth. Cell growth should be monitored using a more sensitive method. In addition, another aim of this chapter was to find a substrate that could emphasize the difference between S and L variants. Such a substrate could be used for a selective medium in later genetic experiments (Chapter 7). Therefore, the substrate concentration studied was within the range for medium components.

Preliminary studies were also carried out at substrate concentrations as low as 0.1 % (1 g/l). Although the difference between S and L was greater (data not shown), it was still not statistically significant. This concentration of substrate may still be too high compared to the concentration that was used by Marshall *et* al. 1971 (7 mg/l).

In a few cases, attached cells had higher activity than free cells, even at high concentrations of soluble low-molecular weight substrates, such as sugars, amino acids, fatty acids or carbon substrates (Kjeleberg *et al.* 1982, Fletcher 1985, Gordon *et al.* 1983, Morisaki 1983, Fletcher 1986), and effects were monitored using various type of measurements such as growth or cell number, respiration of carbon substrate or heat production. In such cases, the rate of cell uptake for the specific substrate may have been very much higher than the rate of diffusion, and the effect of attachment could therefore still be observed. This may be the case for the casein experiment, where attached cells (S) seemed to grow faster. The nitrogen (casein) uptake may have been greater than the rate of diffusion, and the effect of attachment could therefore still be observed. On the other hand, the slight difference in growth of S and L variants at a high concentration of other substrates (carbon source) may suggest that the rate of their uptake for soluble substrates that were used in the experiment was lower than the rate of diffusion.

Microbial adhesion has been suggested to a have significant effect on nutrient depletion. Another approach, by treating the cell under starvation conditions prior to inoculation has been reported by Doran and Bailey (1986, 1987). By monitoring the changes in intracellular NADH using fluorescence spectrophotometry, it was found that when *Saccharomyces cerevisiae* was first starved and then provided with glucose (3.6-89 mM), the immobilized cells had 5-6 times more storage material than suspended cells. Similar studies have been reported by Galazzo *et al.* (1987), using NMR to monitor intracellular components of *S. cerevisiae*. In contrast to this experiment, growing *B. fibrisolvens* in BHI medium prior to defined medium meant that the cells were in

a condition of excess rather than starvation. Secondly, washing with basal medium (medium containing all components except the carbon or nitrogen source) involved holding the cells under essentially a starvation regime. This was reflected by a longer lag phase of washed cells compared to unwashed cells before establishment of logarithmic growth. For growth curve comparison, a longer period in minimal medium may be required before transfer to rich medium in order to observe the effect of starvation. Further studies are required to explore this possibility.

The diffusion effect would be less for complex soluble substrates (starch), since the substrate is not transported directly into the cell and must be hydrolyzed by extracellular enzymes. It is conceivable that bacterial attachment could promote access of bacterial enzymes to this macromolecular substrate, which would enhance bacterial access to nutrient. However, during degradation of the complex substrate, some readily utilizable low-molecular-weight substrates may be released to the medium. It has been pointed out that in such cases, cells tend to assimilate them as rapidly as they are generated, regardless of the presence of complex substrates (Fletcher 1991). Therefore, the difference in S and L growth in starch that was obtained in this experiment was still not show significance due to the presence of low-molecular-weight intermediates during starch degradation.

For insoluble complex substrates (xylan and cellulose), diffusion effects are not likely to occur. This may explain the difference in growth rate of *B. fibrisolvens* S and L cells in xylan compared to the soluble carbon sources. Unfortunately the growth in cellulose was too low to be used for comparison.

Cell mobility is another factor that should be taken into account, since it could facilitate attachment by increasing the number of bacterial collisions with substratum, and therefore increase the statistical probability of attachment (Fletcher 1980a,b). An attached cell would be less mobile than an unattached cell, and this could be a disadvantage for S cells growing on soluble substrates, compared to L cells. This should result in better growth of the L cells. However, during the measurements (OD 600 nm or total protein estimation) the cultures were shaken to make them as homogeneous as possible. This meant increasing the effective mobility of S cells, possibly eliminating any difference. The shaking effect could be avoided by comparing growth over a fixed period of time without disturbance. With insoluble substrates, the mobility factor may not be as important as with soluble substrates, since the substrate was not distributed evenly in all parts of the medium.

For enzymatic activities, lack of cell mobility or substrate-diffusion effects may affect the experimental conditions. These measurements may therefore be better than growth comparisons by emphasizing differences between S and L variants. However, the results from enzymatic proteolytic assays showed that the polymer-associated protein was the most active. This confirms previous reports that proteolytic activity of *B. fibrisolvens* was associated with high molecular weight EP (Cotta and Hespell 1986). In a living cell, the distribution of EP-associated proteins to the surrounding environment may be via secretion from the cell, but under enzyme assay conditions, the migration of EP-associated proteins would only depend on diffusion. The association of EP-protein may become a size barrier for the protein complexes to diffuse within agar, therefore resulting in no

difference in proteolytic activity between S and L cells. EP-protein association has also been reported to affect protein migration in PAGE (Cotta and Hespell 1986). However, the difference between these two variants may not be in their proteolytic activity. In addition, a comparison of protein profiles (Chapter 3) indicated that most of the extracellular proteins were EP associated, and that there were no significant differences between S and L secreted or EP-associated proteins. Possibly proteolytic, xylanolytic and cellulolytic enzymes of *B. fibrisolvens* E14 may all be EP associated, which would explain why there was no significant difference in xylanolytic and CMCase levels between S and L variants. Glycanase acivity in R. albus SY3 have been reported to be 5-10 fold higher than that of the adhesion-defective mutant (Miron *et al.* 2001). However, the enzyme seemed not to be firmly associated with EP or other extracellular structures.

In conclusion, under these experimental conditions, the S phenotype appeared to have little impact on carbon or nitrogen utilization, compared with the L variant.