COMPARATIVE ANALYSIS OF TWO ATTACHMENT VARIANTS OF BUTYRIVIBRIO FIBRISOLVENS

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CHAPTER 6

CHARACTERISATION OF EXTRACELLULAR POLYMER

6.1 Introduction

Most strains of *B. fibrisolvens* have been reported to produce extracellular polymer (EP). In the EP of various strains of *B. fibrisolvens*, the presence of extracellular polysaccharides (EPS), as well as wall (WTA) and lipoteichoic acids (LTA) have been reported (Sharpe *et al.* 1975, Hewett *et al.* 1976, Anderson *et al.* 1993, Ferreire *et al.* 1995, Stack *et al.* 1988a). The monosaccharide composition of various strains of *B. fibrisolvens* EPS comprised mainly glucose and galactose (Stack 1988, Stack *et al.* 1988b, Stack and Ericson 1988, Stack and Weisleder 1990, Wachenheim and Patterson 1990, Ha *et al.* 1991). Further detailed structural studies have also been reported for strains X6C61 (Anderson *et al.* 1993) and 49 (Ferreire *et al.* 1995). The composition of *B. fibrisolvens* LTA was similar to that produced by other organisms (*Lactobacillus* or *Streptococcus*), and comprised mainly phosphorus glycerol, glucose, galactose, ester-linked amino acids and lipid (Sharpe *et al.* 1975, Hewett *et al.* 1976).

EPS alone has been reported to mediate attachment of *Hyphomonas* to surfaces (Quintero and Weiner 1995), while LTA, have been shown to be involved in the attachment of various strains of *Lactobacillus* or *Streptococcus* to surfaces (Hewett *et al* 1976, Duckworth 1977, Wicken 1980). For *B. fibrisolvens*, the involvement of EP (either as LTA or EPS) in its attachment to surfaces remains unknown.

Nili (1995) demonstrated that both S and L cells produced extracellular polysaccharides (EPS), with higher levels being produced by the S cells. However, the analysis was based on glucose content, which reflected the polysaccharide portion of EP. The lipid content of EP was not analysed.

The present chapter aims to study the differences between the EP of S and L cells. The difference in adhesion properties of the two cells may be associated with the level of EP production or with differences in the properties of their EP. This may be supported by data in Chapter 3 (cell-cell communication experiment), where S EP was of higher molecular weight than that of L EP. Therefore, besides the level of EP production, the monosaccharide and fatty acid compositions of S and L EP are compared using gas chromatography (GC).

EP was isolated by striping plate grown cultures using 2 N NaOH, to obtain the total EP (cell associated and secreted EP). However, previous studies on *B. fibrisolvens* EP used samples isolated from cell free medium (secreted EP). To compare between the total and the secreted EP, both methods were therefore used for isolating EP. In addition, to study the effect of carbon source on S and L EP production as well as the EP composition, the EP preparations were carried out using S and L cultures grown on defined medium containing various carbon sources. For comparing the level of EP production between *B. fibrisolvens* cells S and L, three isolation methods were used, gradient centrifugation, plate and cell free medium methods. For characterization, only the last two isolation methods were used. Various fractions, (Figure 6.1), were analysed for monosaccharide and fatty acid compositions.

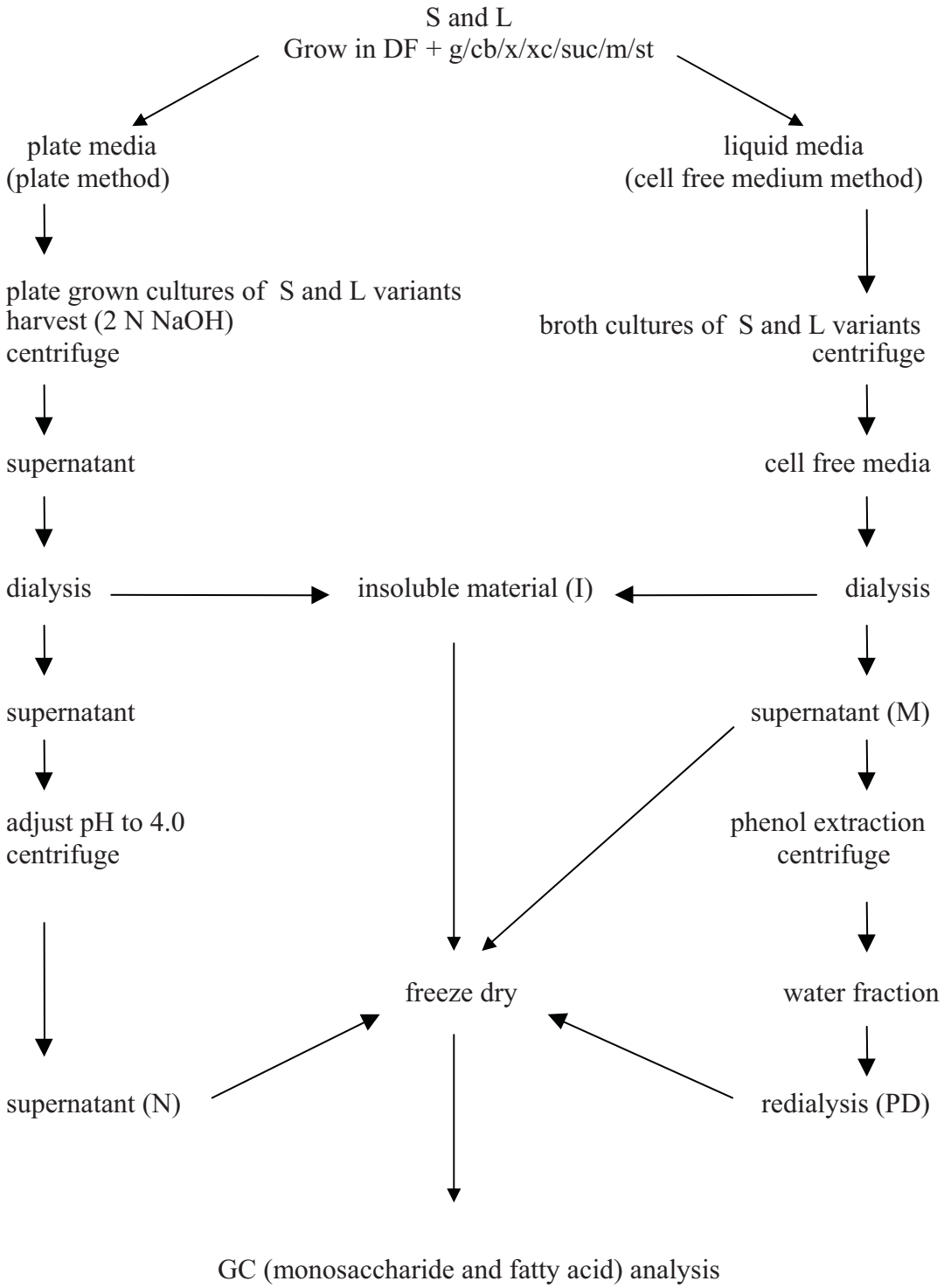

Figure 6.1 Diagrammatic outline of procedures for isolating various EP fractions.

DF, g, cb, x, xc, suc, m and st were defined media, glucose, cellobiose, xylan, cellulose, sucrose, maltose and starch, respectively. The samples obtained from plates represents total EP (cell associated and secreted EP), while those obtained from cell-free medium method represents secreted EP.

6.2 Results

6.2.1 Extracellular polymer production

Gradient centrifugation method

EP from S and L cells was isolated using gradient centrifugation, and the level of EP production was compared using phenol-sulphuric acid analysis as described in the methods. The yields were 11.0 ± 1.0 and 6.4 ± 0.5 x 10^{-2} mg EP/mg dry cell weight for S and L cells respectively. The ratio of S:L EP was 1.7 ± 0.1. Values represent the mean and standard error of two independent experiments carried out in triplicate.

Plate method

EP from S and L cells was isolated from plates (Berri and Rollings 1995), and analysed gravimetrically. The effect of carbon source on EP production was also compared. The plate grown cultures of S or L cells were harvested using 2 M NaOH (Methods and Figure 6.1) to obtain total (cell associated and secreted) EP. On all carbon sources S cells produced more EP than L cells (Figure 6.2). For both cell types, EP production was lower when cellobiose or cellulose were used as carbon sources, compared with glucose, xylan, sucrose, maltose or starch.

During this experiment, plate-grown cells were stripped and EP was harvested under acid (sodium acetate pH 3) or neutral (0.9 % NaCl or phosphate buffer pH 6.8) conditions. However the yields were very low. EP seemed to dissolve better under alkaline conditions, and better yields were obtained when 2 M rather than 1 M NaOH was used (data not shown).

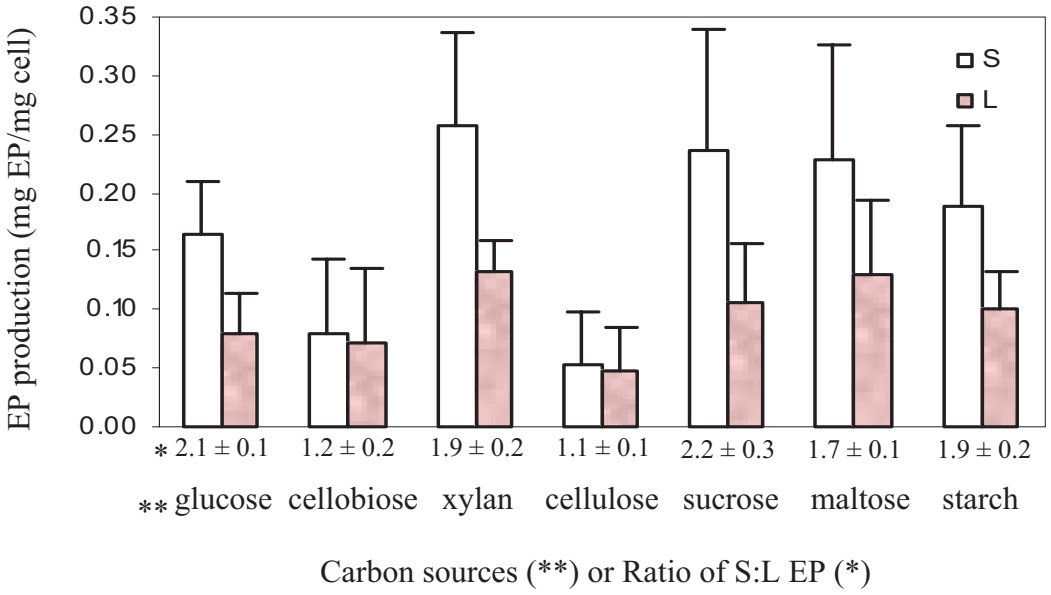

Figure 6.2 Total EP production from *B. fibrisolvens* E14 S and L cells grown on solid medium plus various carbon sources.

S and L were samples isolated from *B. fibrisolvens* cells S and L, respectively. EP was isolated from solid medium grown cultures as described in the methods. Values (EP production and ratio) represent the mean plus standard error of three independent experiments.

Insoluble flocculent material, as described by Stack (1988), was also obtained during dialysis. This fraction was separated by centrifugation, freeze dried, and kept as fraction I, and the EP sample obtained from this method was kept as fraction N (Methods and Figure 6.1).

Cell free medium method

EP from S and L cells was isolated from the cell free medium (Stack 1988), and the effect of carbon source on EP production was also compared, by growing the two cell types in defined medium plus glucose, cellobiose, xylan, cellulose, sucrose, maltose or starch. The culture was fractionated by centrifugation and EP

was prepared from the cell free medium as described in the methods, to obtain secreted EP. S cells produce more EP than L cells, in all carbon sources (Figure 6.3). For both cell types, EP production was lower when cellobiose or cellulose were used as carbon sources, compared with glucose, xylan, sucrose, maltose or starch.

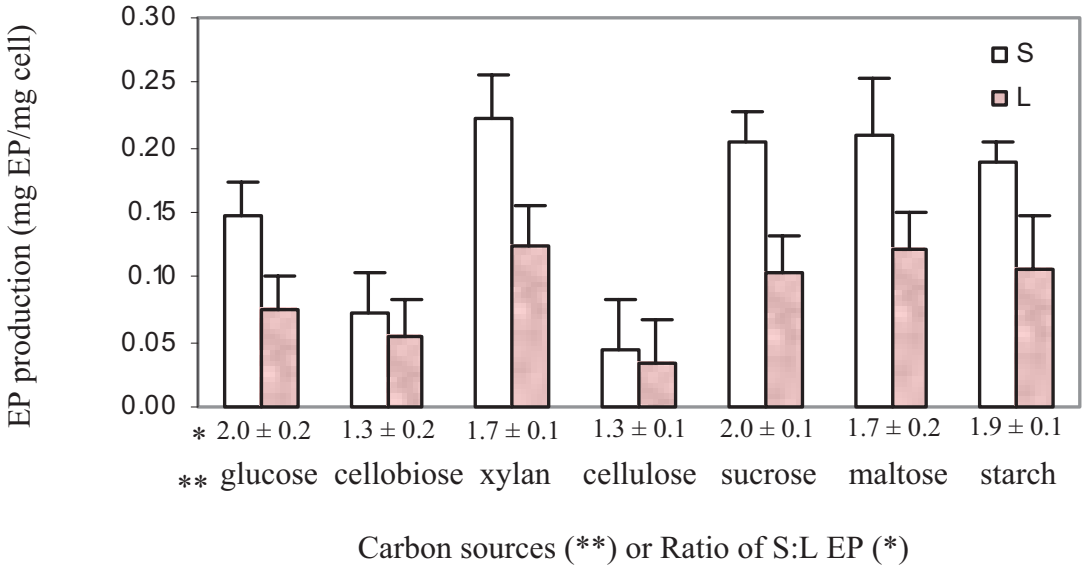

Figure 6.3 Secreted EP from *B. fibrisolvens* E14 S and L cells grown in liquid medium containing various carbon sources.

S and L were samples isolated from *B. fibrisolvens* S and L cells, respectively. EP was isolated from the cell free medium as described in the methods. Values (EP production and ratio) represent the mean plus standard error of three independent experiments.

Flocculent material was also obtained after dialysis, and the precipitate obtained after centrifugation was freeze dried and kept as fraction I. The supernatant was divided into two fractions, M and PD (Methods and Figure 6.1).

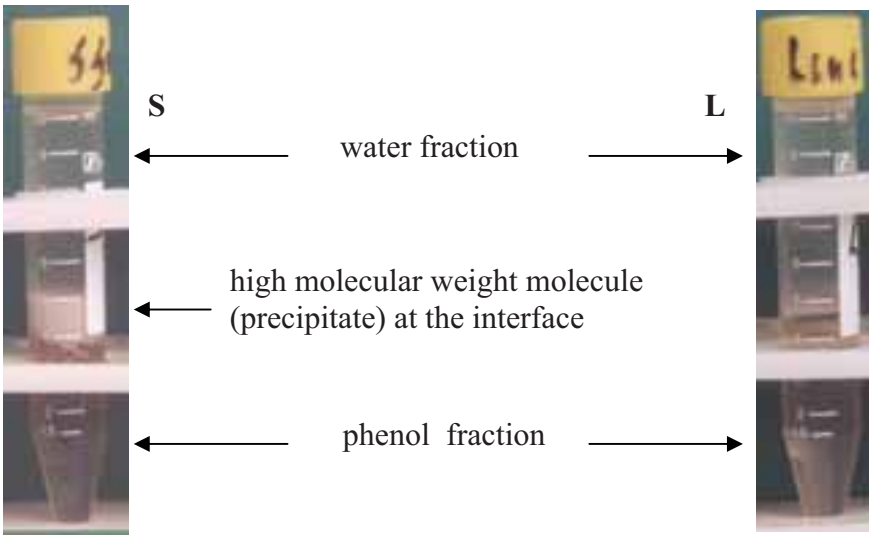

Figure 6.4 Differences between S and L fraction M-EP samples during phenol extraction.

S and L represent EP samples of S and L cells, respectively. The presence of material is shown at the phenol-water interface of all S samples, but not L samples. The brown colour of fraction M was extracted into phenol.

During phenol extraction of fraction M, the presence of high molecular weight molecules was observed at the phenol-water interface in all S samples, but not in L samples (Figure 6.4). The same results were obtained when either water or TE saturated phenol (commonly prepared for nucleic acid phenol extraction) was used for extracting the samples. In addition the S sample layer was maintained as an emulsion, for at least a further two days at room temperature. The phenol and water fractions could only be separated by centrifugation. On the other hand, the L samples formed two layers (phenol and water fractions) after approximately two hours, without centrifugation. Since S cells produced more EP than L cells, the difference between S and L fraction M during phenol extraction might have been due to the higher EP content of S samples. However, this was not the case since

the effect was still observed when the same amount of dried fraction M from S and L cells was dissolved in the same volume of water then phenol extracted similarly. This suggested that the properties of S and L EP were different. However, whether differences in attachment between S and L cells were related to these different properties is not clear. To elucidate the properties of S and L EP, their composition was analysed by gas chromatography. Only a summary of this data will be presented in this chapter. The complete monosaccharide and fatty acid chromatograms and proportion for all fractions of S and L samples isolated from various carbon sources using the plate and cell free medium methods are presented in the appendix A.

6.2.2 Monosaccharide composition of extracellular polymer

The monosaccharide composition of various EP fractions isolated from S and L cells grown in various carbon sources were compared. Various fractions of EP isolated using the plate (fractions N and I) and the cell free medium (fractions M, PD and I) methods (Figure 6.1) were analysed.

Monosaccharide composition of total EP

The monosaccharide composition of total (cell associated and secreted) EP (fraction N) were as follows; unknown 1 (u1), rhamnose, unknown 2 (u2), mannose, glucose and galactose (Figure 6.5 and appendix A 2-3). Based on the retention time, u1 and u2 may be glycerol phosphate and ribitol phosphate, respectively. u1, u2, glucose and galactose were the major constituents, while rhamnose and mannose were only present in trace amounts.

The composition of fraction I was similar whether obtained from plates or cell free medium. The monosaccharide composition of fraction I (Figure 6.6 and appendix A 4-5) was similar to fraction N (Figure 6.5 and appendix A 2-3) but there was less of it. However, unlike fraction N, the major monosaccharides (u1, u2, glucose and galactose) were only present in trace amounts.

There was no major effect of carbon source on the monosaccharide composition of S or L EP, although the proportions of monosaccharides were slightly affected, but the differences were not consistent across all samples (appendix A 2-5).

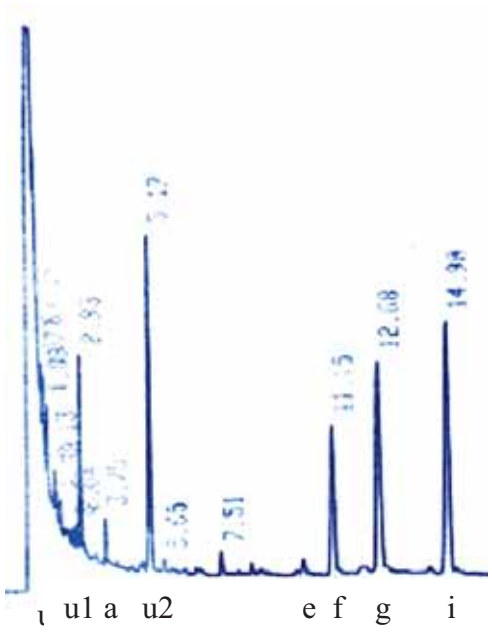

Figure 6.5 Monosaccharide composition of fraction N.

u1, a, u2, e, f, g, and i were unknown 1, rhamnose, unknown 2, mannose, galactose, glucose and internal standard inositol, respectively. Other small peaks were not identified. The chromatograms of fraction N isolated from cultures grown in the presence of various carbon sources are presented in the appendix A 4-5.

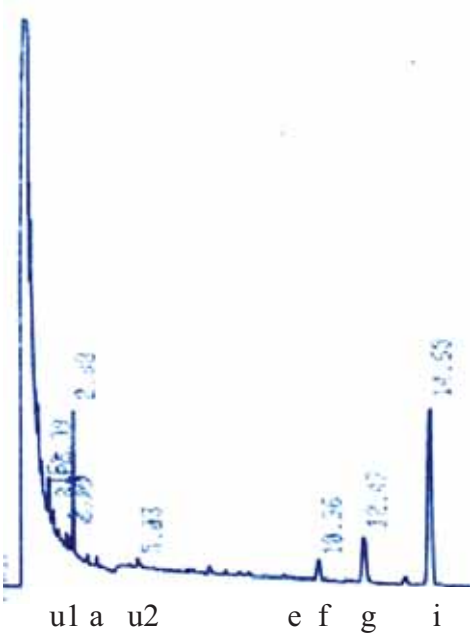

Figure 6.6 Monosaccharide composition of fraction I.

Peaks (u1, a, u2, e, f, g, and i) are as described in Figure 6.5. The chromatograms of fraction I isolated from cells grown in various carbon sources are presented in the appendix A 2-3.

Monosaccharide composition of secreted EP

The monosaccharide composition of fraction M (Figure 6.7 and appendix A 6-7) was similar to fraction N, while fraction PD (Figure 6.8 and appendix A 8-9), was also similar, except for two peaks (glucose and galactose), that were greatly reduced compared to fraction M. The effect of phenol extraction on the proportions of glucose and galactose are shown in Table 6.1.

The effect of carbon source on monosaccharide composition or proportion in fraction M (appendix A 6-7) was similar to those of fraction N (appendix A 2-3).

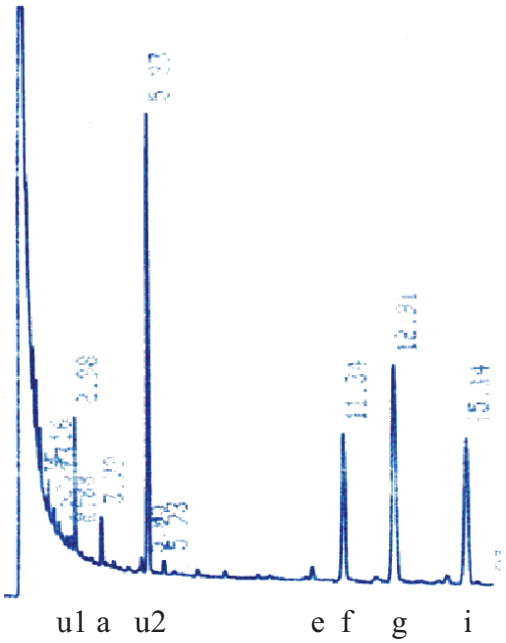

Figure 6.7 Monosaccharide composition of fraction M.

Peaks (u1, a, u2, e, f, g, and i) are as described in Figure 6.7. The chromatograms of fraction M isolated from various cultures of S and L cells, as well as the proportions are presented in the appendix A 6-7.

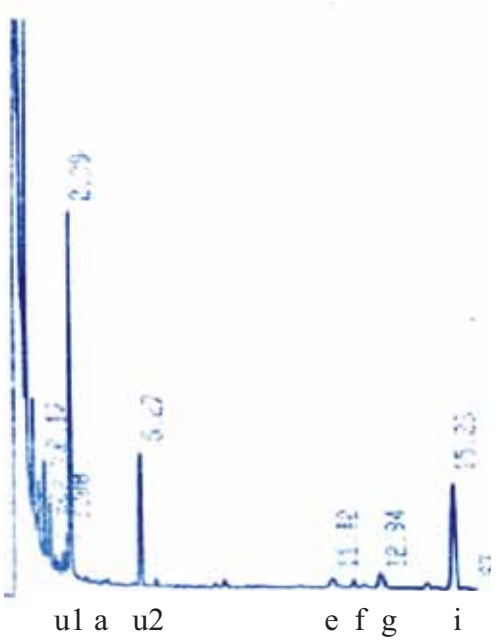

Figure 6.8 Monosaccharide composition of fraction PD.

u1, a, u2, e, f, g, and i were unknown 1, rhamnose, unknown 2, mannose, galactose, glucose and internal standard inositol, respectively. Other small peaks were not identified. The chromatograms of fraction PD isolated from various cultures of S and L cells, as well as the proportions are presented in the appendix A 8-9.

Table 6.1 Galactose and glucose contents of fractions M and PD.

EP	Monosaccharides (nmol/mg EP sample)					
	Fraction M			Fraction PD		
	Exp	Galactose	Glucose	Exp	Galactose	Glucose
Sg	3	639 ± 25	967 ± 23	2	78 ± 6	83 ± 12
Lg	2	445 ± 18	717 ± 17	2	47 ± 5	37 ± 6
Scb	2	451 ± 75	979 ± 190	2	28 ± 4	30 ± 1
Lcb	2	556 ± 86	864 ± 114	2	22 ± 3	69 ± 5
Sx	2	568 ± 49	1222 ± 103	2	38 ± 4	75 ± 11
Lx	2	528 ± 15	1707 ± 113	3	30 ± 5	69 ± 27
Sxc	2	31 ± 4	1032 ± 231	2	24 ± 1	50 ± 4
Lxc	2	6 ± 1	1184 ± 220	2	29 ± 3	50 ± 7
Ssuc	3	576 ± 40	2217 ± 157	2	65 ± 7	67 ± 24
Lsuc	3	408 ± 33	698 ± 56	2	28 ± 3	73 ± 39
Sm	2	497 ± 24	822 ± 25	2	21 ± 2	99 ± 7
Lm	2	217 ± 42	413 ± 76	2	20 ± 3	67 ± 40
Sst	2	842 ± 14	2147 ± 125	2	39 ± 7	102 ± 58
Lst	2	571 ± 19	1343 ± 229	2	53 ± 5	92 ± 37

EP, Exp, M and PD are; EP samples, experiment number, and fractions M and PD, respectively. Sg, Scb, Sx, Sxc, Ssuc, Sm and Sst were EP samples isolated from S cells grown in glucose, cellobiose, xylan, cellulose, sucrose, maltose and starch, respectively. A similar labelling scheme is used for EP isolated from L cells. Values represent the mean plus standard error of 2 or 3 independent experiments, as shown in the table. A large decrease in glucose and galactose was observed in fraction PD, compared to fraction M.

High xylose and arabinose peaks (Figure 6.9) were observed in fraction M of S and L EP isolated from cell free medium of xylan-grown cultures. However, these peaks were not observed in fraction N (Figure 6.9) isolated from xylan-containing plates or fractions M and N isolated from cultures containing other carbon sources.

One question not answered by monosaccharide analysis was the identity of material at the water-phenol interface of S but not L samples during phenol

extraction (Figure 6.4). Preliminary observations suggest that S EP had a more "oily" character than L EP. An experiment was therefore carried out to examine the lipid composition of S and L EP.

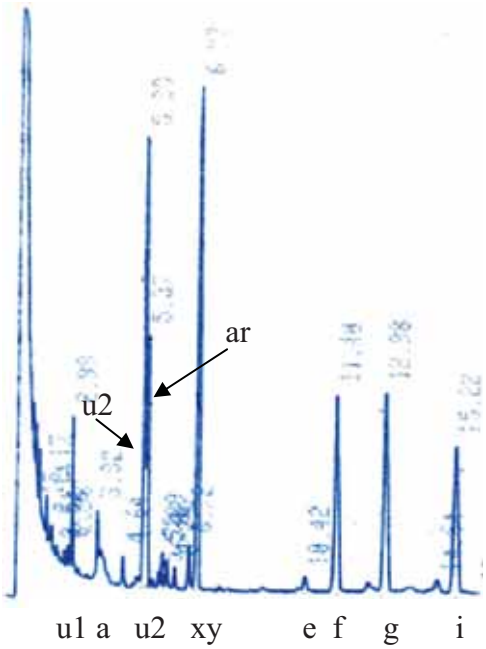

Figure 6.9 Monosaccharide composition of fraction M EP isolated from xylan-grown cultures.

u1, a, u2, e, f, g, and i were unknown 1, rhamnose, unknown 2, mannose, galactose, glucose and internal standard inositol, respectively. Other small peaks were not identified. Arrows indicate arabinose (ar) which was very close to u2.

6.2.3 Fatty acid composition of extracellular polymer

The fatty acid composition of various EP fractions isolated from S and L cells grown in various carbon sources were compared by gas chromatography. The analysis was carried out for various fractions of S and L EP isolated using the plates or cell free medium (Methods and Figure 6.1). Due to the poor growth and

low EP yield from cellulose grown cells, fatty acid analysis was excluded for these samples.

Fatty acid composition of total EP

The fatty acid composition of fraction N was C16:0 (palmitic acid), C16:0:1, unknown 3 (u3), C18:0, C18:1 and unknown 4 (u4) (Figure 6.10). U3 and u4 may be C17 and C22:5(3), respectively. There was no difference in the overall fatty acid composition of S and L EP (appendix B 2-3), however, the C16:0 content of S EP was much higher than that of L EP (Table 6.2).

The effect of carbon source on the fatty acid composition of EP was not significant (appendix B 2-3). However, the C16:0 content was affected, where it was lower in samples isolated from cellobiose compared to that of other carbon sources (glucose, sucrose, maltose or starch) (Table 6.2).

Surprisingly, the insoluble flocculent polymer (fraction I), did not contain much monosaccharide (Figure 6.6), but appeared to contain significant amounts of fatty acid (Figure 6.11). The fatty acid composition was similar to that of fraction N.

Due to the small amounts fatty acid recovered, the analysis was only carried out once, and samples were collected and pooled from several EP isolations. Although not as much as in fraction N, the C16:0 content in fraction I of S EP tended to be higher than that of L EP, and the effect of carbon source was similar to fraction N.

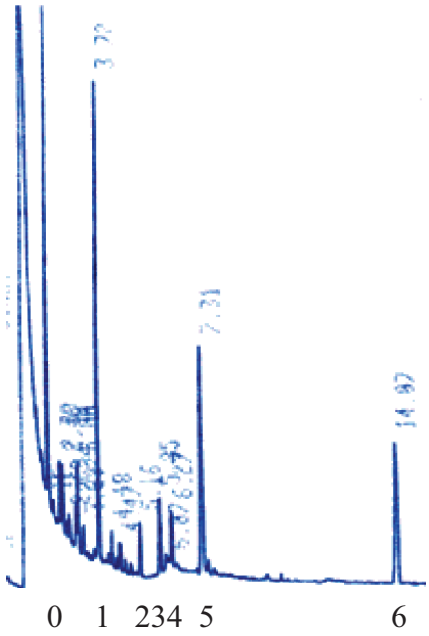

Figure 6.10 Fatty acid composition of fraction N.

0, 1, 2, 3, 4, 5 and 6 were antioxidant, C16:0, u3, C18:0, C18:1, internal standard C19, and u4, respectively. Unknown 3 (u3) and 4 (u4) may be C17 and C22:5(3), respectively. Longer chains fatty acids and other small peaks were not identified. The chromatograms of fraction N isolated from various cultures of S and L cells, as well as the relative proportions are presented in the appendix B 2-3.

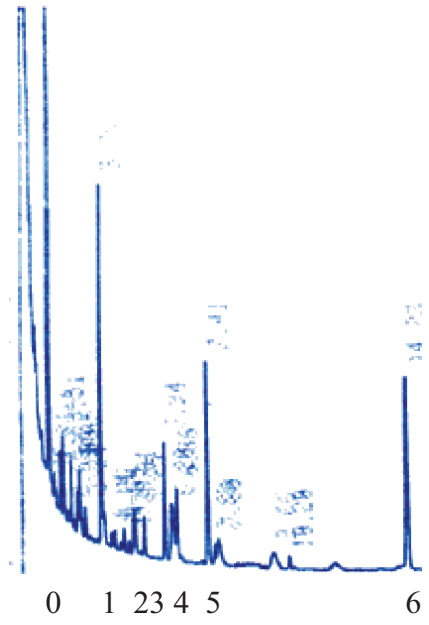

Figure 6.11 Fatty acid composition of fraction I.

Peaks (0, 1, 2, 3, 4, 5 and 6) are as described in Figure 6.10. The chromatograms of fraction I isolated from various cultures of S and L cells, as well as the relative proportions are presented in the appendix B 4-5.

Fatty acid composition of secreted EP

The fatty acid composition of fraction M was identical to that of fraction N (Figure 6.12). A higher C16:0 content was shown in S EP than L EP, and the effect of carbon source seemed to be similar to that of fraction N.

Table 6.2 C16:0 content of fractions N, M and PD

EP	C16:0 content (pmol/mg EP sample)		
	Fraction N	Fraction M	Fraction PD
Sg	328 ± 40	295 ± 14	50 ± 4
Lg	56 ± 6	38 ± 2	28 ± 2
Scb	58 ± 2	95 ± 19	44 ± 3
Lcb	45 ± 2	36 ± 2	32 ± 5
Sx	120 ± 8	138 ± 12	19 ± 2
Lx	59 ± 3	42 ± 8	22 ± 3
Sxc	not determined		
Lxc	not determined		
Ssuc	191 ± 8	259 ± 26	28 ± 5
Lsuc	25 ± 2	46 ± 22	21 ± 1
Sm	504 ± 39	421 ± 32	28 ± 4
Lm	27 ± 1	50 ± 3	20 ± 1
Sst	157 ± 15	168 ± 15	39 ± 4
Lst	38 ± 11	22 ± 4	18 ± 2

EP, N, M and PD were EP samples, fractions M, N and A, respectively. Sg, Scb, Sx, Sxc, Ssuc, Sm and Sst were EP samples isolated from S cells grown in glucose, cellobiose, xylan, cellulose, sucrose, maltose and starch, respectively. Similarly for EP isolated from L cells (Lg, Lcb, Lx, Lxc, Lsuc, Lm and Lst). Values represent the mean and standard error of 2 independent experiments.

The fatty acid composition of fraction PD (Figure 6.13) was identical to that of fraction M. However, in fraction PD, the difference in C16:0 content between S and L, as well as the effect of carbon source on C16:0 content were not significant (Table 6.2). The C16:0 content of S fraction PD was less than fraction M, while that of L EP were similar between fractions M and PD (Table 6.2).

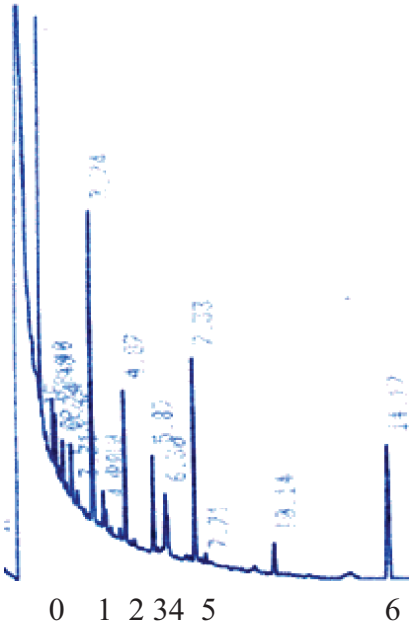

Figure 6.12 Fatty acid composition of fraction M.

0, 1, 2, 3, 4, 5 and 6 were antioxidant, C16:0, u3, C18:0, C18:1, internal standard C19 and u4, repectively. Unknown 3 (u3) and 4 (u4) may be C17 and C22:5(3), respectively. Longer fatty acids and other small peaks were not identified. The chromatograms of fraction M isolated from various cultures of S and L cells, as well as the relative proportions are presented in the appendix B 6-7.

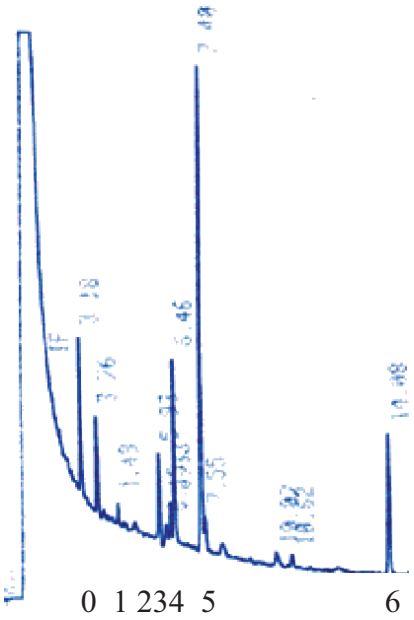

Figure 6.13 Fatty acid composition of fraction PD.

Peaks (0, 1, 2, 3, 4, 5 and 6) are as described in Figure 6.12. C16:0 (2) was decreased compared to fraction M (Figure, 6.12).The chromatograms of fraction PD isolated from various cultures of S and L cells, as well as the relative proportions are presented in the appendix B 8-9.

During the preparation of fractions M and N for fatty acid analysis, the presence of material at the water/methanol-chloroform interface of S but not of L samples was noted (Figure 6.14). The effect was observed in all samples (fractions M and N) from cells grown in various carbon sources. This phenomenon was similar to that observed during phenol extraction (Figure 6.4).

Since more lipid was identified in S cells, attempts were undertaken to differentiate between S and L cells using Sudan Black as described by Liu *et al.* (1998). It was tested in order to develop a selective medium for cloning genes associated with attachment. The concentration of Sudan Black was varied. However it was not successful and both cells produce similar results. This may be due to the fact that the L variant still produced fatty acids, other than C16:0.

6.3 Discussion

Results from various EP isolation methods showed that the level of EP production from S cells was approximately twice that of L cells. This supports previous observations that the S cells produced more EP than the L cells (Nili 1996). Although less than the S cells, the L cells still produced significant amounts of EP, unlike EPS-dependent microorganisms that attach to surfaces, where adherent mutants produced little or no EPS (Quintero and Weiner 1995). Therefore, the difference in attachment between S and L cells is unlikely to be due to the level of EP production. The fact that some high-EPS-producing-strains of *B. fibrisolvens* (eg. H17c, and A49) do not attach to surfaces, may support this suggestion (Stack *et a.* 1988a,b; Stack and Weisleder 1990; Ha *et al.* 1991). The difference in

attachment properties between the two cell types may be due to the composition of their EP, which may therefore affect their properties.

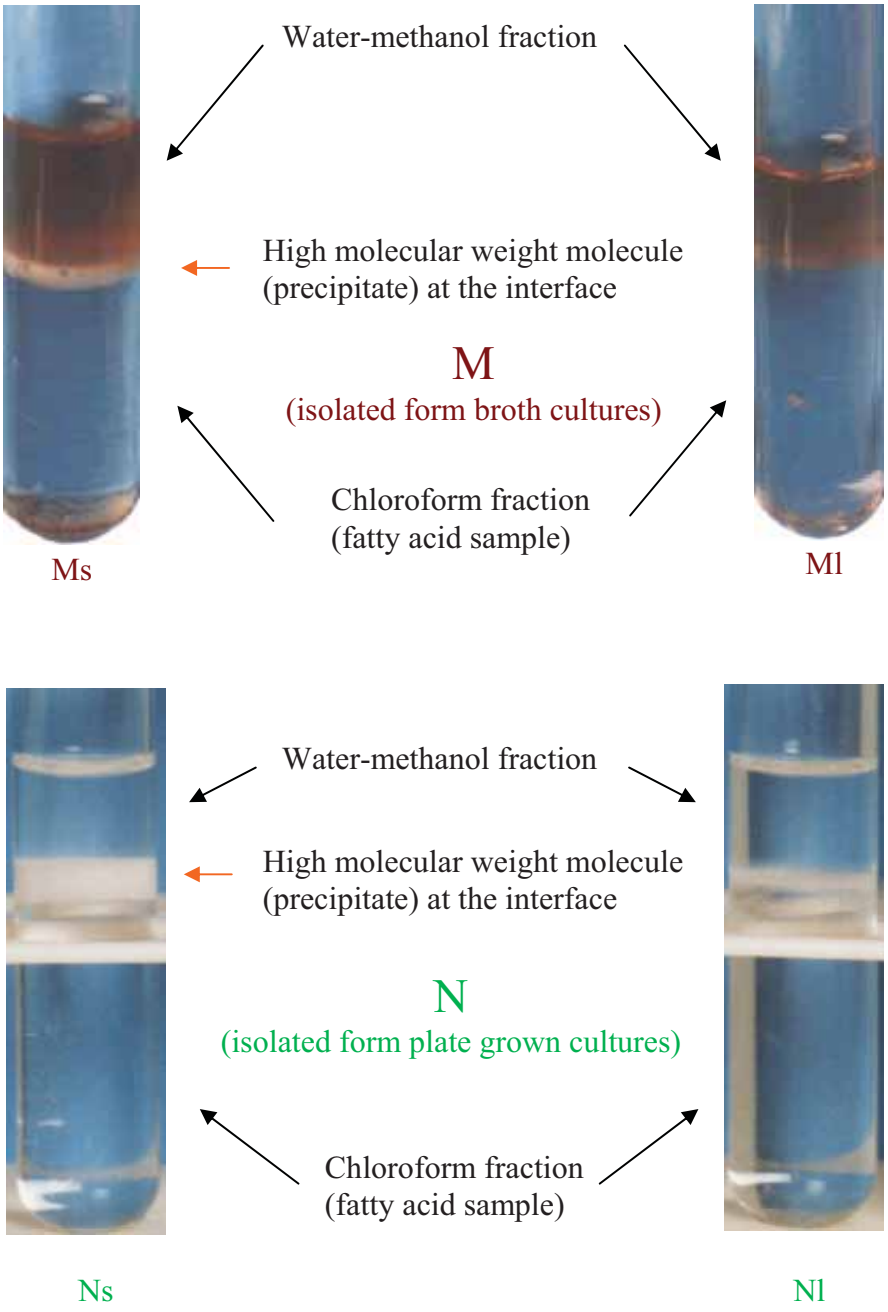

Figure 6. 14 Differences between water/methanol-chloroform extracted S and L EP samples.

M and N indicate fractions M and N, respectively. Ms, Ml, Ns and Nl indicate S and L EP samples of fractions M or N, respectively.

The presence of unknown materials at the interface during phenol extraction and fatty acid preparation also suggests that the S and L EP behave differently under extraction conditions.

The monosaccharide compositions were similar for S and L EP, and comprised rhamnose, mannose, glucose, galactose, trace amounts of acidic monosaccharide and 2 unknowns. The lack of difference in the monosaccharide composition of S and L EP suggested that the difference in the adhering properties between S and L cells was not due to the carbohydrate content of the EP. In addition, although EP alone has been reported as the adhesive factor for *Hyphomonas* (Quintero and Weiner 1995), it was not clear whether its EP comprised carbohydrates alone. Although adhesion to surfaces has been mentioned among the functions of EP to enhance bacterial survival in the environment, this was seldom conclusively demonstrated (Decho 1990, Quintero and Weiner 1995).

Culture conditions (carbon source) affect the level of EP production, but not the composition, and the effect of carbon source on EP production was similar for both S and L cells. This supports previous reports that the amount of EP produced from various *B. fibrisolvens* strains was influenced by carbon or nitrogen source, but not the monosaccharide composition (Stack 1988, Wachenheim and Patterson 1990, Ha *et al*. 1991). Similar results have been reported for *Streptococcus thermophilus* (Degeest and De Vuyst 2000), where its EPS production was also influenced by carbohydrate source, but not the composition (galactose:glucose 4:1). The level of EPS production was correlated with the activity of phosphoglucomutase, UDP-galactose 4-epimerase, and UDP-glucose

pyrophosphorylase enzymes. The correlation of EPS production with EPS biosynthetic enzyme activity has been previously reported (Grobben *et al*. 1995, 1996, 1997; Escalante et al.1998). In most cases, medium composition (carbon sources, nitrogen sources, metal etc.) affected the amount of EPS production, but not the composition. This may suggest that the level of EPS production in most bacteria is under inducible catabolite control, and is probably controlled by an operon, as has been reported for EPS-gene clusters from various bacteria (Jiang *et al*. 1991, Brown *et al*. 1992, Glucksmann *et al*. 1993, Morona *et al*. 1994, Guidolin *et al*. 1994, Arakawa *et al*. 1995, Bugert and Geider, 1995, Huang and Schell 1995, Allen and Maskel 1996, Kolkman *et al*. 1996, Stingele *et al*. 1996, Karenburg *et al*. 1997). Alternatively, since most nutrient alteration hardly affected the composition of EPS, this may reflect a natural mechanism for maintaining cell stability and identity through subsequent generations. If the composition of cell surface-polymer components can be easily changed by environmental conditions, such as the availability of nutrients, then very high rates of phenotypic changes would be expected in various EPS-producing microorganisms. This is clearly not the case. The phenotypic stability concept is also supported by the fact that many organisms attach to surfaces through specific ligand-receptor interactions. Changes in cell surface polymers (ligand) involved in such interaction would change the ligand-receptor specificity, and therefore alter the ability of the microorganism to attach to specific surfaces, and hence could result in high rates of change in microbial populations on surfaces. This does not seem to occur. Attachment inhibitor/activator substances may repress or induce attachment, but usually by blocking the specific site required for attachment or by

altering molecular configuration of the ligand/receptor, but not by changing the molecular composition of the ligand/receptor (Ofek and Beachey 1980).

Fatty acid compositions (C16:0, C16:0:1, C18:0, C18:1 and 2 unknown compounds) were identical in S and L EP, as well as in various fractions of EP. However the proportion of each fatty acid, especially C16:0 was different. The C16:0 content of S was higher than that of L EP. In S EP the C16:0 content was affected by carbon source, while in L EP this effect was not significant. This suggests that C16:0 content was the major factor that differed between S and L EP. Various studies on cell surface-polymer biosynthesis (Duckworth 1977, Sutherland 1990, Kranenburg *et al*. 1997) indicate that in most cases, endogenous lipid acceptors that anchor EPS to the cell surface are required, although it is still not known whether the polysaccharide is assembled on the anchor molecule or whether this component is added after the polymerisation is complete (Duckworth 1977, Troy 1979, Ofek and Beachey 1980, Sutherland 1982, Whitfield 1988, Fletcher 1991, Griffin *et al*. 1995, Stingele *et al*. 1996, Kranenburg *et al*. 1997). The C16:0 may be associated with the anchor molecule, and therefore the difference between S and L may be in the availability or biosynthesis of the lipid acceptor. Since the EPS composition of both cells was identical, this may suggest that the anchor molecule was added after polymerisation was complete.

The monosaccharide and fatty acid compositions, as well as the EP content of fractions N and M were identical. This suggests that there was no additional component in fraction N compared to fraction M, and therefore that there was no

fraction attached to cells at all stages of growth. Most EP seemed to be secreted during the final stages of growth. This is supported by the fact that most EPS analyses reported for *B. fibrisolvens* were prepared from cell free medium (Stack 1988; Stack *et al.* 1988a,b; Stack and Ericson 1988; Stack and Weisleder 1990; Wachenheim and Patterson 1990; Ha *et al.* 1991; Andersson *et al.* 1993; Ferreira *et al.* 1995).

The monosaccharide analysis of fractions M (before phenol extraction) and PD (after phenol extraction) showed that phenol extraction seemed to extract polysaccharide (especially galactose and glucose) from EP. The decrease in sugars as a result of phenol extraction was similar for both S and L EP. This data could not explain the presence of material at the water-phenol interface, during extraction of S but not L samples (unless the glucose or galactose was associated with proteins as glycoproteins). This question may be answered by the data from fatty acid analysis, where in S samples, a large decrease in C16:0 content was observed in fraction PD compared to fraction M. In contrast, in L samples, no significant difference was observed. In S EP, besides extracting monosaccharides, phenol extraction also extracted some C16:0, resulting in a large apparent decrease in C16:0 content, between M and PD fractions. In L EP, only monosaccharides were extracted (Table 6.2). Besides differences in properties, the presence of material (in S samples) at the water-phenol interface suggests that there may be an association between the extracted and the retained (at the interface) materials, but not in L EP. This suggestion is also supported by the presence of material at the water/methanol-chloroform interface during fatty acid preparation of fractions N and M of S but not L samples. The association in S EP

may be between hydrophobic and hydrophilic polymers. The lipid content (C16:0) may reflect the hydrophobic polymer fraction, with carbohydrate (EPS) being hydrophilic. Such an association may not be present in L EP, and this may be the factor that affects their attachment properties. This suggestion may be supported by the indication that S EP was of greater molecular size than L EP.

The presence of C16:0 has been reported in the *B. fibrisolvens* membrane. The membrane lipids of *Butyrivibrio* were unlike those of other bacteria, particularly the presence of cross-links formed by esterification with $(C16:0)_2$ dicarboxylic acid (diabolic acid) in almost all of the phospholipid (Hazlewood *et al.* 1980, Clarke *et al.* 1980, Dibbayawan *et al.* 1985). The diabolic acid bridge has been suggested to increase the fluidity of the membrane lipids, since most fatty acids available in the rumen are saturated (Hauser *et al.* 1979), or to protect from powerful lipases present in the membrane (Clarke *et al.* 1980, Hazlewood *et al.* 1983, Dibbayawan *et al.* 1985). During this study, it was also observed that S cell free medium was more viscous than L, probably due to more fatty acids being secreted from S cells, thus decreasing the fluidity of the medium. The viscosity difference was also observed during sample preparations for GC analysis.

B. fibrisolvens produced both wall and membrane LTA. However, the membrane rather than wall LTA have been suggested to be involved in the attachment of some strains of *Lactobacilli*, *Streptococcus*, or marine *Pseudomonas* to surfaces (Duckworth 1977, Fletcher 1980a, Wicken 1980). The fact that the S and L EP contained lipids (fatty acid) as well as monosaccharides suggested the possibility of LTA. Based on the retention time, u1 may be glycerol phosphate, which

together with glucose, galactose, and fatty acids suggests the presence of LTA. The LTA composition of some *B. fibrisolvens* (Sharpe *et al.* 1975, Hewett *et al*. 1976), *Lactobaciilus* and *Streptococcus* strains (Duckworth 1977, Wicken 1980 and Figure 1.4) have been reported, and comprised mainly glycerol phosphate, glucose, galactose, ester-linked amino acids and lipid. *B. fibrisolvens* EP may be very complex and be comprised of wall and membrane LTA and EPS (Figure 1.6). The difference between S and L EP may be at the level of LTA, which would be reflected in the fatty acid content, especially C16:0. The polymer association that has been suggested above may be between LTA and EPS. Based on what has been discussed, a model is proposed as outlined in Figure 6.22.

LTA may act as the anchor molecule in cell-surface polymer biosynthesis (Duckworth 1977, Wicken 1980, Kranenburg *et al*. 1997), and be required as the precursor of the bridging region between peptidoglycan and other wall polymers. In most cases, the assembly (association) between EPS and LTA or other anchor molecules is catalysed by a specific enzyme, such as glycosyltransferase (Duckworth 1977, Ofek and Beachey 1980, Fletcher 1991, Kranenburg *et al*. 1997). 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). Examples are the regulation of cellulose biosynthesis in *A. xylinum* by an unusual cyclic diguanylic acid molecule, and indirectly by the levels of intracellular Ca^{2+} (Ross et al. 1987); positive and negative control of the colanic acid production in *E. coli* by more than three discrete regulatory genes, one of which (rcsA) is auto-proteolysis regulated (Torres-Cabassa and Gottesman 1987 Whitfield 1988). Therefore,

besides the lower C16:0 content in L compare to S EP that may reflect the reduced amount of LTA in their cell-surface polymer, glycosyltransferase activity in L EP may also be lower. However this was not measured in this study. In addition, it is also possible that the LTA biosynthesis itself is lower in L compared to S cells, due to the action of unknown regulatory reactions.

The extent to which LTA normally occurs (approx. 2/3 % of the cell weight) and the fact that some wall LTA-lacking bacteria still have LTA, make it unlikely that being an anchor molecule is their only function. It is also not clear whether all LTA fractions can act as an acceptor or whether carrier molecules differ in location or structural aspect (Duckworth 1977). The overall negative charge of wall LTA may also serve a special function- the repulsive charges on the surface of unicellular organisms may be a desirable property enabling cell populations to disperse in the medium and hence use nutrients more efficiently. Removal of LTA from a wall preparation causes it to settle from suspension as a flocculent mass (Heptinstall *et al*. 1970, Duckworth 1977). In L cells, the overall negative charge of their wall LTA is maintained and this keeps the cells hydrophilic, and therefore dispersed in liquid medium at all stages of growth. While in S cells, the negative charge may be neutralised by combination with LTA, possibly making the cells hydrophobic, and therefore clumpy in liquid medium at early stages of growth. After longer period of incubation, the complex cell surface polymers are secreted to the medium and the cells may become more hydrophilic, and therefore dispersed through out the liquid medium.

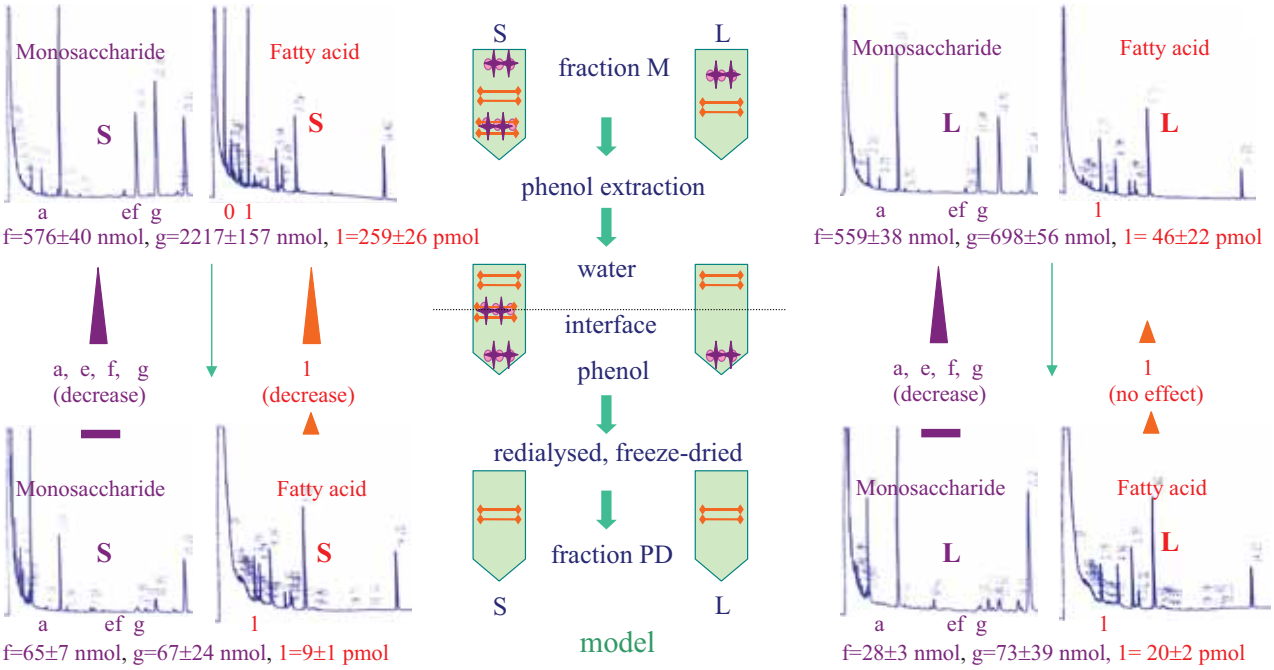

Figure 6.15 Phenol extraction effect on monosaccharide and fatty acid contents of S and L fraction M EP.

a, e, f, g, 0 and 1 indicate rhamnose, mannose, galactose, glucose, antioxidant and C16:0, respectively. Other peaks as described in Figures for monosaccharides (6.5-6.9 and appendix) and fatty acid (6.10-6.13 and appendix). Phenol seemed to extract most of the monosaccharides (a, e, f, g) from fraction M of both S and L EP. In S sample, it was also followed by a large decrease in C16:0 (1) in fraction PD, compared to fraction M, but with little effect on L samples. There may be an association between C16: and polysaccharides in S but not in L EP, and this may explain the presence of material at the phenol-water interface in S but not L samples (model). ✦ = EPS; = LTA; ● = protein (prot); = EPS + prot; = EPS + prot + LTA.

Recent studies on *B. fibrisolvens* EP only report on its monosaccharide composition, and often only as polysaccharide (Stack 1988; Stack *et al.* 1988a,b; Stack and Ericson 1988; Stack and Weisleder 1990; Wachenheim and Patterson 1990; Ha *et al.* 1991). There have been no reports on the fatty acid composition, although some of the strains used have been reported to produce LTA (Sharpe *et al.* 1975, Hewett and Wicken 1975). The EPS of some strains contained trace amounts of acidic monosaccharides, such as 4-O-(1-carboxyethyl)-D-galactose (Stack *et al*.1988b) and 4-O-(1-carboxyethyl)-L-rhamnose (Stack and Weisleder 1990). The LTA composition of some *B. fibrisolvens* strains (Sharpe *et al.* 1975, Hewett *et al*. 1976) have been reported to be similar to some *Streptococcus* or *Lactobacillus* strains (Figure 1.4). The glycerol phosphate of LTA was mostly attached to a glucose or galactose moiety of EPS (Figure 1.4) and the carboxyethyl group may be from the glycerol of LTA that became attached to the monosaccharides as a result of random degradation during sample preparation. The EP of S and L cells also contained acidic monosaccharides. However, these were present in trace amounts and did not differ between the cell types.

The monosaccharide composition of S and L EP was similar to that reported for various strains of *B. fibrisolvens* (Stack 1988, Mannarelli *et al.* 1990b, Ha *et al.* 1991). Taxonomic relatedness of *B. fibrisolvens* as determined by monosaccharide composition of EPS, has been suggested (Stack 1988, Mannarelli *et al.* 1990b), and based on previous studies, strain E14 used in this project was related to group IVC.

The insoluble flocculent material (fraction I), which contains low sugar but significant amounts of lipid other than C16:0, has been described previously (Stack 1988, Stack *et al.* 1988a, Ha *et al.* 1991), and may be a nonmicellar LTA. It has been reported that a lower molecular weight fraction is usually obtained in the purification of LTA on sepharose 6B (Duckworth 1977, Wicken 1980). This fraction is non-dialyzable and yields glycerol, mono and diphosphates, and traces of fatty acids on acid hydrolysis (Wicken and Knox 1975, Wicken 1980). However, the precise nature of this fraction is unclear (Wicken 1980). A similar fraction has also been obtained from *S. faecalis* (Garfield and Pieringer 1975) during the preparation of stable protoplasts by lysozyme digestion in the presence of Mg^{2+} and sucrose. This fraction was found in the supernatant, suggesting that it was not as firmly bound to the cytoplasmic membrane, as was the "true" LTA, which was found on the protoplast. Wicken and Knox (1975) also reported this nonmicellar LTA contained less fatty acid than that of "true" LTA. It was suggested to be non-acetylated LTA and may represent a partial degradation product or a completely separate polymer (Wicken and Knox 1975, Duckworth 1977).

A special case was the presence of xylose and arabinose peaks in fraction M (cell free medium) isolated only from xylan-grown cultures. However the two peaks were not observed in fraction N (plates) isolated from xylan or other carbon source-grown cultures. This suggested that the two peaks were contaminants from the remaining xylan substrate, due to incomplete degradation. It may have become trapped in the EP network or its size was still too big and was retained during dialysis. For some *B. fibrisolvens* strains, substitution of xylose for glucose

appeared to have little effect on the EPS composition (Stack 1988). This phenomenon may suggest that both *B. fibrisolvens* E14 cell types only partially degraded the xylan substrate. Similarly, this might have taken place when EP was isolated from the cell free medium of starch or cellulose-grown cultures, however would not be observed because the remaining glucose would be indistinguishable from that of EP.

During dialysis, the brown colour of the concentrated cell free medium decreased over the first two days but there was no further decrease after five days, although the dialysis was extended for up to seven days. This may due to a medium component that attached to fraction M EP. This is supported by the fact that during phenol extraction of fraction M, the brown colour was also extracted to the phenol fraction, together with most of the monosaccharides. On the other hand, during fatty acid preparation of fraction M (chloroform-water/methanol extraction), the colour did not follow the fatty acid (chloroform) fraction. This may suggest an interaction between a medium component and carbohydrate, but not with the lipid fraction of EP.

In conclusion, S cells produced more EP than L cells. The composition of S and L EP were identical, with no missing or additional monosaccharides and fatty acids, but the relative proportions of EP substituents were different. The difference was greatest with lipids, especially C16:0 content, which was much higher in S- than L- EP. Carbon sources did not affect the composition of EP. The presence of fatty acids, especially C16:0, probably reflects the existence of LTA in S and L EP and S EP possibly contains more LTA than L EP. More work is required to clarify

how EPS and LTA interact with each other to form the extracellular matrix (glycocalyx).

CHAPTER 7

GENETIC COMPLEMENTATION

7.1 Introduction

In general, attachment gene(s) can be defined as the gene(s) responsible for extracellular structures such as fimbriae, flagella, attachment proteins or extracellular polymer (EP). However, organisms bearing extracellular structures are not necessarily attached to surfaces (Christensen 1989, Kranenburg *et al*. 1997). Therefore genes for extracellular structures are not always equivalent to attachment genes.

Protein has been found to be the ligand for adherence in certain strains of *E. coli* (Salit and Gotschlich 1977a,b), gonococci (Buchanan and Pearce 1976), mycoplasma (Collier 1980) and *Azospirillum brasilense* (Bashan and Levanony 1988, Dufrêne *et al.* 1996). For EP, the ligand may be the lipid portion of glycolipid molecules such as lipoteichoic acid in group A *Streptococci* (Ofek *et al.* 1975, Beachey and Ofek 1976), *Staphylococcus aureus* (Aly *et al.* 1979) and marine *Pseudomonas* (Fletcher 1980a). It may also be sugars or other carbohydrates such as those found in *Hyphomonas* (Quintero and Weiner 1995), and in some marine (Jones *et al*. 1969, Fletcher and Floodgate 1973) as well as fresh water (Allison and Sutherland 1987) bacteria. Fimbriae have been reported for *Klebsiella sp.* (Korhonen *et al.* 1983), enteric bacteria (Haahtela 1985), *Pseudomonas fluorescens* (Vesper 1987) and *Bradyrhizobium japonicum* (Vesper

and Bauer 1986). Flagella have been studied extensively in *Vibrio paraemolyticus* (Belas *et al.* 1986, Macnab 1987).

It is also important to bear in mind that the ability of bacteria to adhere not only depends on the ability to synthesize the ligand but also to express it in an accessible configuration on the surface. Surface components other than the ligand may influence the configuration, such as by altering the hydrophobicity of the cell surface, or by masking the ligand itself. Genetic alteration may affect surface components other than the ligand, and indirectly affect the expression of the ligand on the bacterial cell surface (Silverblatt and Ofek 1978, Ofek and Beachey 1980). Beside genetic control, the synthesis and expression of bacterial ligands appears to be under phenotypic control, such as source of the bacterium, the phase and condition of growth, number of laboratory passages, and other variations that may not exist *in vivo* (Ofek and Beachey 1980). The expression of bacterial ligands in *Salmonella typhimurium* was found to be under the control of c-AMP dependent catabolite repression (Saier *et al*. 1978, Ofek and Beachey 1980). Glucose has been reported to suppress hemagglutination activity and fimbriae formation (Ottow 1975, Ofek and Beachey 1980). Phenotypic variations are more complex however, and are difficult to generalize from different environments.

Although the attachment characteristics were different between S and L cells, various comparisons from previous experiments at the protein and genetic levels showed no significant difference. As described in previous chapters, for the strain used in this study, the possible ligand may be an attachment protein or EP or both. The results from morphology and EP analysis (Chapter 5 and 6) strongly suggest

the involvement of EP in the attachment process. The cytoplasmic (Nili and Brooker 1995) and extracellular protein profiles (Chapter 3) indicated no significant difference, and involvement of a single major protein seems unlikely. However, it is possible that small differences may not have been detected by PAGE. In addition, differences may be at the post-translational level, or in the structural sequence, rather than at the regulatory level. In this case, all proteins are still expressed in both variants, but one or more protein(s) may not carry out its proper function(s).

In this chapter, an attempt was made to clone attachment gene(s). Attachment genes, either as extracellular structures or attachment protein genes have been reported for a number of bacteria. Unfortunately, there has not been a detailed study of attachment of *B. fibrisolvens* to surfaces. The composition of its EP has been reported for a number of *B. fibrisolvens* strains. However, only the physical and rheological properties of the EP were studied and none of the EP of various *B. fibrisolvens* strains were proved to be related to attachment properties. In addition, there has not been any attempt to isolate gene(s) responsible for EP biosynthesis in *B. fibrisolvens*.

I explored two possibilities for elucidating the differences in S and L cells attachment properties at genetic level. Firstly, attachment gene(s) were being expressed in S but not in L cells. Therefore, to isolate attachment gene(s), L cells could be transformed with S chromosomal DNA. To obtain sticky transformants through genetic complementation. Secondly, gene(s) that inhibit attachment may be expressed in L but not in S cells. This possibility can be explored by

transforming S cells with L chromosomal DNA to obtain S transformants that become loose through genetic complementation.

Various genes of *B. fibrisolvens* have been successfully cloned (Chapter 1). However most of them were cloned in *E. coli* due to a lack of stable vectors and an efficient DNA delivery system for *B. fibrisolvens*. Among a few available vectors, pBHerm (Beard *et al*. 1995), was the most appropriate for this project, although it has been reported to be incompatible with some *B. fibrisolvens* strains (Hefford *et al*. 1997).

Since there is no defined activity associated with attachment of *B. fibrisolvens* to surfaces, transformants were screened for antibiotic resistance and morphological changes. In preliminary studies, *E. coli* was transformed with S chromosomal DNA using pUC19 or pBherm. For further experiments, L cells were transformed with S chromosomal DNA and vice versa using pBHerm. In addition, it was necessary to check whether pBHerm was compatible with the strains used in this study, especially since both S and L variants already contained endogenous plasmids.

7.2 Results

7.2.1 Plasmid maintenance

pBHerm was electroporated into *E. coli* ED8299. Plasmid DNA was isolated from transformants, and analysed by restriction mapping. The restriction pattern of the plasmid obtained from transformants (Figure 7.1) was similar to the map described by Beard *et al.* (1995).

7.2.2 Plasmid compatibility

Plasmid pBHerm was electroporated into L cells, to test compatibility. pBHerm was compatible with L cells, although the transformation efficiency (± 10^2 transformant/μg of plasmid DNA) was low (Figure 7.2). The restriction pattern of plasmid isolated from L transformants (Figure 7.2) was identical to that isolated from *E.coli* (Figure 7.1), suggesting no rearrangement had occurred and that the plasmid was stable. The presence of pBHerm in L cells seemed to inhibit replication of the endogenous plasmid, since it was not clearly seen in low (Figure 7.2) but clearly at higher concentrations of plasmid DNA (Figure 7.3). The intensity of staining of the indigenous plasmid bands was also less in the transformed compared to that of untransformed cells (figure 7.4). This is also supported by the results of hybridization analysis as described in section 7.2.11.

7.2.3 Transformation of *E. coli* with *B. fibrisolvens* S chromosomal DNA

E. coli was transformed with *B.fibrisolvens* S chromosomal DNA using pUC19 or pBHerm as vectors. Transformants were screened using ampicillin (50 μg/ml for pUC19 and 20 μg/ml for pBHerm). Each time transformation experiments were carried out, several colonies that mimicked the sticky phenotype were observed. These *E. coli* colonies were spherical, shiny, and different from other colonies, but did not stick to an agar plate. They also grew faster than the remaining other colonies. However, these colonies were not stable and did not grow when streaked or inoculated into liquid medium. When the transformation mixture was examined by SEM before plating on selective medium, the presence of *E. coli* producing excessive amounts of extracellular polymer was observed (Figure 7.5).

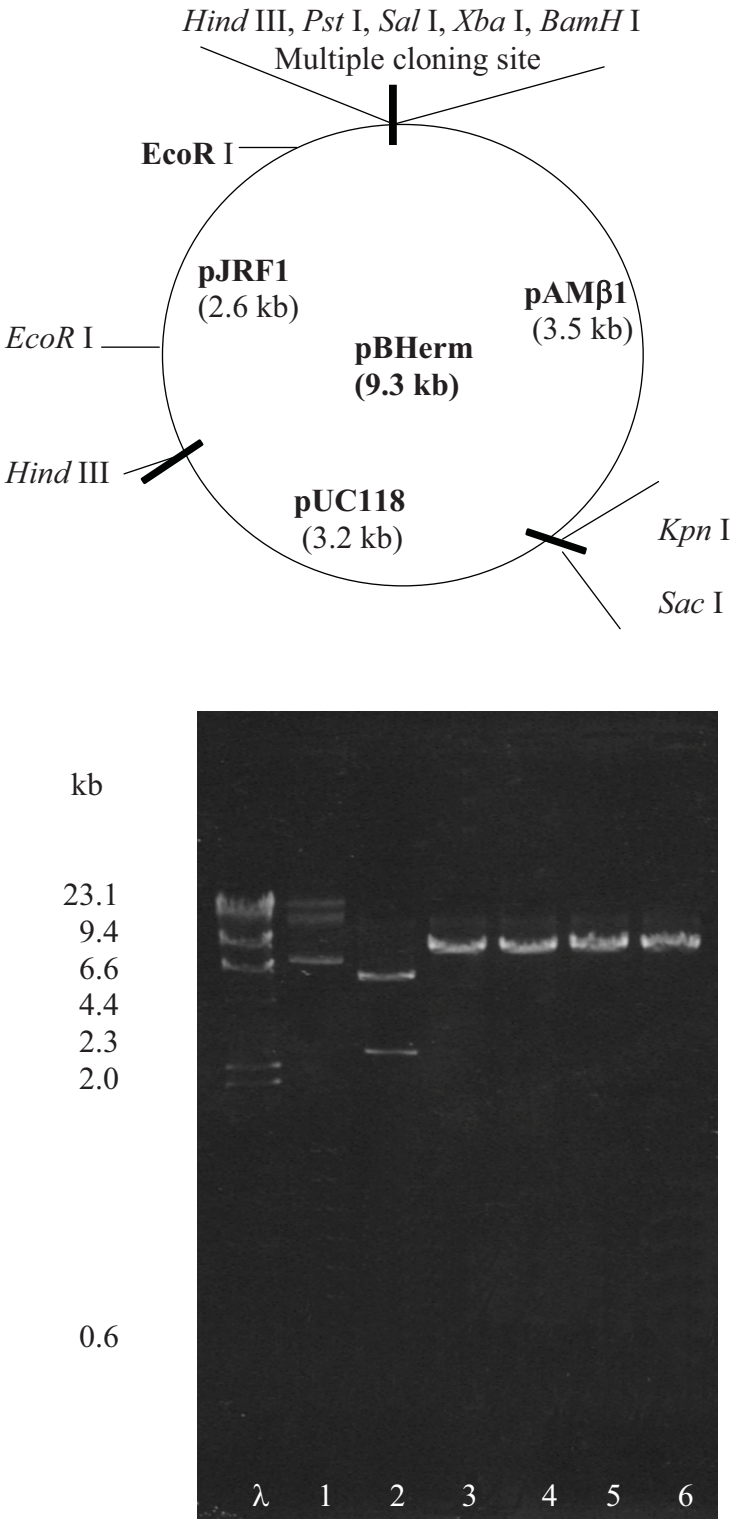

Figure 7.1 Restriction analysis of pBHerm isolated from *E. coli*.

pBHerm was isolated from *E.coli* ED8299 and restricted at the multiple cloning site. λ was Hind III cut λ DNA. 1, 2, 3, 4, 5, and 6 were uncut, *Hind* III, *Pst* I, *Sal* I, *BamH* I and *Xba* I cut pBHerm plasmid, respectively. The above diagram indicates the pBHerm plasmid map as described by Beard *et al*. (1995).

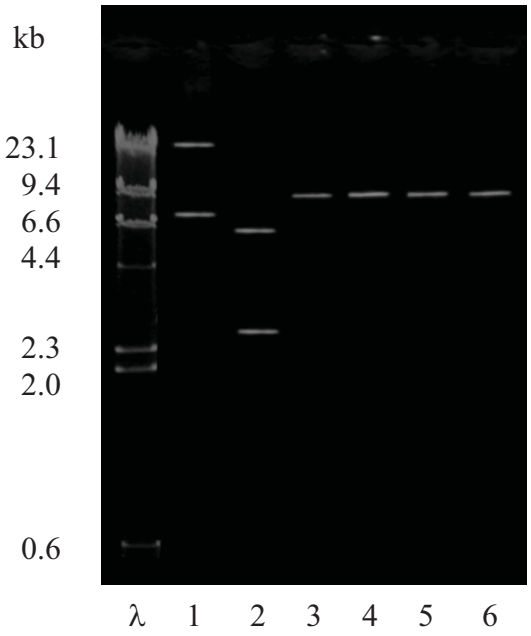

Figure 7.2. Restriction analysis of pBHerm isolated from *B. fibrisolvens*.

pBHerm was isolated from *B. fibrisolvens* L and restricted at multiple cloning site. 1, 2, 3, 4, 5, and 6 were uncut, *Hind* III, *Pst* I, *Sal* I, *BamH* I and *Xba* I cut pBHerm, respectively. The size marker was *Hind* III cut λ DNA

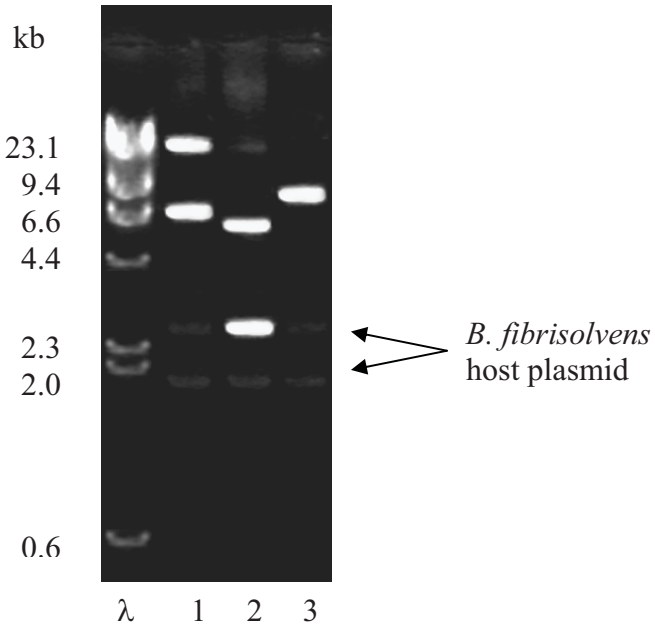

Figure 7.3 The presence of host plasmid in *B. fibrisolvens* transformed by pBHerm.

pBHerm was isolated from *B. fibrisolvens* variant L and restricted at its multiple cloning site. 1, 2 and 3 were uncut, *Hind* III and *Sal* I cut pBherm, respectively. The size marker was *Hind* III cut λ DNA. The arrows indicate the host plasmid of *B. fibrisolvens* L, which was only seen when high concentrations of plasmid were used, and visualized by the Biorad gel documentation system.

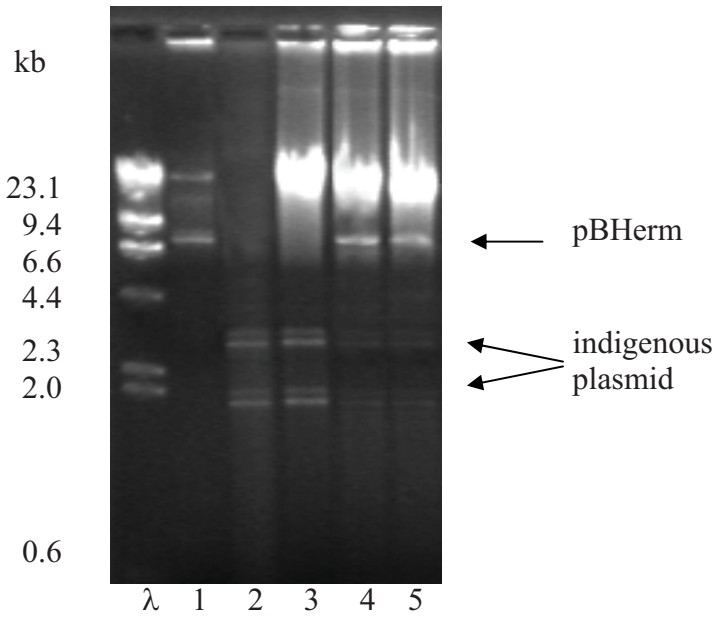

Figure 7.4 Effect of pBHerm on *B. fibrisolvens* indigenous plasmid levels.

B. fibrisolvens was transformed with pBHerm. The total DNA (4 and 5) was isolated and compared to that of untransformed cells (3). 1 and 2 were pBherm and indigenous plasmid respectively. The size marker was *Hind* III cut λ DNA.

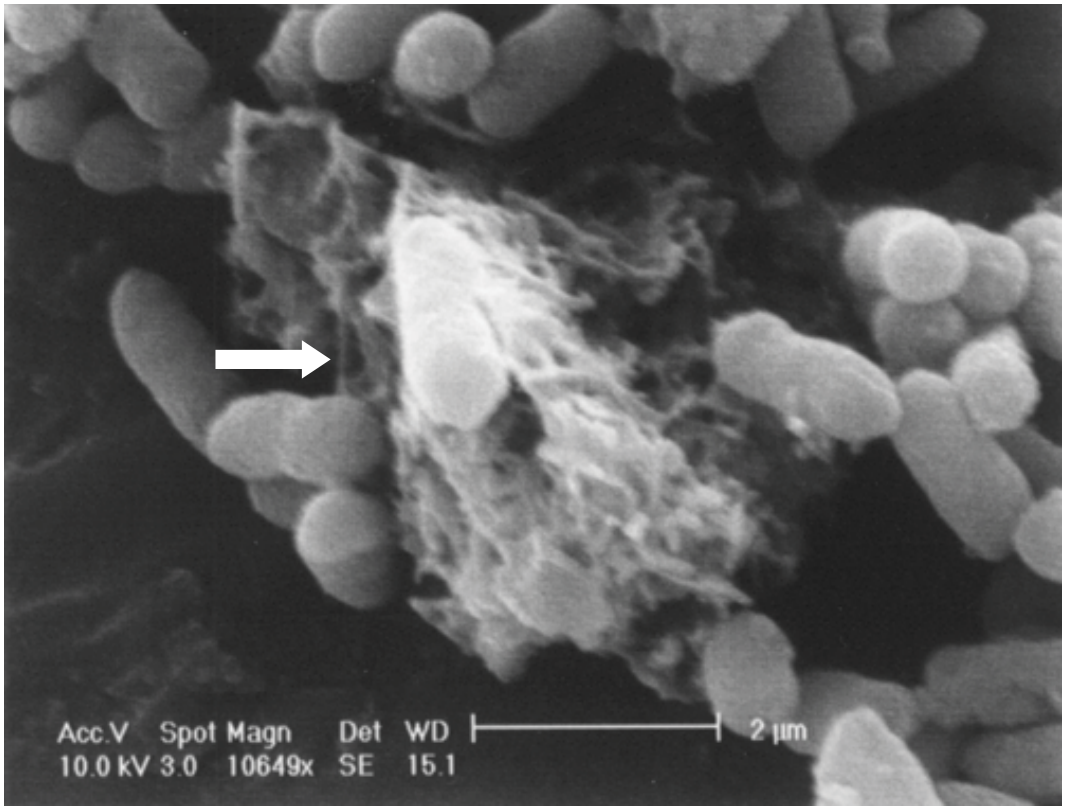

Figure 7.5 SEM of *E. coli* transformed by S DNA.

Escherichia coli ED8299 was transformed with S chromosomal DNA and prepared for SEM. The arrow indicates transformed *E. coli* producing extracellular polymer, but not the other transformed cells.

7.2.4 Complementation of *B. fibrisolvens* S with L DNA

If the attachment gene(s) were not expressed in L cells, it may be possible to transform L cells with S chromosomal DNA and obtain sticky transformants through genetic complementation. *Sal* I fragment of *B. fibrisolvens* S DNA was inserted into pBHerm plasmid. The recombinant plasmid was then electroporated into *B. fibrisolvens* L cells, and transformants were selected for erythromycin (10 μg/ml) resistance and examined for changes in cell morphology. As control, *B. fibrisolvens* L cells were transformed with pBherm plasmid. Since the transformation efficiency was relatively low, several electroporation parameters were varied, such as voltage, resistance, DNA concentration and plasmid source. Growing the plasmid within the same species (*B. fibrisolvens*) before transformation may increase transformation frequencies.

The results are shown in Table 7.1 to 7.4. The optimal electroporation condition was found to be 1.25 kV, 400 Ω and 0.35 μg of DNA. The time constant was 4-5 or 8-9 msec when 200 Ω or 400 Ω resistance was used, respectively. The transformation efficiency was 10^2 or 10^3/μg of DNA when pBHerm used was isolated from *E. coli* or *B. fibrisolvens*, respectively.

During these experiments, erythromycin resistant transformants were obtained, but none were sticky. However, some sticky-like colonies (Figure 7.6) similar to those described in section 7.2.3 were observed. The colonies were more yellow, spherical and tended to stick together, but did not attach to agar surfaces. Unlike

those in *E. coli*, these colonies were able to grow when reinoculated and further purified. Some colonies were further purified and stored as glycerol stocks.

Table 7.1 Effect of voltage on transformation efficiency (from S to L).

Number of experiment	Voltage (kV)	Efficiency (transformant/μg DNA ($\times 10^{-2}$)	
2	1.00	no growth	
2	1.25	1.9	*
2	1.50	0.6	
2	2.00	0.2	
1	2.50	no growth	

The resistance (400 Ω) and DNA concentration used for transformation (± 1.5 μg) were the same. Only the voltage was varied. * indicates the same data as in Table 7.2 and 7.3.

Table 7.2 Effect of resistance on transformation efficiency (from S to L).

Number of experiment	Resistance (Ω)	Efficiency (transformant/μg DNA) ($\times 10^{-2}$)	
2	200	1.7	
2	400	1.9	*

The voltage (1.25 kV) and DNA concentration used for transformation (± 1.5 μg) were the same. Only the resistance was varied. * indicates the same data as in Table 7.1 and 7.3.

Table 7.3 Effect of DNA concentrations on transformation efficiency (S to L).

Number of experiment	DNA (μg)	Efficiency (transformant/μg DNA) ($\times 10^{-2}$)	
1	2.00	2.0	
2	1.49	1.9	*
1	0.85	1.5	^
2	0.5	0.7	
2	0.35	2.2	#

The resistance (400 Ω) and the voltage (1.25 kV) used for transformation were the same. Only the DNA concentration was varied. * indicates the same data as in Table 7.1 and 7.2. ^ and # indicate the same data as in Table 7.4.

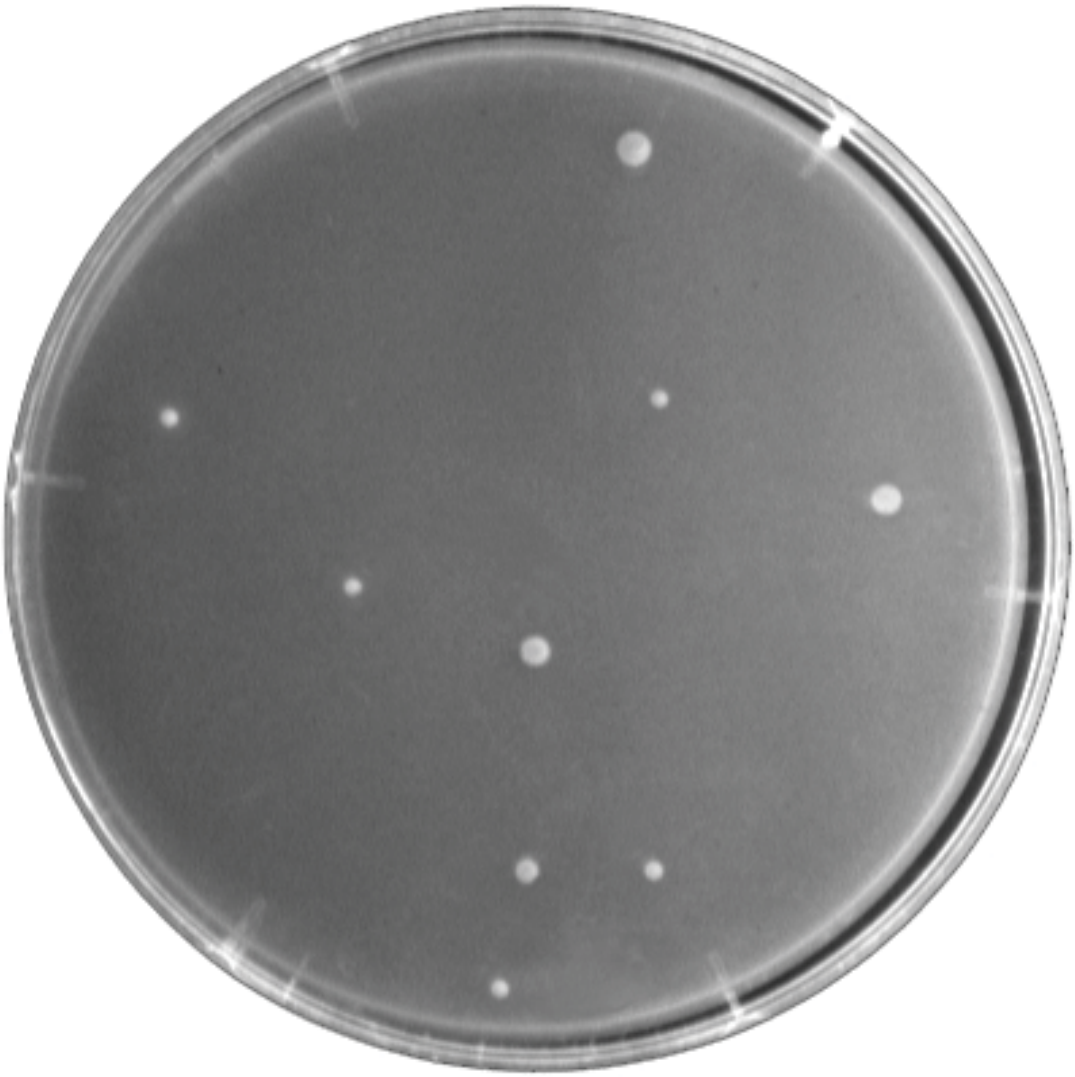

Figure 7.6 Example of sticky-like *B. fibrisolvens* transformants.

B. fibrisolvens transformants were grown in defined liquid medium (Nili and Brooker 1995) containing glucose and erythromycin (10 μg/ml), and incubated anaerobically at 39^{o}C until OD 1.0. The culture was diluted (1. 10^{6} x) and streaked onto defined medium containing glucose and erythromycin (10μg/ml), and incubated anaerobically at 39^{o}C for 36 hours.

7.2.5 Transformation of S with L chromosomal DNA

B. fibrisolvens S was transformed with L chromosomal DNA inserted into the *Sal* I site of pBHerm, to seek S transformants that became L-like. The transformants were selected for erythromycin (10 μg/ml) resistance and changes to cell morphology. The electroporations were carried out under optimised conditions obtained above (section 7.2.4). However, some variations were still undertaken, including competent cell preparation, DNA concentration and resistance. The results are shown in Tables 7.5 to 7.7. The transformation efficiency was similar to that obtained for transformation of *B. fibrisolvens* L with S chromosomal DNA. The optimal amount of DNA was about 0.9 μg, but the difference was not significant. At low levels of DNA (0.05 μg) the efficiency increased up to 10 fold. Attempts to use competent cells that were prepared at an earlier growth point (OD<1) did not increase transformation efficiency significantly. None of the S transformants showed the same morphology as the L cells.

Table 7.4 Effect of plasmid source on transformation efficiency (from S to L).

Number of experiment	DNA (μg)	Plasmid source	Efficiency (transformants/μg DNA) (x 10^{-2})	
2	0.50	*E. coli*	0.7	^
2	0.50	*B. fibrisolvens*	6.7	
2	0.35	*E. coli*	2.2	#
2	0.35	*B. fibrisolvens*	21.5	

The resistance (400 Ω) and the voltage (1.25 kV) used for transformation were the same. Only the DNA concentration was varied (0.5 and 0.35 μg) and the ligation mixtures were prepared using pBHerm isolated from *E. coli* and *B. fibrisolvens*. ^ and # indicate the same data as in Table 7.3.

Table 7.5 Effect of competent cell preparation on transformation efficiency (from L to S).

Number of experiment	OD at the time harvested	Efficiency (transformants/μg DNA) ($\times 10^{-2}$)
2	± 0.5	3.1 ◊
1	± 1.0	1.9
1	± 1.3	1.5

The resistance (400 Ω), the voltage (1.25 kV) and DNA (1.8 μg) were the same. Competent cells were prepared at different ODs. ◊ indicates the same data as in Table 7.6.

Table 7.6 Effect of DNA concentrations on transformation efficiency (L to S).

Number of experiment	DNA (μg)	Efficiency (transformants/μg DNA) ($\times 10^{-2}$)
2	1.8	3.1 ◊
1	0.9	3.3
1	0.5	2.5
1	0.1	2.8
1	0.05	26.6 ∞

The resistance (400 Ω), the voltage (1.25 kV) and OD of competent cells (± 0.5) used for transformation were the same. DNA concentration was varied. ◊ and ∞ indicate the same data as in Table 7.5 and 7.7, respectively.

Table 7.7 Effect of resistance on transformation efficiency (from L to S).

Number of experiment	Resistance (Ω)	Efficiency (transformants/μg DNA) ($\times 10^{-2}$)
1	200	25.0
1	400	26.6 ∞

The voltage (1.25 kV), DNA and OD of the competent cells (0.5) used for transformation (0.05 μg) were the same. The resistance was varied. ∞ indicates the same data as in Table 7.6.

7.2.6 Stability of sticky-like transformants

To further examine *B. fibrisolvens* sticky-like transformants (section 7.2.4), 3 transformants (no. 3, 4 and 6) were selected for further analysis. The transformants (glycerol stock) were inoculated into defined medium containing glucose and erythromycin as described in Methods and as outlined in Figure 7.7. Throughout the subsequent 5 passages (representing more than 15 generations), the sticky-like transformants maintained their behaviour (cells were shiny, spherical and tended to stick together but did not attach to surfaces).

7.2.7 Growth ability of transformants under various conditions

The ability of *B. fibrisolvens* transformants to grow on plates and in liquid medium containing various carbon sources (glucose, cellobiose, xylan, cellulose, sucrose, maltose or starch) or nitrogen sources (casein or casein + ammonium chloride) were checked. Growth of transformants in these media was slightly slower (0.6 x) than the original variant, possibly due to the erythromycin added to the medium.

7.2.8 Reversion of transformants

B. fibrisolvens transformants were tested to determine whether they would revert to the original variant when grown in medium without selection pressure. The experiments were carried out as described in Methods and as outlined in Figure 7.8. After the fourth passage with and without erythromycin selection the sticky-like transformants were not stable and showed some reversion to the original type (L), as reflected by a decrease in growth after the third passage (Figure 7.9). An

example of plates containing transformants and revertants is shown in Figure 7.10.

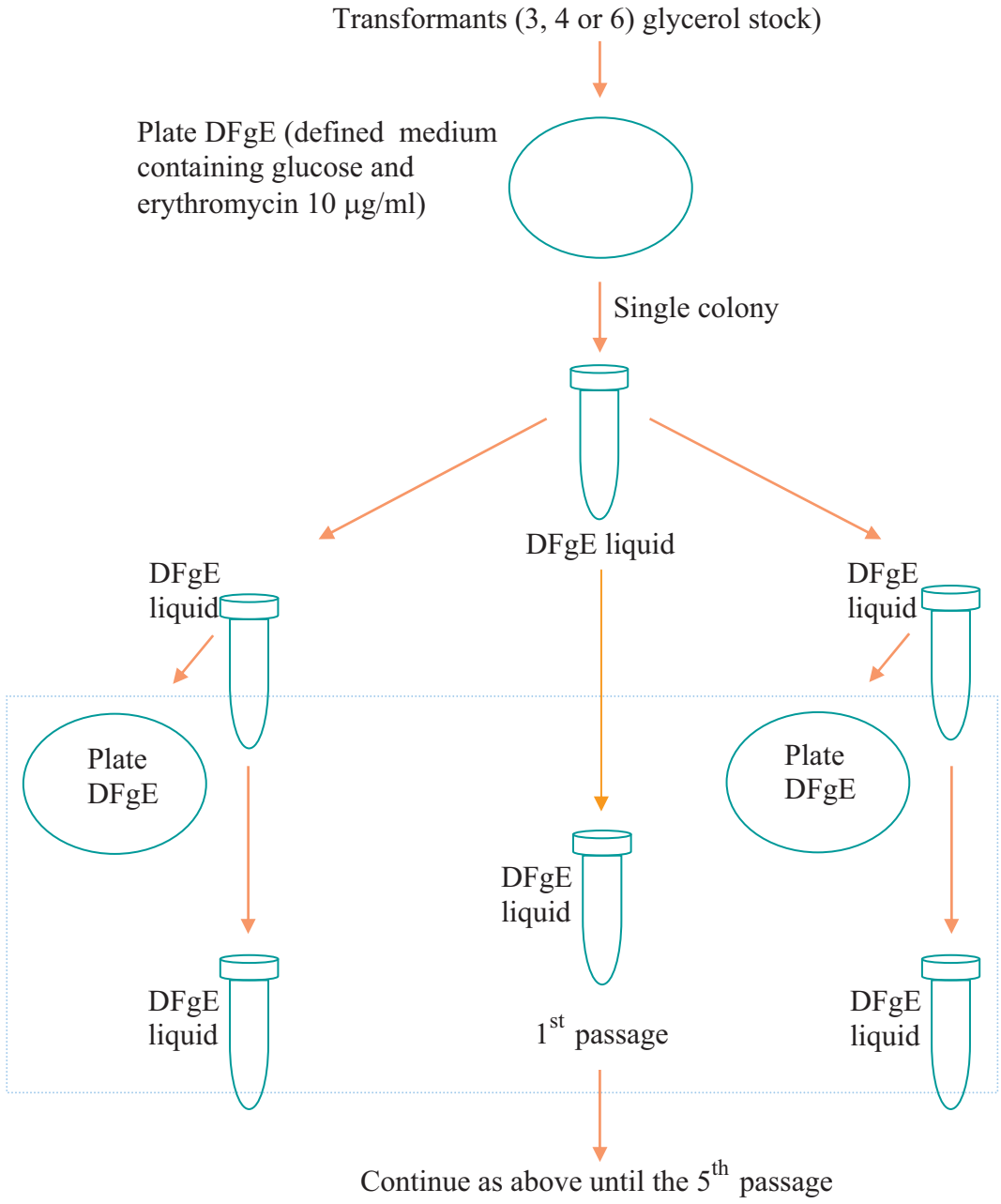

Figure 7.7 Diagrammatic procedures of transformant stability experiment.

Cultures were incubated anaerobically at 39°C for 16-24 hours. All inoculation were done using 1 % starting culture for broth medium and ± 5 µl (of 1000 x dilution) for streaking on agar plates. Three independent experiments were done and carried out in duplicate.

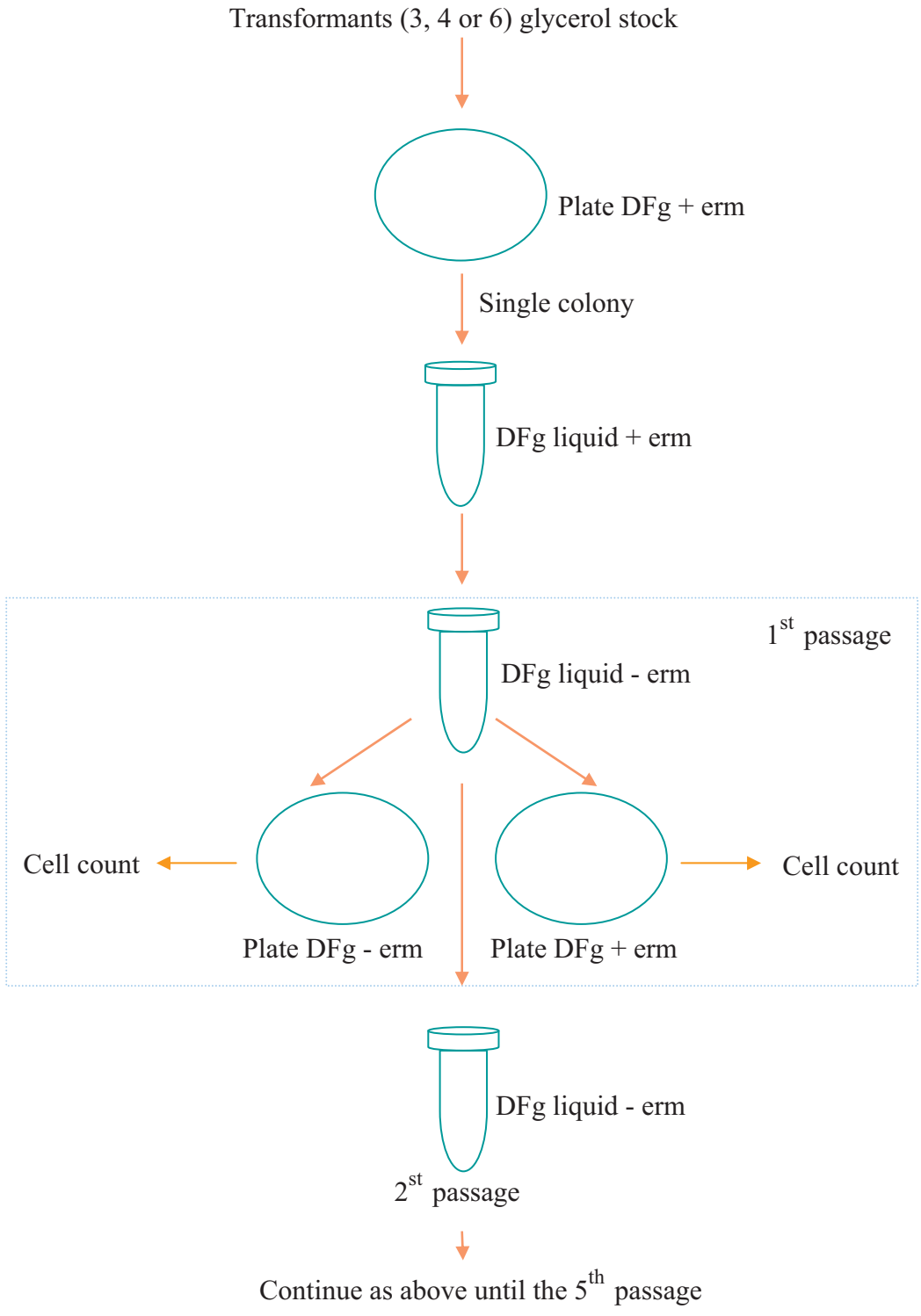

Figure 7.8 Outline of procedures to examine reversion of transformants

Plates were incubated anaerobically at 39°C for 16-24 hours, while liquid cultures were incubated similarly until an OD 600 nm 1.0. All inoculations were carried out using a 1 % starting culture for broth medium and 5 µl of a 1000 x dilution of the culture for streaking onto nutrient agar plates. DFg, + erm and -erm were defined medium (Nili and Brooker 1995) containing glucose, with and without erythromycin, respectively.

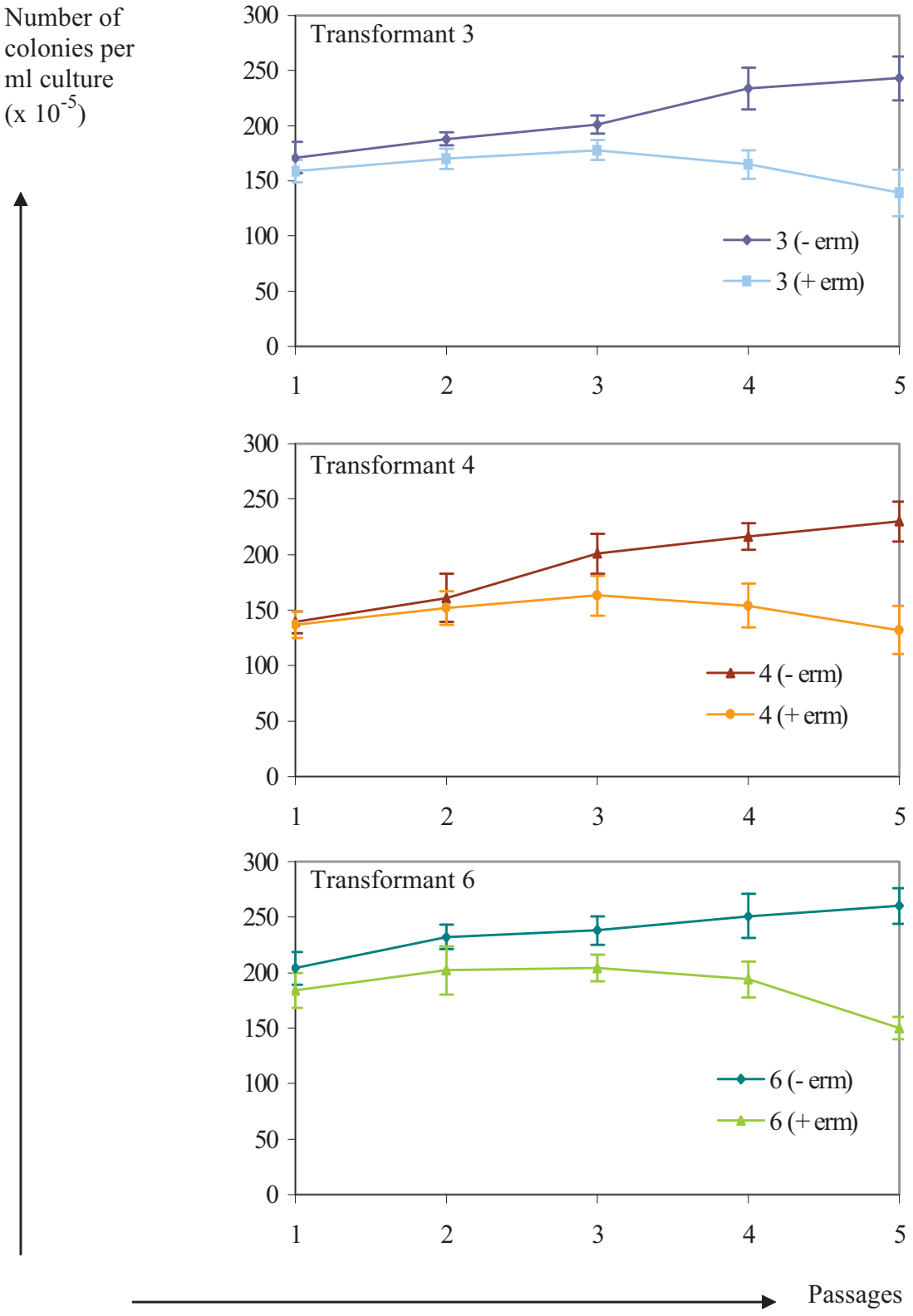

Figure 7.9 Growth of transformants in medium with and without erythromycin.

3, 4 and 6 were the numbers of transformants. – erm and + erm indicate with and without erythromycin. Cultures were treated as described in the method and as outlined in Figure 7.7. Values represent the mean and standard error of two independent experiments carried out in duplicates.

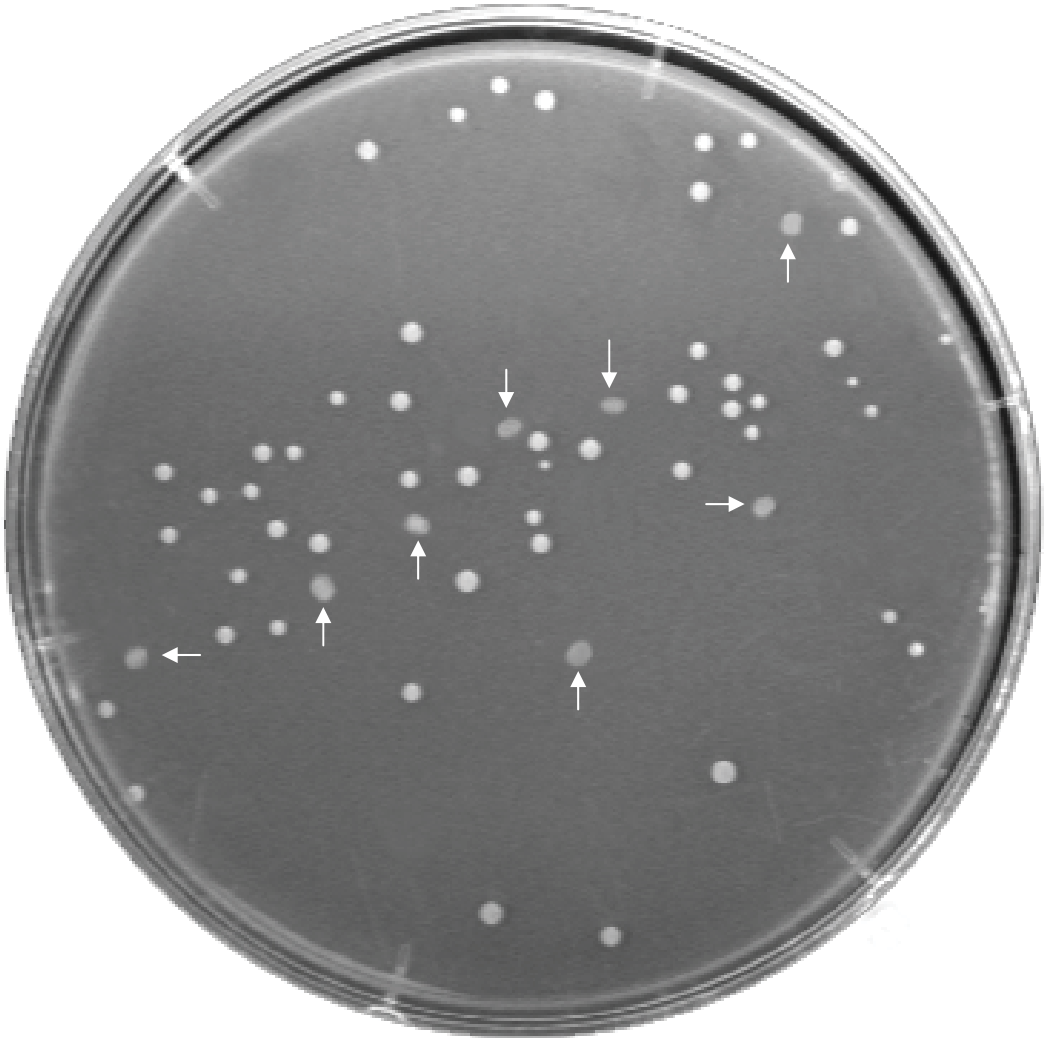

Figure 7.10 Reversion of transformants.

Transformants were grown in defined liquid medium containing glucose, and incubated anaerobically at 39^{o}C until OD 600 nm 1.0. Cultures were diluted x10^{-5}, streaked onto defined solid medium containing glucose and erythromycin (10μg/ml), and incubated anaerobically at 39^{o}C for 24 hours. Arrows indicate sticky-like transformants that revert to the original variant (L).

7.2.9 Recombinant plasmid

An attempt was made to isolate recombinant plasmid from the transformants. However, this was not successful. It was not known whether this was due to the low copy number or to the size of the recombinant plasmid. Total plasmid or chromosomal DNA preparations of the transformants were electroporated into *E. coli* ED8299 with selection for ampicillin^r or DB11 with selection for erythromycin^r. No transformants were obtained. Control transformants using standard plasmid yielded transformants in both hosts.

7.2.10 Total DNA profiles of transformants

Since I was not successful in isolating a recombinant plasmid, it was necessary to check the authenticity of transformants and their relationship to the original variants. The uncut and *Sal* I-cut chromosomal DNA of transformants (3, 4, and 6) were compared with the original S and L variants.

The *Sal* I-cut total DNA profiles of the transformants were identical to that of S and L variants (Figure 7.11). In addition, the same endogenous plasmid, as in S and L variants was observed in the transformant DNA samples. These results show that the transformants are derived from the original S and L variants.

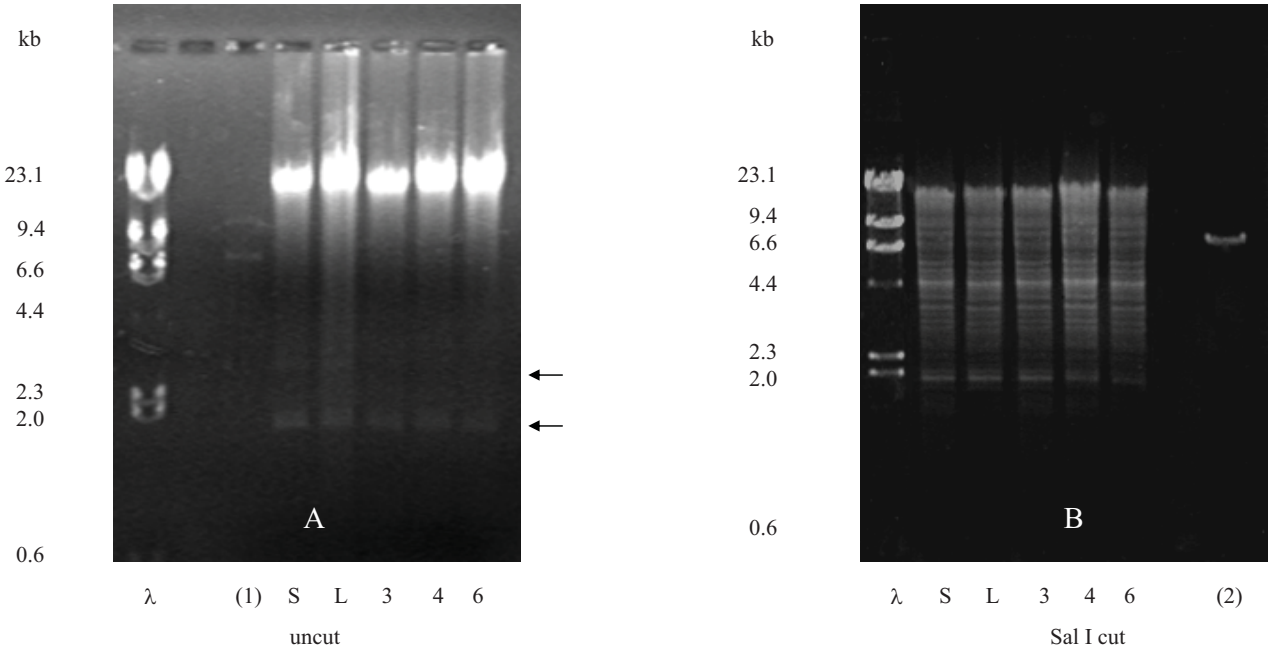

Figure 7.11 Comparison of DNA profiles between variants and transformants.

The total chromosomal DNA of *B. fibrisolvens* E14 S and L as well as the transformants (3,4 and 6) were isolated and compared on 0.8 % agarose gels. λ was Hind III cut λ DNA. (1) and (2) were uncut and *Sal* I cut pBHerm plasmid, respectively. Arrows (←) indicate the indigenous plasmid bands. A, uncut DNA; B, *Sal* I cut DNA.

7.2.11 Hybridisation analysis of transformants

Since S and L are related, it is possible that chromosomal integration may have occurred when L cells were transformed with S chromosomal DNA, due to DNA homology between the two variants. The plasmid vector (pBHerm) may have been included during chromosomal integration. If this had occurred, plasmid DNA would be detectable in chromosomal DNA of the transformants. This possibility was explored by hybridising chromosomal DNA of the transformants with pBHerm as a probe. Chromosomal DNA of S and L cells (which should not contain pBHerm DNA) were included as controls. The probe was isolated from *E. coli* to avoid any *B. fibrisolvens* DNA contaminant that might positively hybridise with the samples. In addition, if pBHerm was isolated from *B. fibrisolvens*, it might also be contaminated with the host plasmid, which could hybridise with the samples. The probe was linearized and further purified from an agarose gel, as described in the Methods.

The uncut, *Sal* I-, EcoR I- and *Pst* I- cut total DNA of transformants hybridised with the pBHerm probe, while that of S and L variants as controls did not (Figure 7.12). It was also observed that the indigenous plasmid in the variants (S and L) and transformants (3, 4 and 6) positively hybridised with the pBHerm probe. In addition, the presence of recombinant plasmid showed similar results as demonstrated previously with pBHerm (section 7.2.2); i.e. reduced replication of the endogenous plasmid as reflected by the lower endogenous plasmid band intensity of the transformants compared to that of untransformed S and L cells.

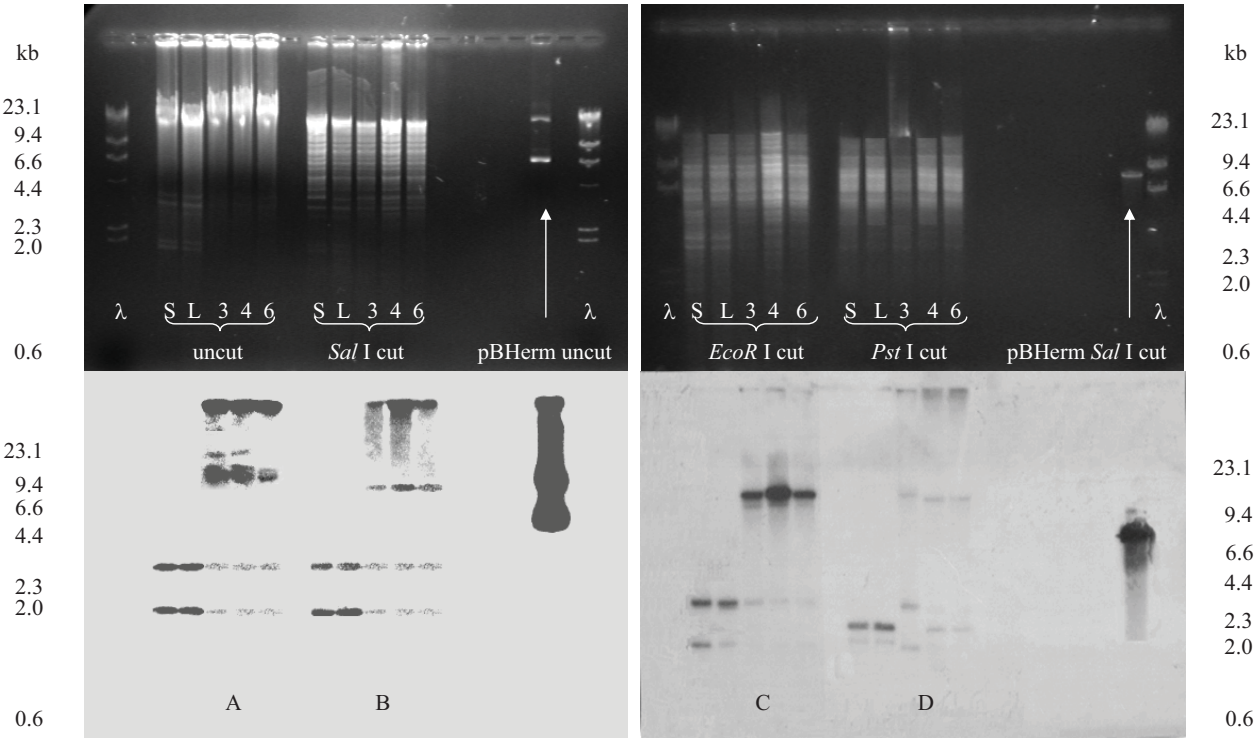

Figure 7.12 Hybridisation analysis of variants and transformants.

The total chromosomal DNA of *B. fibrisolvens* E14 S and L, as well as the transformants (3,4 and 6) were isolated. The uncut (A), Sal I (B), EcoR I (C) and Pst I (D) cut DNA was fractionated by 0.8 % agarose gel electrophoresis. After transferring to a nylon membrane by Southern blot, it was hybridised with *Sal* I cut pBHerm (isolated from *E. coli*), and was further purified from an agarose gel. λ, *Hind* III cut λ DNA.

7.2.12 Phylogenetic analysis of transformants

To further confirm that the transformants were derived from S and L cells, the 16S rDNA profiles of the transformants were compared with the original cells (S and L). The total DNA of S and L cells and transformants were isolated, and the 16S rDNA was amplified PCR as described in the Methods. The 16S rDNA of S, L and transformants were restriction digested with *Hae* III, *Alu* I or *Taq* I, and then fractionated by agarose gel electrophoresis. The results showed that The *Hae* III-, *Alu* I- or *Taq* I–digests resulted identical profiles for the variants S, L and the transformants 3, 4 and 6 (Figure 7.13).

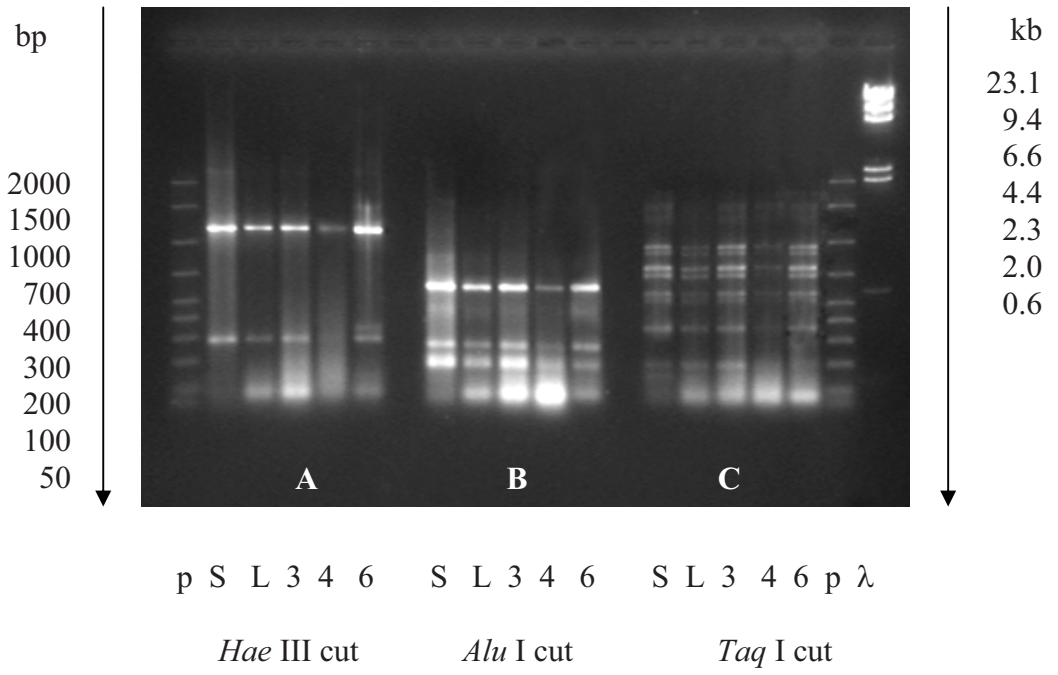

Figure 7.13 Comparison of 16S rDNA profiles between S, L and transformants.

P and λ were Amplize and Hind III cut λ DNA markers, respectively. S and L were DNA samples of *B. fibrisolvens* E14 S and L, and 3, 4, 6 were that of transformants 3,4 and 6, respectively. The 16S rDNA was restricted with *Hae* III (A), *Alu* I (B) and *Taq* I (C), and fractionated by (1.2 %) agarose gel electrophoresis.

7.3 Discussion

Although with low transformation efficiency, pBHerm seemed to be compatible with *B. fibrisolvens* E14 S and L. The transformation frequency of pBHerm plasmid into *B. fibrisolvens* obtained during this study (10^3/μg of DNA) was slightly lower than what has been previously reported (10^4/μg of DNA) for pBHerm plasmid without additional DNA (Beard *et al.* 1995), and the lower frequency may due to the plasmids larger size when ligated with S chromosomal DNA. The transformation frequency into *B. fibrisolvens* strains is usually low (Teather 1982, Mann 1986, Gennaro *et* al. 1987, Ware *et al.* 1992, Hefford *et* al. 1993, Beard *et al*. 1995, Kobayashi *et al*. 1995) compared with other bacteria, especially *E. coli* (10^6/μg of DNA). The higher viscosity EPS produced by *B. fibrisolvens* strains may interfere with their ability to be transformed using electroporation, and could explain the low transformation frequency (Ha *et* al. 1991, Clark *et al*. 1994, Beard *et* al. 1995). The transformation frequency was also affected by plasmid source; lower transformation frequencies (10^2/μg of DNA) were obtained when pBHerm was isolated from *E. coli* rather than *B. fibrisolvens* (10^3/μg of DNA). Growing the plasmid within the same species (*B. fibrisolvens*) before transformation may avoid methylation and other DNA modification reactions, and therefore give higher transformation frequencies. Varying the conditions of electroporation did not increase the efficiency significantly, suggesting that the value obtained was maximal for this system. Higher efficiencies may be possible using other techniques eg. heat shock, or another host strain.

Various *B. fibrisolvens* genes have been successfully cloned in *E. coli*, but none, which expressed extracellular structures. It is possible that *E. coli* might not be an appropriate host due to differences in its membrane structure compared with *B. fibrisolvens*. This may be one of the reasons why no stable sticky *E. coli* were obtained when S chromosomal DNA was electroporated into *E. coli*. In addition, such gene(s) may be lethal when expressed in *E. coli*, due to differences in membrane structure. After expressing the gene(s) at early growth, the *E. coli* cell may no longer be able to maintain its cell integrity. This was supported by the observation that sticky-like *E. coli* were shiny, spherical and grew much faster than neighbouring colonies, but were not stable and did not grow when further inoculated. Furthermore, EP is usually the product of a multi-enzyme system, and therefore encoded by several genes, possibly located on an operon (Gottesman 1985, Fett *et al.* 1992, Gervais and Drapeau 1992, Stingele *et al.* 1996, Kranenberg *et al*, 1997). To obtain sticky *E. coli*, the inserted DNA fragment in the recombinant plasmid may need to encode several genes for EP biosynthesis. The results from SEM examination of S and L cells (Chapter 5) showed that their EP structure was very complex, and comprised carbohydrate and lipid. These facts strongly suggest that a complex EP mechanism is involved in the biosynthesis of EP. In addition, it is not known whether *B. fibrisolvens* EP-genes are located in single or multiple clusters. In *X. xampestris*, EP genes are organized into more than one cluster (Harding *et al*. 1987, Thorne *et al*. 1987), and similarly in some *E. coli* strains (Diderichson 1980, Sanderson and Roth 1983, Bacmann 1983) and *Klebsiella* K20 (Whitfield 1988). The regulation of colanic acid biosynthesis in *S. typhimurium* (Markovittz 1977, Whitfield 1988) and EPS-genes

in *R. meliloti* (Leigh *et al.* 1985) are also divided between plasmid and chromosomal DNA.

The phenotypic difference between S and L cells may be a spontaneous mutation such as error in DNA replication, spontaneous lesion or indigenous transposable element. Such mutations are found to occur naturally at low frequencies in most hosts (Griffin *et al.* 1995). Although EP biosynthesis involves a large DNA fragment with multiple genes, EPS-deficient phenotypes are often obtained by a single gene disruption. A plasmid-encoded EPS gene cluster in *Lactococcus lactics* has been found to be a 12 kb region containing 14 genes, but an EPS-deficient phenotype was obtained by a single gene disruption (Kranenberg *et al*, 1997). The product of the essential gene was glycosyltransferase, which is involved in linking the first sugar (glucose or galactose) of the sugar repeating unit to a lipid carrier. Glycosyltransferase export and polymerisation genes have been identified within gene clusters in the biosynthesis of *Salmonella* LPS (lipopolysaccharide) (Jiang *et al*. 1991, Brown *et al*. 1992, Morona *et al*. 1994, Allen and Maskel, 1996); *Streptococci* CPS (capsular polysaccharide) (Rubens *et al*. 1993, Guidolin *et al*. 1994, *et al*. 1994, Arakawa *et al*. 1995, Kolkman *et al*. 1996); and *Rhizobium meliloti* EPS (Glucksmann *et al*. 1993, Bugert and Geider, 1995, Huang and Schell 1995, Stingele *et al*. 1996). A spontaneous mutation may have occurred in S cells, to yield L cells. Since L cells still produced EP, the mutation may have only partially deactivated EP biosynthesis rather than causing a complete loss of activity. In most cases, disruption of the glycosyltransferase gene results in complete deactivation of EP biosynthesis (Rubens *et al*. 1993, Kolkman *et al*. 1996, Reuber and Walker 1993, Jiang *et al*. 1991, Wang and

Reeves, 1994). This is unlikely to be the case of S and L cells, since both variants produce EP, it is also unlikely that the mutation occurs within the export genes. The mutation may have occurred within the polymerisation genes and, since I observed much higher lipid content in S than L EP, it may be within the lipid-carrier biosynthetic genes.

In some cases, the synthesis and expression of bacterial ligand appears to be under phenotypic control (Saier *et al*. 1978, Ofek and Beachey 1980), such as catabolite repression of ligand biosynthesis in *Salmonella typhimurium* and fimbriae formation of other bacteria (Ottow 1975, Saier *et al*. 1978, Ofek and Beachey 1980). However, this is unlikely to be the case of S and L cells, since they were both treated similarly under all experimental conditions.

Since the sticky-like *B. fibrisolvens* transformants also contained endogenous plasmids (as that of S or L cells), and the (uncut, *Sal* I-, *EcoR* I- and *Pst* I-cut) DNA profiles (Figure 7.11 and 7.12) as well as the 16S rDNA (*Hae* III-, *Alu*- and *Taq* I-cut) profiles (Figure 7.13) were identical to those of S and L cells, I can conclude that the transformants were derived from the S or L cells. The fact that pBHerm probe hybridised with the total chromosomal DNA of the sticky-like transformants but not with the original cells (S and L) (Figure 7.11), suggested that pBHerm was integrated into the hosts DNA but was not present in the original chromosomal DNA. It is likely that the recombinant plasmid integrated into the chromosomal DNA due to DNA homology, since S and L were of the same species. The cryptic plasmid pOM1 of *B. fibrisolens* has been found to contain sequences encoding a plasmid recombination protein and a recombination

site (RS_A) (Hefford *et al*. 1997). The sequence was also found to be homologous with a site-specific recombination function in *Staphylococcus aureus* plasmid (Gennaro *et al.* 1997), and with several other plasmids (van der Lelie 1989, Hefford *et al*. 1997). If such a sequence was present within the indigenous plasmid of the L cells, it may also induce the integration of the recombinant plasmid into L chromosomal DNA. In addition, the large size of the recombinant plasmid (9.3 kb of pBHerm + unknown size of insert DNA) requires energy for replication and expression, which may induce a cellular response to keep a minimal copy number by integrating into chromosomal DNA, although plasmid copy number is usually encoded on the plasmid.

The fragment of S DNA within the recombinant plasmid may contain a partial sequence of the EP gene cluster or attachment genes, which may partially compliment the L cell genotype, and result in a partially sticky phenotype. The S-like *B. fibrisolvens* transformants obtained during this study were stable for up to 3 passages (Figure 7.9) without selection pressure, and this was reflected in a decrease in growth in medium containing erythromycin after the third passage. This may be due to rearrangement and/or deletion of the recombinant plasmid that integrated into L chromosomal DNA. The cryptic *B. fibrisolens* plasmid pOM1 also appeared to be unstable and subject to rearrangement and/or deletions when cloned in pBR322 (Hefford *et al*. 1997). The probability of this occurring would be higher in this experiment, which was within a species, compared to the above example, which was between 2 species. It is also possible that the host cell itself induced the DNA rearrangement, as a cellular response to stabilise its chromosomal DNA, to reach the most stable base sequence.

The pBHerm probe also hybridised with the indigenous plasmid within the variants and the transformants, suggesting homology, possibly between the pJRF1 fragment of pBHerm plasmid and the indigenous plasmid. pJRF1 was also derived from *B. fibrisolvens,* but from strain OB156 (Figure 7.1) (Beard *et al*. 1995) rather than E14. The indigenous plasmid of *B.* fibrisolvens E14 S or L was very similar in size to pJRF1 or pJRF2 (2.8 kb) of *B. fibrisolvens* OB156, and may either represent a common plasmid for some *B. fibrisolvens* strains or have a common component. The size of pOM1 plasmid in strain Bu49 and pBF1 in strain AR10 were also about 2.8 kb in size (Teather 1982, Mann *et al.* 1986, Hefford *et al.* 1997). Indigenous plasmids among *B. fibrisolvens* strains may therefore share common functions.

For future studies, the cloned fragment of complementing S DNA should be isolated and sequenced, and the flanking region should be examined through plasmid rescue experiments. This approach may yield a full EP or attachment gene cluster from S cells. The known pBHerm sequence may facilitate oligonucleotide synthesis that can be used as a primer for PCR amplification and chromosomal walking experiments, particularly in the S-complemented L cells.

During this study, an attempt was made to use plasmid pUB110, a common vector for *Bacillus subtilis*. However this was not continued since both *B. fibrisolvens* E14 S and L cells were resistance to neomycin and kanamycin (the selection marker of pUB110).

Attempts to isolate gene(s) of *B. fibrisolvens* S that confers the altered phenotype were not successful for various reasons, including;

- The complexity of S EP biosynthesis that may involve multi enzymes and therefore be coded by a large fragment of DNA. These genes may reside at different regions of the chromosomal DNA.
- The size of pBHerm plasmid (9.3 kb) may limit the size of DNA fragment that can be accommodated.
- The homology between donor and host DNA, which were of the same species, would in high probability, promote chromosomal integration.
- Both *B. fibrisolvens* E14 S and L contain indigenous plasmids that may reduce transformation efficiency, and increase the complexity of DNA analysis.
- No defined activity associated with attachment of *B. fibrisolvens* E14 variant S to surfaces has been identified.
- The time limitation of the study.

In conclusion, pBHerm was compatible with S and L cells, and hybridised with S and L endogenous plasmids, sticky-like transformants that exhibit some of the S variant characteristics were obtained and there were indications of chromosomal integration within these transformants. These data support the hypothesis that S and L cells vary probably by only one gene function, and this may be complemented through gene transfer. The existence of S and L cells however, provides a useful model system to study attachment mechanisms in *B. fibrisolvens*

and further work is needed to stabilise the gene complementation assay and, or by subtraction hybridisation, to identify and isolate the gene involved.

CHAPTER 8

GENERAL DISCUSSION AND FUTURE STUDIES

8.1 General discussion

The morphology of S and L cells showed differences in their EP structure. The lipid content of their EP was also different. Therefore, it might be expected that these differences would be reflected in some observable changes in at least one protein involved in EP biosynthesis. However, the similarity between S and L extracellular and cytoplasmic protein profiles (Nili 1996) suggested that differences in attachment ability did not involve major changes in protein synthesis. Although EP genes normally occur in an operon structure and may be arranged in a single or multiple clusters, EP-defective mutants are often obtained by disruption of a single essential gene (Rubens *et al*. 1993, Kolkman *et al*. 1996, Reuber and Walker 1993, Jiang *et al*. 1991, Wang and Reeves, 1994, Kranenburg *et al*.1997), and therefore should not incur major changes in protein synthesis. It is also possible that a mutation in the structural gene sequence may be responsible for the effect, rather than in regulatory sequences. The protein(s) may still be expressed but without proper function. In addition, the difference may be during post-translational processing of newly synthesised protein. If this was the case, PAGE would not resolve the differences, although 2 dimensional gel electrophoresis may detect single amino acid changes if they involved structural changes to the protein. Similarly, agarose gel electrophoresis would not resolve differences of a few bases in the genome. The similarities between S and L

chromosomal and plasmid DNA, as well as 16S rDNA may also suggest the absence of protein(s) that differ between S and L cells, but did not eliminate the possibility that a mutation may be within a structural sequence. Further experiments are required to confirm this point.

The protein(s) of interest may not necessarily be an attachment protein *per se* in S, but may be an attachment-inhibitor protein in L cells. For this reason, in chapter 3 cell to cell communication experiments were carried out to explore the possibility that S/L cells may produce molecules that could mediate or inhibit cell attachment to surfaces. Certain proteins may harbour determinants which bind specifically to bacterial ligands and inhibit epithelial adhesion. M protein has been found on the cell surface of *Streptococci,* and it is this that confers resistance to phagocytic attachment (Stollerman 1975, Ofek and Beachey 1980). LTA receptors have been shown to be involved in the phagocytic attachment of *Streptococci* which lack M protein on their surface (Ofek and Beachey, 1979). M protein may mask surface LTA and therefore prevent the organism from binding to receptors on the phagocytic cell membrane of epithelial cells (Ofek et al. 1975, Ofek and Beachey 1980). The binding of LTA to epithelial-cell membranes is mediated by its ester-linked fatty acids. Albumin is also known to possess binding sites for fatty acids; this protein binds ester-linked fatty acids of LTA, but not deacylated LTA. The albumin-LTA binding blocked the interaction between membrane and LTA, and the attachment between epithelial cells and *Streptococci* was restored by albumin removal. This suggested a reversible inhibition and albumin did not irreversibly alter ligand or receptors (Peters 1975, Ofek and Beachey 1980). L cells may produce such a protein that masks their EP and hence prevents the cells from

attaching to a surface. In contrast, S cells may express this protein without proper function. However these were not detected, due to the limitations of sensitivity of agarose and polyacrylamide gel electrophoresis.

SEM examination showed that in S cells, there were associations between EP and surfaces at a distance from the cell body. However, it is still questionable whether it is EP alone or in combination with specific proteins that mediates or inhibits an association between S/L cells and surfaces. If protein(s) were involved in such associations, either mediating (in S cells) or inhibiting (in L cells) attachment, the protein(s) may be EP associated (type 1) or secreted to the medium (type 2), but not cell associated (type 3) as outlined in Figure 8.1.

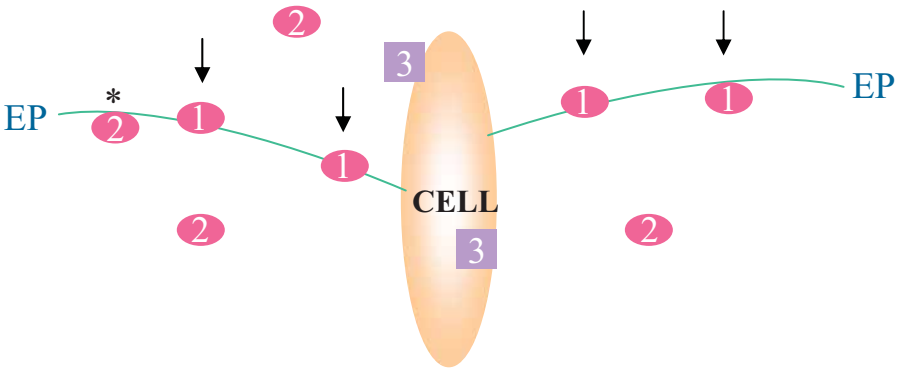

Figure 8.1 Possible locations for attachment proteins.

1, 2 and 3 indicate EP associated, secreted and cell associated proteins, respectively. EP indicates extracellular polymer, while arrows (↓) indicate EP-surface association. If protein(s) mediate or inhibit such association, it should be type 1 or 2, but not type 3. Instead of type 1, secreted protein(s) (type 2) may also mediate such association (*), but with much lower efficiency, since it is not already attached to the EP, and depends on statistical-collision probability. Type 3 is too far from the EP-surface association (↓).

The similarity between total extracellular and secreted protein profiles also suggested that the major extracellular proteins were EP associated (Chapter 3). A previous report (Cotta and Hespell 1986) that some protein bands were observed when EP was fractionated by PAGE, may also support this suggestion. Therefore, the protein(s) of interest are likely to be EP associated. Further investigations on protein(s) involvement should be focused on EP associated protein.

Differences in intracellular or cell-associated protein(s) between S and L cells are still possible, since their differences could be in protein(s) responsible for EP biosynthesis. Most EP-defective mutants are the results of biosynthetic alterations (Quintero and Weiner 1995, Rubens *et al*. 1993, Kolkman *et al*. 1996, Reuber and Walker 1993, Jiang *et al*. 1991, Wang and Reeves, 1994, Kranenburg *et al*.1997), which are mostly catalysed by intracellular enzymes. The fact that Nili (1996) found no difference in S and L cytoplasmic protein profiles may be due to limitations in methodology or a structural mutation, as mentioned above. However, such an alteration often results in an almost complete inhibition of the biosynthetic process. This may not be the case for S and L cells, since L cells still produce EPS. Compositional analysis showed that the fatty acid content, especially C16:0, of L-EP was much lower than that of S-EP and this seemed to be the major difference between S and L EP. Therefore, based on the lipid content, alterations during the early steps of lipid biosynthesis may still be possible. Further comparisons between S and L EP biosynthesis should be more focused on lipid biosynthesis.

In contrast, cell-surface cellulases have been suspected to mediate attachment of *R. albus* to cellulose, and adhesion-defective mutants appeared to be blocked in exocellular transport of this enzyme (Miron *et al.* 2001). Wood *et al.* (1982) reported that this enzyme was associated with cellulosome-like complexes. Although Miron *et al.* (2001) also reported that this enzyme was not associated with cellulosome-like complexes, it is still questionable whether the enzyme was dissociated from the complexes during isolation or whether it was a non-cellulosomal glycanasesimilar to that previously reported (Karita *et al.* 1997, Nagamine *et al.* 1997, White *et al.* 1997, Ohara *et al.* 2000). In addition, glycocalyx exopolysaccharides, cellulosome-like structures, cell-surface glycanases/glycoproteins, and fimbrial proteins have been suggested to be involved in the adhesion of *R. albus* to cellulose (Kim *et al.* 1999, Larson *et al.* 1999, Miron *et al.* 2001).

The fact that the L cells still produced some EP suggests that a gene mutation may not have inactivated EP biosynthesis completely. Since *B. fibrisolvens* synthesises several different polymers, possible mutation sites may be within sequences that code for one (or more) of the polymers which contain or are associated with lipid (C16:0) or LTA. Genetic alterations may also affect surface components other than the ligand and indirectly affect the expression of the ligand on the bacterial surface, thus affecting the hydrophobic properties of the bacterial surfaces, as well as the bacterial ability to adhere to fibre or animal cells (Peres *et al.* 1977, Kihlstrom and Edebo 1976). In *Proteus mirabilis*, which appears to adhere to epithelial cells via surface fimbriae (Silverblatt 1974, Silverblatt and Ofek 1978), mutants that are defective at various stages of lipopolysaccharide biosynthesis are

unable to form or express fimbriae, or to adhere to epithelial cells under growth conditions that promote both activities in the parent strain (Ofek and Rottem, 1978, Ofek and Beachey 1980).

It has been discussed previously that the difference in attachment properties between S and L may at least be partly due to properties of the EP rather than the amount. This suggestion is supported by the presence of high molecular weight molecules at the phenol-water (during phenol extraction) or chloroform-water/methanol interfaces (during sample preparation for fatty acid analysis) in S but not in L samples. In addition, S EP could not pass through a 0.22 μm membrane filter while L EP could. This suggests a fundamental size difference in EP between S and L cells. The EP of *B. fibrisolvens* comprises polysaccharide (Stack 1988; Stack *et al.* 1988a,b; Stack and Ericson 1988; Stack and Weisleder 1990; Wachenheim and Patterson 1990; Ha *et al.* 1991; Andersson *et al.* 1993; Ferreira *et al.* 1995), WTA (Sharpe *et al.* 1975, Duckworth 1977, Fletcher 1980a, Wicken 1980), and LTA (Sharpe *et al.* 1975, Hewett *et al.* 1976, Wicken 1980). LTA is also known to be associated with EPS (Wicken 1980), and these two polymers have been identified as being involved in various microbial attachment processes (Wicken 1980, Fletcher 1991, Quintero and Weiner 1995). Beside size and property differences, the recovery of material at the water-phenol or chloroform-water/methanol interfaces suggests that in S EP, there may be an association between different types of polymers, but not in L EP. Such an association may be between EPS, WTA, and LTA to form a more complex structure than in S EP. This may be seen as polymer spreading in the SEM analysis. Such LTA is known as high molecular weight LTA, and LTA that is not

associated with other polymers is known as low molecular weight LTA (Knox *et al.* 1970, Wicken and Knox 1970). S EP may contain more high molecular weight LTA than L EP, and this may explain the size difference between S and L EP.

The presence of high as well as low moleculer weight LTA have been reported for *B. fibrisolvens* NOR37, IL6-31 and 49 (Hewett *et al.* 1976). The composition of high moleculer weight LTA was found to be similar to that reported for various strains of *B. fibrisolvens* LTA, mainly glycerol phosphate, glucose, galactose, ester-linked amino acids and lipid, while low moleculer weight LTA was similar in composition, but lacked fatty acid esters (Sharpe *et al.* 1975). The presence of 2 forms of LTA in extracts of other bacteria as well as in the external media used for their growth has also been reported (Markham *et al.* 1975, Wicken and Knox 1975, Joseph and Shockman 1975). In L EP, most of its LTA may be low moleculer weight LTA (deacylated LTA), and therefore the lipid (C16:0) content of L EP was low. The fact that L cell EP still contained lipid, especially other than C16:0, it may be from another type of polymer, such as lipopolysaccharide or wall teichoic acids.

Micelle formation (Figure 1.4 D) may explain the apparent large molecular size of high moleculer weight LTA and the presence of material at the interface in phenol and water-methanol-chloroform extractions. It may indicate an amphipathic structure in which the hydrophobic lipid portion of the complex was buried as well as firmly bound to the hydrophilic teichoic acids (Wicken and Knox 1970). Such micellar formations have been reported for phospholipids and lipopolysaccharides from gram-negative organisms (Rothfield and Horne 1967,

Wicken and Knox 1970). The presence of a lipid-teichoic acid complex was consistent with it being present as an integral component (with phospholipid) of the common leaflet structure of the cell membrane (Wicken and Knox 1970). For gram-negative organisms lacking teichoic acid, lipopolysaccharide fulfils a similar role in the outer cell envelope (Rothfield and Horne 1967).

SEM examination indicated that in cellobiose grown S and L cells, more globular EP and less polymer spreading occurred, compared to other carbon sources. The C16:0 content of EP isolated from cellobiose grown cultures was also relatively low compared to those of cells grown on other carbon sources. The level of C16:0 may indicate the amount LTA synthesised and may correspond to polymer spreading. In other carbon sources (glucose, sucrose, maltose, starch) where more polymer spreading was observed in the S variant, the C16:0 content of S EP was much higher than that of cells grown in the presence of cellobiose. It is known that LTA is closely associated with polysaccharide. The free acid-phosphate of LTA has been suggested as the active site for attachment of organisms (Markham *et al*. 1975, Rölla 1976, Fletcher 1980a,b). For various bacteria, it is known that the phosphate group of glycerol phosphate within LTA attaches to glucose or galactose in EPS (Figure 1.4 A). In the adhesion of dextran-LTA complexes to the tooth surface, LTA also binds through its ionised phosphate to hydroxyapatite (Markham *et al*. 1975, Rölla 1976, Wicken 1980). However, the site where the hydroxyl phosphate of LTA attaches to glucose or galactose moiety of EPS has not been further studied. The free acid of the phosphate group would require a hydroxyl function of glucose or galactose (Figure 8.2) to form an ester bond, but which hydroxyl group is unknown.

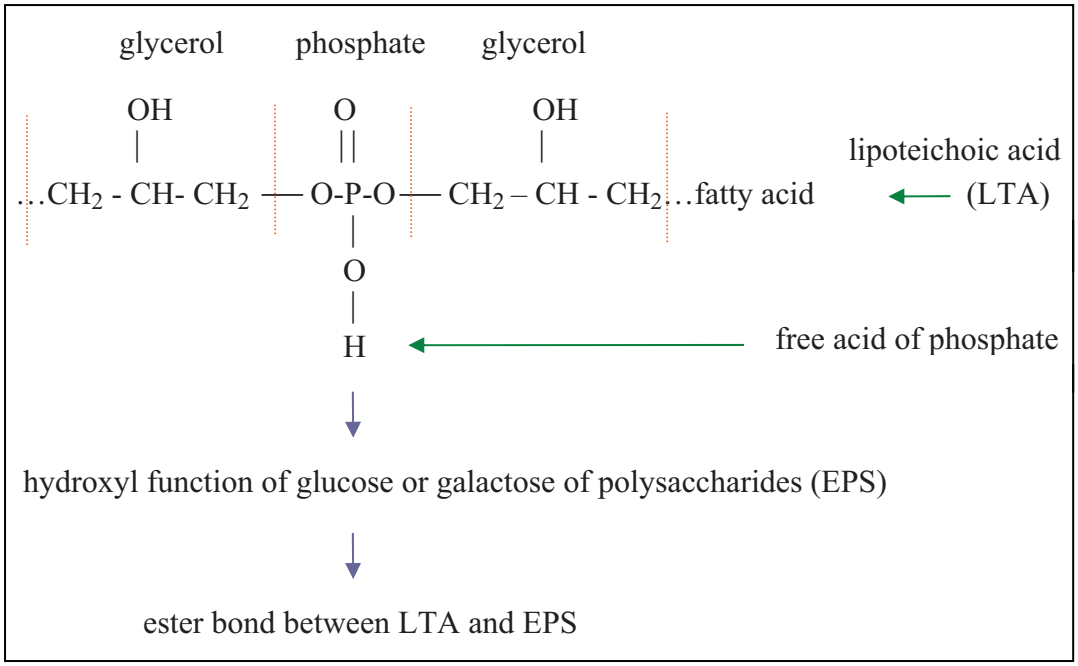

Figure 8.2 Possible linkage between LTA and EPS

Based on the results and the above discussion, the difference in EP between S and L cells is proposed as outlined in Figure 8.3. In S cells, the polymer network may be established between LTA and EPS, through the free acid functional group of the phosphate in LTA and an unknown side hydroxyl function of glucose or galactose. In L cell EP, this association is not established, possibly due to the lower amount of LTA present compared to S cells. The L cell EP may comprise mostly polysaccharides and possibly wall teichoic acids. Therefore the overall negative charge on their WTA is maintained and this keeps the cells hydrophilic, enabling cell populations to disperse in the medium. In S cells, the negative charge may be neutralised by combination with LTA and keeps the cells hydrophobic, and therefore clumped together in liquid medium at early stages of growth. After longer periods of incubation, the complex cell surface polymers are

secreted to the medium and the cells become hydrophilic, and therefore disperse throughout the liquid medium. Removal of teichoic acid from a wall preparation causes cells to settle from suspension as a flocculent mass (Heptinstall *et al*. 1970, Duckworth 1977).

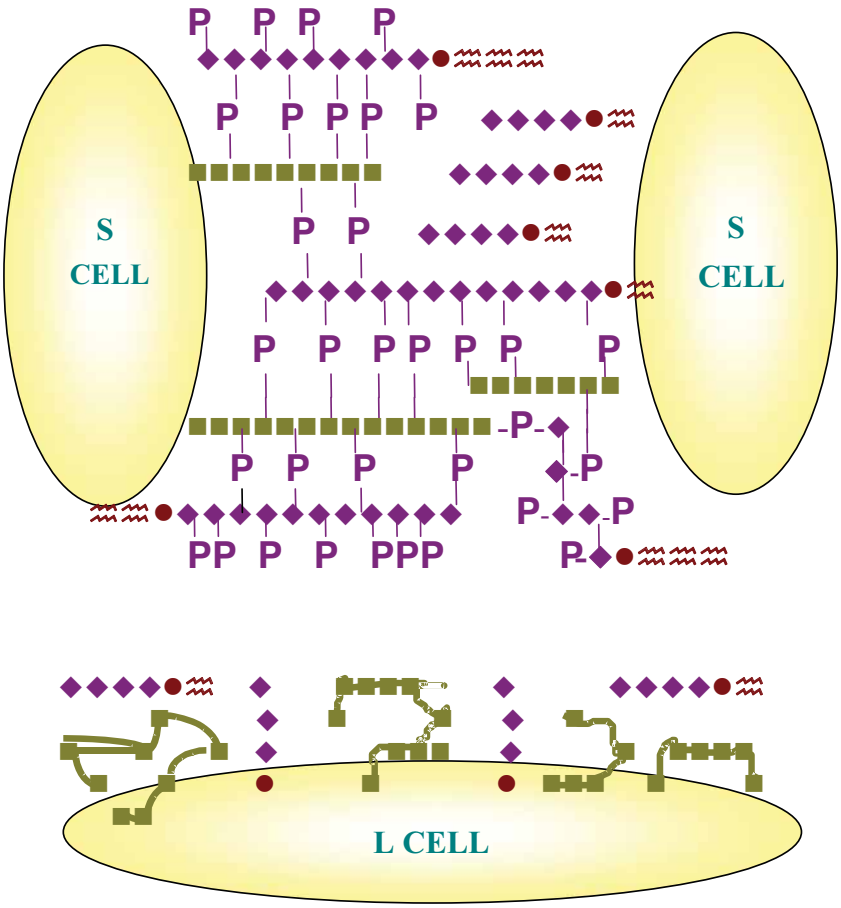

Figure 8.3 Possible difference in the extracellular polymer network of S and L cells.

■■■■ = extracellular polysaccharides (EPS), ■ = glucose or galactose residue, ≈●◆◆◆◆ = lipoteichoic acids (LTA), ◆ = glycerol phosphate, P = phosphate, ≈ = fatty acid.

Beside palmitic acid (C16:0), smaller amounts of other fatty acids (C16:1, u3, C18:0, C18:1, u4) were also present in S and L EP. These may be derived from other components of the cell wall, such as wall teichoic acids (WTA). WTA displays a full range of structures including glycerol phosphate, ribitol phosphate and sugar 1-phosphate (Figure 1.3 and 1.5), and is covalently linked to peptidoglycan (Wicken and Knox 1975, Duckworth 1977). Based on the retention time, u2 may be ribitol phosphate, derived from WTA. The WTA may not be strongly associated with the polysaccharide-lipoteichoic acid network, and is possibly retained as the PD fraction. What was described as the remaining LTA (↔) of the PD fraction may actually be WTA. The WTA was not included in Figure 8.3, since its involvement in attachment is unknown and it may be just trapped in the network and easy to separate from LTA-EPS network as the PD fraction.

The LTA-EPS association and lipid effect (Figure 8.4) would permit the S EP to be spread to surfaces or neighbouring cells, thus giving a wider surface area to be exposed for attachment. Through hydrophobic effects, the lipid portion would drive the polymer to be stretched and direct the free acid of the phosphate to react with other EPS units like those in β-pleated sheet protein, resulted in EP spreading (network). The polymer stretching would be less favourable to the free acid phosphate reacting with hydroxyl groups of EPS, resulting in globular EP, as with globular protein structures. This may explain the absence of polymer spreading in L EP, due to the lack (or at least much lower concentration) of acetylated LTA. It has been suggested that adhesive properties are also dependent on the conformational states of the polymer and the arrangement of functional groups

(Christensen 1989). Therefore the ability of bacteria to adhere not only depends on the ability to synthesize the ligand (EP) but also to express it in an accessible configuration on the surface (Ofek and Beachey 1980). Surface components other than the ligand may influence the configuration, such as by altering the hydrophobicity of the organism (Ofek and Beachey 1980). The hydrophobic effect or micelle formation (Figure 8.4) may direct the conformational states of the polymer (EP) and the arrangement of functional groups to an accessible configuration on the surface.

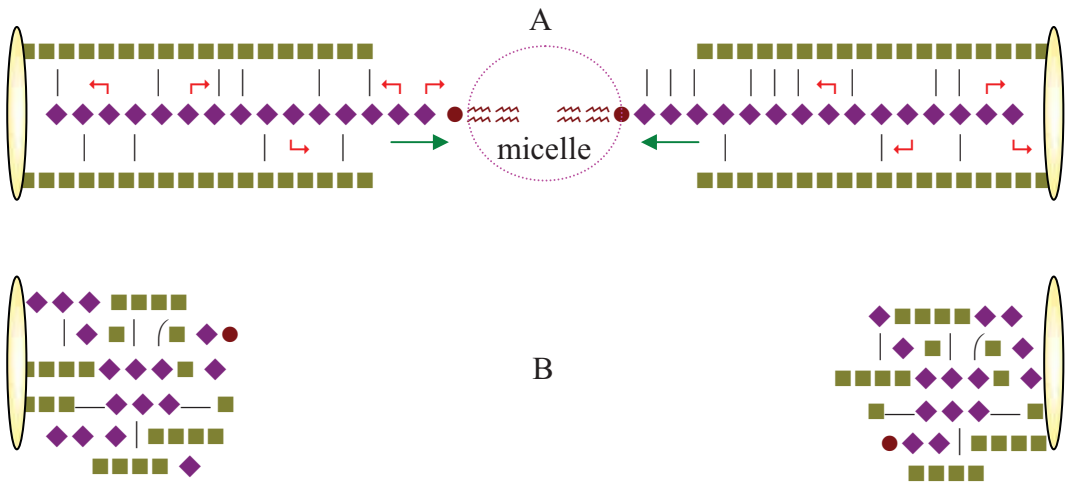

Figure 8.4 Possible effect of fatty acid on extracellular polymer spreading.

= cells; = extracellular polysaccharides (EPS); = lipoteichoic acids (LTA); = free acids of phosphate that form ester bonds with EPS, while and indicate those that are not associated with EPS, and may be used for attaching to surfaces or substrate within the medium (such as glucose or galactose). and indicates the direction of hydrophobic forces that drives the fatty acid to form a micelle, and results in polymer stretching (A), which may be an advantage for the orientation of free acid phosphate to establish attachment to surfaces, substrate or to other EPS units, as in the β-pleated sheet structure of protein. Without fatty acids (B), there is no force to drive the polymer stretching, and the free acid phosphate may bind to the same polysaccharides, forming globular structures, as in the secondary (α-helix) and tertiary structures of globular proteins.

The polymer stretching or EP spreading (Figure 8.4) could only occur if the polymer was held from both ends, in this case, the cell on one end and the hydrophobic force directed by fatty acids on the other end. When the cell secretes its EP, the polymer stretching would no longer exist, and the cell would no longer attach to surfaces. SEM examination and EP analysis suggested that the cell associated EP would be secreted during the final stage of growth. This may be the reason the S variant only attached to agar surfaces (or clumped in liquid medium) during the early stages of growth.

If the free acid phosphate could react with the hydroxyl group of EPS, it would be possible to react with carbohydrate substrates, resulting in attachment to surfaces. Most of the carbohydrates were glucose-based compounds, such as glucose, cellobiose, cellulose, sucrose, maltose, or starch. The higher glucose content of S EP (fractions N and M) than L EP may be the result of attachment to the glucose moiety of the substrate. Some of the glucose may not exactly be a component of the EP, but could be derived from the attached substrates, as may be the xylose and arabinose components of M fraction isolated from the cell free medium of xylan grown cultures.

The EP-associated brown colour of fraction M that tended to follow the EPS (Figure 6.4) and not the fatty acid (Figure 6.14) fraction may suggest that the lipid (fatty acid) fraction was not involved in the mode of action of EP. The fatty acid component of LTA may only direct the polymer stretching (Figure 8.4) through hydrophobic effects (or micelle formation), but not be involved in the association

with EPS, medium components or surfaces. Such interactions may involve the free acid phosphate of LTA (Figure 8.2).

Another possibility is that LTA in L EP was acetylated with fatty acids other than palmitic acid (C16:0). Those fatty acid esters would be less reactive for agglutination compared with the C16:0 ester. It has been shown that bacterial polysaccharides would not agglutinate red blood cells (Davies *et al.* 1958), but agglutination could be achieved when the polysaccharide was esterified (Hammerling and Whestphal 1967, Slade and Hammerling 1968). It has been demonstrated that among polysaccharides esters of C12 to C18, palmitic acid (C16:0) was the most reactive in its ability to sensitize red blood cells to agglutination in the presence of specific antiserum (Pavlovskis and Slade 1969). Therefore, the S variant EP would be more reactive for aggregation than that of the L variant EP, due to its higher C16:0 content. The more C16:0, the more favourable for aggregation, and the more favourable for polymer spreading, or clumping in liquid medium. In glucose, sucrose, maltose or starch, where the C16:0 content was high, more polymer spreading was observed under SEM, while in cellobiose where the C16:0 content was low, less polymer spreading or more globular EP was observed. This is similar to that of the marine *Pseudomonas*, where there is no polymer network (spreading) in unattached cells, and its EP was condensed (globular EP) (Fletcher 1980a, Figure 5.20).

Attachment of the S cells seemed to be related to the level of EP production and its fatty acid content, as well as its morphology, compared with L cells. However, the ability of S cells to attach to a surface had little effect on growth and

enzymatic activities, compared with L cells. The lack of effect on attachment, growth and activity may be due to the high concentration of substrates (0.25-0.5 %, and trials to as low as 0.1 %) used in the medium. Most significant differences in growth and activity between attached and free floating cells were reported at very low concentrations (0.01 %) of substrates (Fletcher 1991). It may be necessary to re-examine these cells at a very low substrate concentration (0.01 %). However, this concentration is beyond the common substrate concentration used in the medium, and a sensitive method to measure growth, rather than OD 600 nm or total protein, needs to be developed.

Studies on cell-surface polymer biosynthesis indicate that assembly of the sugar repeating unit is a common process. This process is catalysed by glycosyltransferase and may use UDP-glucose as a substrate (Kranenburg *et al* 1997, Degesst and De Vuyst 2000), and therefore requires glucose as a precursor. Cellobiose is usually utilised directly by the cell (Bequin 1990), and therefore must be metabolised prior to use as a precursor for UDP-glucose, while glucose can be used directly without prior processing, and this may induce more cell surface polymer biosynthesis, hence higher EP production. Sucrose is normally digested by extracellular enzyme to produce glucose and fructose (Costerton *et al*. 1981). Degradation of other carbon sources (maltose, starch, or cellulose) also generate glucose. These may be the reason for lower EP production in cellobiose compared to other carbon source grown-cultures. In cellulose-containing medium, lower EP production may be due to limited levels of glucose or cellobiose, since *B. fibrisolvens* E14 is only weakly cellulolytic. It has been reported that the amount of EPS produced by *S. thermophylus* was influenced by the type of

carbohydrate source used and the levels of activity of phosphoglucomutase, UDP-galactose 4-epimerase, and UDP-glucose pyrophosphorylase (Degesst and De Vuyst 2000). In addition, fructose could only be fermented when it was used in combination with glucose (Degesst and De Vuyst 2000).

From what has been discussed in this thesis, I hypothesise that *B. fibrisolvens* E14 synthesises several different polymers, and one of them (LTA) is not correctly synthesised or processed in L cells. The lack of this polymer affects the cells ability to attach to substrates, possibly by reduced spreading on the cell surface. Spontaneous mutations, which occur at low frequencies in most bacteria, may result in disruption of essential gene(s) encoding LTA biosynthesis. The mutation may affect structural sequences or post translational processing.

Both attached and free cells have advantages as well as disadvantages in terms of nutrient utilisation, and these may be eliminated under laboratory conditions involving shaking or nutrient diffusion effects, and hence the correlation between attachment and nutrient utilisation may not be detected.

Culture-age dependent attachment may be one of several natural mechanisms for maintaining microbial populations on a substrate surface, such as within the rumen or marine/fresh water environments where occasional flushing may remove the old/dead cells.

The S and L phenomenon may be one of various avenues of evolution. Survival of spontaneous mutations may be selected for by environmental conditions, thus

generating different cells, such as the sticky and loose cells studied in this thesis. These cells have different nutrient preferences, and after being exposed through many generations, may become different strains. For *B. fibrisolvens* within the rumen, where there is no shaking effect, S cells are less motile than L cells, which would result in less growth of S compared to L cells, and this may be the reason that most *B. fibrisolvens* strains do not attach to surfaces.

8.2 Future studies

Future studies on the attachment of *B. fibrisolvens* to surfaces should be carried out at very low concentrations of substrate, and methods should be developed to monitor growth and activity at such low concentrations. Fermentation activity ie. gas production to measure growth may be used for such experiments. In addition, methods to measure the strength of attachment are also required. To identify proteins involved in EP biosynthesis or attachment, work should be focused on intracellular or EP associated proteins, respectively. The study of EP biosynthesis and EP structure should be focused on lipid biosynthesis and the interaction between EP components (WTA, LTA and EPS). Genetic studies should focus on genes involved in the synthesis of LTA or fatty acids, and on the isolation of genes involved in EP biosynthesis. Further work on genetic complementation may reveal genetic details of *B. fibrisolvens* attachment gene(s).